A Histological and Morphometric Assessment of Endocrine and Ductular Proliferation in the Adult Rat Pancreas using an Occlusive Pancreatic Duct Ligation Model

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work, and has not previously in its entirety or in part, been submitted at any university for a degree.

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SUMMARY

Diabetes Mellitus (DM) is synonymous with "ß-cell failure". Ligation of the pancreatic duct distally to its confluence into the bile duct has been shown to induce endocrine tissue regeneration from a number of probable sources. The cells responsible for regeneration are supposed to possess either dormant pluripotent stem cell ability and/or the plasticity to undergo metaplasia to form new and surplus endocrine tissue able to replace pathologically and/or experimentally compromised pancreas. The sequence of events (cell lineage) during this process of neogenesis, has been the source of controversy for quite some time as various studies suggest that the cell lineage differs from *in vivo* and *in vitro* studies, according to experimental model and species of laboratory animal.

The object of this study was to utilise an established experimental laboratory animal model to study islet morphological changes, neogenesis and or both *in vivo*. Further aims of the study were to determine the extent, sequence and magnitude of pancreatic duct ligation (PDL) induced endocrine neogenesis/morphogenesis in a laboratory rat model using occlusive pancreatic duct ligation.

PDL's were performed on six groups of 25 normal adult Sprague-Dawley (SD) rats (300g+) according to the method of Hultquist and Jönsson (1965). Experimental animals were sacrificed at 12 hr intervals from day one post-PDL to day 10 and every 24 hrs thereafter to day 14 as described by Wang, Klöppel, Bouwens (1995). Animals received BrdU (a thymidine marker and cell proliferation indicator) 50mg/kg intraperitoneally as described by Wang et al. (1995), one hour prior to removal of the pancreas after which it was fixed in Bouin's solution and histologically processed. Seven consecutive 3-6 μm thick serial sections were sequentially stained with H & E, insulin (I), glucagon (G), somatostatin (ST), pancreatic polypeptide (PP), neuropeptide tyrosine (NPY) and peptide tyrosine tyrosine (PYY). Immunolabeling was done according to the method of Guesdon, Ternynck , Avrameas (1979). Double immunolabeling for BrdU and each pancreatic peptide was performed on the sections on days 3, 5, 7, 9 and 11 as described by Wang et al (1994).

Cellular transformation between one and 3½ days was characterised by simultaneous total deletion and/or transdifferentiation of the acinar compartment and the appearance of immunoreactive cells for I (11.53 ±1.5%), G (1.85 ±0.8%), PP (1.50 ±0.09%), and ST (1.96 ±0.24%). Changes in the endocrine composition in existing islets, occurred along a pathway that saw PP- and ST-cells invading the islet core, islet mantle glucagon deletion and random appearance of all endocrine cell types within the inter-islet interstitium on day 3½. Days 4 to 6½ saw further endocrine expansion while days 7 to 14 were distinguished by islet reconstitution and consolidation. NPY immunoreactivity appeared on day 4½ and persisted intermittently throughout while PYY first appeared on day 4 and disappeared after day 7½.

The results suggest that PDL firstly induced the development of endocrine tissue distributed haphazardly throughout the space previously occupied by acinar parenchyma. Secondly, the appearance of insulin is preceded by the appearance of PP, glucagon and somatostatin by 24-hours. A still to be determined proportion of the ligation induced endocrine formation appeared to be associated with existing islets, resulting in a number of very large islets, some of which might have secretory access through the glomerular-like capillary network known to occupy the islet core. The remainder appeared to form separate "new" islets, which have a dubious access to the blood stream.

In conclusion, if it is true that the pancreas can regenerate some of its endocrine tissue then it has potential clinical implication for the stabilising of diabetes mellitus. Ligated exocrine pancreatic tissue, devoid of its acinar component, has been shown to contain notable quantities of insulin positive cells. This presents intriguing possibilities as an alternative for donor tissue, usually obtained from rat foetuses, during foetal rat pancreas transplantation studies. Pancreas tissue harvested from duct ligated rats could replace the foetal tissue currently used in the treatment of experimental diabetes mellitus in laboratory animals in this laboratory.

OPSOMMING

Diabetes Mellitus is sinoniem met ß-sel disfunksie. Endokriene regenerasie kan segmenteel bewerkstellig word deur eksperimentele afbinding van die pankreasbuis distaal tot sy samesmelting met die gemene galbuis. 'n Verskeidenheid van selle word vermoedelik by hierdie proses betrek. Dormante stamselle besit die vermoë en/of plastisiteit om 'n aantal vorms van metaplasie te ondergaan om nuwe en/of oortollige endokriene weefsel te vorm wat patologiese en/of eksperimenteel gekompromiseerde weefsel vervang. Die selontwikkelings volgorde wat tydens hierdie proses plaasvind is al vir 'n geruime tyd die middelpunt van 'n meningsverskil. Sommige studies dui daarop dat die *in vivo* sel ontwikkelingsvolgorde verskil van in vitro, volgens eksperimentele model en tipe proefdier gebruik.

Die doel van die studie was die gebruik van 'n bestaande eksperimentele laboratorium proefdier model om pankreas eiland morfologiese verandering en/of neogenese of beide *in vivo* te evalueer. Die oogmerk van die studie was om die omvang en volgorde van veranderings in die endokriene kompartement (neogenese/morfogenese) te bepaal deur gebruik te maak van 'n pankreas buis afbindings (PBA) model wat totale afsnyding van die buis tot gevolg het.

PBA's is uitgevoer op ses groepe van 25 volwasse normale Sprague-Dawley (SD) laboratorium rotte (±300g) soos beskryf deur Hultquist en Jönsson (1965). Proefdiere is elke 12 uur geoffer vanaf dag een post-PBA tot dag tien en elke 24 uur daarna tot dag 14 soos beskryf deur Wang, Bouwens, Klöppel (1995) na die toediening van 50 mg/kg 5-Bromo-2-deoksi-uridien intraperitoneaal ('n selproliferings aanduider) soos beskryf deur Wang et al. (1995). Die pankreas is werwyder, in Bouin se oplossing gefikseer en histologies geprosesseer. Sewe openvolgende seriesnitte (3–6 µm) is alternatiewelik gekleur met H & E, en immunositochemies, soos beskryf deur Guesdon, Terugnck, Avrameas (1979), vir insulien (I), glukagon (G), somatostatien (ST), pankreatiese-polipeptied (PP), neuropeptied tirosien (NPY) en peptied tirosien-tirosien (PYY). BrdU dubbel-immuunkleuring is ingesluit op dae 3, 5, 7, 9 en 11 soos beskryf deur Wang et al. (1994).

Sellulêre transformasie tussen dae een en 3½ dae is gekenmerk deur gelyktydige en totale uitwissing en/of metaplasie van die asinêre kompartement en die verskyning van selle immunorektief vir I (11.53 ±1.5%), G (1.85 ±0.8%), PP (1.50 ±0.09%), ST (1.96 ±0.24%). Metaplasie was verantwoordelik vir merkbare veranderings in bestaande endokriene weefsel langs 'n transformasie weg waar eiland insulien kernselle vervang is deur PP- en ST-selle, glukagon buitelaag uitwissing en die toevallige verskyning van alle endokriene seltipes in the inter-eiland interstitium teen dag 3½. Dae 4½ deur 6½ is gekenmerk deur verdere endokrinetoename terwyl dag 7 deur 14 gekenmerk is deur eiland hersamestelling en konsolidering. NPY immunoreaktiwiteit was vanaf dag 4½, met afwisseling, te bespeur terwyl PYY slegs tussen dae 4 en 7 'n verskyning gemaak het.

Die resultate suggereer eerstens, PBA induseer die ontwikkeling van oortollige endokriene weefsel op 'n lukrake wyse versprei deur die ruimte voorheen deur asinêre parenchiem beset. Tweedens, dat die verskyning van insulien deur dié van PP, glukagon en somatostatien met minstens 24-uur voorafgegaan is. Die verhouding, van nuutgevormde endokriene weefsel wat met bestaande eilande assosieer om 'n aantal baie groot eilande te vorm, moet nog vasgestel word. Sulke strukture mag moontlik afskeidings toegang hê tot die bloedstroom, deur die glomerulusagtige kapillêre netwerk, in die eilandkern teenwoordig terwyl die oorblywende nuutgevormde endokrine weefsel "nuwe" apparte eilande vorm wat wel of gladnie toegang tot die bloedstroom mag hê nie.

As gevolgtrekking, indien dit waar is dat volwasse pankreas eilandweefsel wel regenerasie kan ondergaan, dan het dit kliniese implikasie vir die stabilisering van diabetes mellitus. Weefsel verkry uit PBA bevat geen asinêre weefsel nie maar wel merkbare hoeveelhede endokriene weefsel, veral insulin positief. Dit bied dan interessante alternatiewe as skenker weefsel by fetal rot pankreas oorplantings. PBA en/of die oorplanting van pankreasbuis afgebinde weefsel, na *in vitro* weefsel kultuur, bied moontlikhede vir die behandeling van diabetes mellitus.

PEER REVIEWED PRESENTATIONS

28TH Annual Conference of the Anatomical Society of Southern Africa, Maputo 1998.

In vivo pancreatic duct ligation induces islet neogenesis and differentiation of duct epithelium into islet tissue – a preliminary light microscopic assessment **by B.J. PAGE, D.F. DU TOIT, C.J.F. MULLER, R. LYNERS, R. MATTYSEN and *J. LOUW.** *Department of Anatomy and Histology, Faculty of Medicine, University of Stellenbosch. Tygerberg 7535, *Experimental Biology Unit, Medical Research Council, Parowvallei, 7530.*

29TH Annual Conference of the Anatomical Society of Southern Africa, Pretoria 1999.

Occlusive Pancreatic Duct Ligation (PDL) induces spontaneous and simultaneous pancreatic endocrine regeneration from ductular-, acinar and endocrine cells by re-, retro-and/or transdifferentiation in a laboratory rat **by PAGE, B.J., DU TOIT, D.F., MULLER, C.J.F., LYNERS, R., MATTYSEN, J., ARENDSE, C .*LOUW, J.** *Department of Anatomy and Histology, Faculty of Medicine, University of Stellenbosch., *Experimental Biology Unit, Medical Research Council.*

In vitro differentiation of isolated pancreatic acinar and non-islet endocrine cells into non-specific cells and subsequent morphogenesis into cells with endocrine characteristics **by B.J. PAGE(1), E.G. HOAL-VAN HELDEN(2), D.F. DU TOIT(1), P.D. VAN HELDEN (2), D. HON(2), C.J.F. MULLER(1), R. LYNERS(1).** *(1) Department of Anatomy and Histology, (2) Department of Medical Biochemistry, Faculty of Medicine, University of Stellenbosch, Tygerberg 7505.*

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TABLE OF CONTENTS

CHAPTER 5

LIST OF TABLES

LIST OF FIGURES

DEFINITION OF TERMS

Anagenesis

Reproduction or regeneration of tissue (Dorland's Medical Dictionary)

Apoptosis

Programmed genetically determined cell death, which is part of normal development in tissue. A cell starts to shrink, losing contact with neighbouring cells. Its chromatin condenses and its DNA breaks into nucleosomal sized fragments. Finally the cell fragments into membrane bound pieces called apoptotic bodies. These fragments are removed by macrophages or neighbouring cells with no inflammatory reaction in the tissue (Abe et al. 1997; Doi et al. 1997; Scaglia et al. 1995, 1997; Wada et al. 1995, Walker 1987).

Atrophy

Wasting away, diminution in size of a cell, tissue or organ or part (Dorland's Medical Dictionary).

Differentiation

The act or process of acquiring completely individual character as occurs in the progressive diversification of cells and tissues of the embryo (Bertelli and Bendayan 1997).

Morphogenesis

Evolution and development of form. As the development of form or shape of a

particular organ or body part (Human Embryology, Langman)

Necrosis

Death of tissue, usually as individual cells, groups of cells or in a small localised area. Cell death due to abnormal circumstances i.e., oedema, ischaemia etc. (Clinical Pathology)

Neogenesis

A form of tissue regeneration that is slower than anagenesis (Bouwens and Kloppel 1996, Bouwens 1994, 1998; Dorland's Medical Dictionary).

Nesidioblastosis

Diffuse proliferation of islet cells in the pancreas (Sempoux et al. 1998; Ueda et al. 1998).

Retrodifferentiation

When differentiated cells undergo retrodifferentiation, specialised functions are interrupted, there is deletion of cytoplasmic structure and the cell undergoes functional and morphological simplification. They acquire an increased capacity to divide and eventually replace the population deficit with cells identical to those that were lost (Scarpelli and Rao 1981).

Transdifferentiation

Given the proper stimulus, regenerating cells in adult pancreas can be redirected into a

totally different pathway of differentiation (Scarpelli and Rao 1981).

Transdifferentiation is a complex aspect of cell physiology taking place in many cell types. The switch in cell phenotype may require several steps including de-differentiation, proliferation and re-differentiation, but direct transdifferentiation without cell division has been described in oesophageal smooth muscle and pancreas. Transdifferentiation is characterised by cells expressing intermediate phenotypes. In the pancreas, acinar cells and islet cells transdifferentiate toward ductal cells. Pancreatic cells may transdifferentiate toward hepatic cells and acinar cells into islet cells via a series of intermediary cells expressing acinar and islet characteristics (dual expression). Intermediary cells have been described by Melmed et al. (1972), Melmed (1978), Bertelli and Bendayan (1997), Gu et al. (1994), Sarvetnick et al. (1996)

In Pancreatic duct ligation acinar cells have been shown to co-express insulin and islet cells (all types) co-express amylase. Cells undergoing a switching process between secretory programs may undergo several transitional stages from one secretagogue to another. Cells in the pancreas with characteristics of up to four cell types have been described (references as above).

ABBREVIATIONS

α-cell	Alpha Cell
ALX	Alloxan
ß-cell	Beta Cell
BrdU	5-Bromo 2-deoxy uridine
G	Glucagon
GIR	Glucagon immunoreactive/immunoreactivity
HGF	Hepatocyte growth factor
HPBT	Hepato-pancreatic biliary tract
I	Insulin
IIR	Insulin immunoreactive/immunoreactivity
IR	Immunoreactivity
IRA	Insulin Rich Area
LIEF	Ligation Induced Endocrine Formation
MI	Mitotic index
NGF	Nerve growth factor
NPY	Neuropeptide tyrosine
PCNA	Proliferation Cell Nuclear Antigen
PDL	Pancreatic Duct Ligation
PP	Pancreatic polypeptide
PP-IR	Pancreatic polypeptide immunoreactive/immunoreactivity
PYY	Peptide tyrosine tyrosine
SOC	Sham Operated Control
ß-cell	Beta cell
ST	Somatostatin
ST-IR	Somatostatin immunoreactive/immunoreactivity
STZ	Streptozotocin
VGEF	Vascular epithelial growth factor

CHAPTER 1

The Problem

Overview of Chapter 2
Literature Survey

Overview of Chapter 3
Materials and Methods

Overview of Chapter 4
Results

Overview of Chapter 5
Discussion

Overview of Chapter 6

Aims and Objectives
Findings
Conclusions
Topics for Further Research

1. THE PROBLEM

Although much has been achieved over the past ten years to enhance our understanding of the development, structure and functioning of the pancreas many fundamental questions, especially in the realm of experimentally induced cell lineage development, as yet, either remain unanswered or are the topic of a difference of opinion/interpretation. One of the contended issues is due to a real or apparent lack of specificity/sensitivity of measuring techniques. As NPY/PYY/PP share a large number of structural similarities, antibodies raised against each of the peptides used for immunocytochemical staining of tissue sections tend to exhibit an amount of cross-reactivity.

The fundamental problem, which is the topic of this study, is whether experimentally induced pancreatic tissue remodelling, arising from one experimental intervention, (duct ligation) develops along the same pathway as that of alternative experimental procedures (cellophane wrapping, 90% partial pancreatectomy, ß-cell destruction by Streptozotocin/Alloxan). Dr Susan Bonner-Weir (1993) suggested that pancreatic recapitulation follows ontogeny.

These questions and issues have been the source of an ongoing dispute and discussion between prominent research scientists such as Herrera et al. (1991, 1994); Rosenberg et al. (1988, 1990, 1998) and Wolfe-Coote et al. (1987, 1990, 1992, 1996), Bouwens (1998); Githens (1993) Jackerott et al. (1996, 1997); Larsson (1998); Teitelman et al (1987a, 1987b, 1993, 1998) and Upchurch et al. 1994, 1996) for various reasons, which will be discussed in detail at a later stage in the study.

Much of the contention surrounds the interpretation of the results obtained under dissimilar experimental conditions from *in vitro* pancreatic duct cell culture, 90% pancreatectomy, cellophane wrapping, occlusive and non-occlusive pancreatic duct ligation *in vivo,* and attempts to compare results with streptozotocin or alloxan induced pancreatic regeneration in neonatal and/or adult experimental animals with developmental events during embryonal development in mice, rats, vervet monkeys, chacma baboons, rhesus monkeys, hamsters to chickens…to mention but a few. It is

the contention of this writer that the dissimilarity in the interpretation of experimental results and the resulting differences of opinion as to pancreatic cell lineage development stems from this divergence of investigative/experimental procedure in a wide variety of experimental animals.

Two further factors that need to be considered in this scenario are, firstly, the retrodifferentiation that occurs in tissue that has been experimentally manipulated (both *in vivo* and *in vitro*) to form pluripotent stem/duct epithelial cells/oval cells, and secondly, the sequence of events that leads to neogenesis which often overlaps the former, resulting in the presence of cells representing both processes, adding to the confusion and resulting disparity in the interpretation of data.

2. OVERVIEW OF CHAPTER 2 – The Literature Survey

Historically, the precise aetiology of diabetes mellitus (DM) has eluded researchers. Although the prevalence of DM is on the increase both in Southern Africa and other countries world wide, no cure has, as yet, been found for the chronic systemic disorder. Treatment is based on the symptomatic treatment of hyperglycaemia and its secondary complications, which include cardiovascular disease, retinopathy, nephropathy to mention but a few. In India, where unofficial estimates put the incidence of DM in some areas at fifteen percent of the population [consultation with Dr Rema Mohan, MV Diabetes Specialities Centre (P) Ltd, Chennai, India. (http://mvdsc.com)] DM is unquestionably not as a result of affluence as is the case in the western world. The history and manifestation of DM is the topic of this chapter, together with the research thrust and endeavours over the past twenty years and focuses on the issues around anagenesis, neo- and morphogenesis induced by a variety of experimental procedures. Attention is also given to histological technique and the principles of immunocytochemistry.

3. OVERVIEW OF CHAPTER 3 - Materials and Methods

The foundation of this study was laid at the University of the North West where the planning and initial experimental protocol was established. This chapter gives the briefest of outlines of that early process. After translocating the study to Stellenbosch University much of the work done at UNW had to be repeated and a course of action decided upon in consultation with Dr Sonia Wolfe-Coote (who had visited Dr Luc Bouwens at his facility in Brussels) as to the mechanism of duct ligation both, in terms of positioning of the suture and how tight it should be.

4. OVERVIEW OF CHAPTER 4 – The Results

The bulk of this thesis is set aside for the presentation of the results of the study and although the section would have been much longer if all the data were presented, the chapter was compiled with brevity in mind without compromising vital information. The reader should be able to follow the line of thought or the argument to follow in the next chapter. An attempt has been made to present the data in a variety of ways, ranging from a brief global overview to a comprehensive assessment on day-to-day basis for each of the parameters under investigation and a composite summary at the end of the chapter.

5. OVERVIEW OF CHAPTER 5 – The Discussion

To blend the diverse variety of opinions on neogenesis considering the divergence in experimental procedure followed (mentioned under THE PROBLEM in point 1 above), as well as the very obvious "*comparing of apples with oranges*" that has unwittingly become an integral part of the problem, has been a forbidding task. The discussion has relied heavily on a schematic/diagrammatic representation of what the various authors, involved in the saga, have said and/or implied from their perspectives with the unique information at their disposal and is compiled into a special appendix at the end of the

chapter. The issue of misinterpretation can therefore not be excluded considering the divergence of the material under consideration. Interpreting the results in the light of the findings in similar studies would have been a far simpler task if only "*oranges or apples*" were under discussion, but as this was not the case, the topic has become an issue. This study proposes a three-tier model whereby experimental models in pancreas research could be evaluated.

6. OVERVIEW OF CHAPTER 6 - Conclusions

Many questions resulting both from this study and from those of the various authors cited in this study have been presented as avenues for possible further future study. In a study such as this, many more questions arise than answers and many aspects pop to the surface that need to be addressed before one can arrive at a truly satisfactory explanation/understanding of the issues surrounding tissue regeneration and their application to diabetes mellitus. So, although the chapter does focus on the issues which are the essence of this study, some attention is also given to the broader theme of uncovering solutions to the mystifying syndrome making life a veritable "living hell" for the countless millions that suffer from diabetes.

7. AIMS AND OBJECTIVES

7.1 Hypothesis:

Studies have found that both endocrine and exocrine pancreatic tissue regenerates after partial obstruction of the pancreatic duct (pancreatic duct ligation, PDL) or cellophane wrapping of the head portion of the pancreas. Current evidence suggests that this regeneration originates from the cells forming the ducts of the pancreas and seems to go through a developmental sequence that mimics the events occurring during embryonic development.

7.2 Project Aims and Objectives:

This study aims to:

1. follow the sequence of cellular changes that are presumed to occur in both the endocrine and exocrine compartments of the pancreas in duct ligated laboratory rats,
2. compare the sequence of cellular events especially in the endocrine compartment with those quoted in literature for similar and/or alternative experimental models,
3. to establish whether any part of the sequence of cellular change is the same as during embryonic development, and in which respects it is so, and
4 propose a model for the evaluation of the sequence of cellular change occurring in the pancreas after occlusive duct ligation.

CHAPTER 2

LITERATURE SURVEY

Historical Overview

The Pancreas

Experimental Models in Pancreas Research

Oval Cells

Diabetes Mellitus

Histological Procedure

2.1 HISTORICAL OVERVIEW

2.1.1 Introduction - 300 BC to 1922

The term pancreas is derived from the Greek where "pan" means all, and "kreas" means flesh. The earliest record of it being described, according to Fitzgerald, and Morrison, (1980) is attributed to Herophilus of Chalkidon at roughly 300 BC while the anatomical description thereof is attributed to a certain Rufus of Ephesus in 100 AD. As early back as mentioned above, practitioners recognised the pancreas as a separate entity but had no concept of its function. Around 1640 AD Wirsung described the main pancreatic duct, and a while later, Vater (1720) described the duodenal ampulla and Santorini (1742) the accessory duct. As recently as the early 20th century, physicians still referred to the pancreas as "the hermit organ" due to its rertroperitoneal position. The medical fraternity was aware of the disease of "diabetes mellitus" and referred to it by terms like "melting down". It was not until around 1889 (a mere 110 years ago) that Oskar Minkowski and Joseph von Mering established the relationship between diabetes mellitus and the pancreas (Fitzgerald and Morrison 1980).

The ß-cell was described by Lane in 1907 (Hellerström 1984) and classified according to the histological characteristics of its cytoplasmic granules. Subsequently, various researchers have described the various symptoms of diabetes and begun more carefully to describe the syndrome. Banting and Best only discovered the relationship between insulin, the endocrine pancreas and diabetes around 1922.

2.1.2 1922 to date

During the past 75 years, the pancreas has been studied and described in infinite detail. Studies have included embryological development, secretory function, establishment of experimental models to looking at intra- and interspecies transplantation (al-Mahrouki, Youson 1998, Cooper 1998, Cortesini 1998) of cultured and uncultured foetal and mature pancreatic islet tissue, both as unencapsulated tissue and in encapsulated form (Andersson, Eizirik, Bremer et al. 1996; Charles, Harland, Opara 1998; Triverdi, Suzuki, Bonner-Weir et al. 1997), with and without various

immunesuppression regimens, in various (including intraportal) sites of the body, and pancreas regeneration *in vitro* and *in vivo* (Vinik 1992). Although much work has been done on the aetiology of diabetes, the issue is still too complex for alternative regimens. For this reason modern treatment protocols still focus on the symptomatic treatment of hyperglycaemia in order to prevent the degenerative secondary complications that are responsible for the morbidity and mortality profile of diabetic patients. Transplantation is not the only treatment option open to medicine and there are a number of impediments to successful islet (and/or foetal pancreatic) transplantation (Weir, Bonner-Weir, 1997). These include "high expectations, controversy surrounding targeted research, the balance between basic and applied research, the roles of industry and academia, the concerns about xenotransplantation and the difficulties in developing a planned approach to the problem."

2.2 THE PANCREAS

2.2.1 Gross Anatomy

The pancreas has a thin, ill-defined fibrocollagenous capsule from which narrow irregular septa penetrate, dividing it into lobules. Each lobule is composed of roughly spherical clusters of secretory exocrine cells (acini) (Fawcett 1986).

The major portion of the organ consists of thin segments of pale yellow tissue suspended in the mesenteries of the duodenal loop and transverse colon on the right. A more mobile larger portion is attached to the splenic vessels, and extends into the hilum of the spleen, dorsal to the stomach in the left flank region.

The righthand boundary of the pancreas is the duodenal loop. Caudally, on the right, it is attached to the distal portion of the duodenum. From the pancreas in the duodenal loop a tuck of pancreas with mesentery extends ventrally to the right half of the transverse colon. The left caudal margin of the pancreas is a mesentery enclosed thick portion about the splenic vessels and hilum of the spleen. Cephalically the pancreas is present in the region of the upper pole of the spleen, along the dorsal aspect of the fundus and the pyloric area of the stomach and adjacent to the inner curvature of the initial portion of the duodenum. Continuous with the rest of the pancreas at the

retropyloric region of the stomach, is a small amount of pancreas surrounding the bile duct and extending into the porta hepatis.

As far back as 1964, Richards, Fitzgerald, Carol, Rosenstock established an anatomical basis on the structure of the rat pancreas. The pancreas was sub-divided into four major segments, namely, splenic, duodenal, gastric and parabiliary based on the structure of the duct and vascular systems (see figure 3.2 in Materials and Methods). The rat has no gall bladder or cystic duct. The duct from the liver to the duodenum is considered to be the bile duct and consists of two sections.

The first is the proximal part, from the junction of the hepatic tributaries to the point of connection with the pancreatic duct from the splenic segment (see figure 3.2). The second is the common duct, which is the distal portion of the duct, and extends from the junction of the bile duct with the splenic segment duct, to the ampullary region of the duct at the duodenum. Richards, Fitzgerald, Carol et al. (1964) also found that the rat pancreas was diffusely distributed throughout the upper quadrants of the peritoneal cavity.

2.2.2 The Ductular System

Duct cells comprise approximately 10% of the cells and 4% of the volume of the pancreas (McEvoy, Madson 1980; Rosenberg, Kahlenberg, Vinik, Duguid 1996). Duct epithelial cells form the tubular structures through which exocrine (enzymatic) secretions find passage to the duodenum (Fitzgerald, Morrison 1980) and contribute substantial amounts of bicarbonate-rich fluid to the enzyme secretions (Wheater, Daniels, Burkett 1987).

The ductal system consists of three to four major ducts, designated by Richards et al. (1964), as splenic, duodenal, gastric and parabiliary, (figure 3.2 on page 81). The first extends from the common duct to the pancreas, in the duodenal loop (duct of Wirsung). The duodenal ducts usually consist of four major ducts, each smaller in diameter than the splenic duct, but larger than the main gastric duct, each with a separate opening into the common duct. The second, is a relatively large duct, from the common duct to the pancreas which is to be found along the splenic vessels and hilum of the spleen. It

arises behind the stomach, near the pylorus, from a dorsal aspect of the common duct and proceeds cephalically and dorsally in the retropyloric area before bending sharply to descend caudally, ventrally and laterally into the pancreas along the splenic vessels into the hilum of the spleen. A third smaller gastric duct arises from the splenic duct and extends laterally to the left to the tissue about the pyloric region of the stomach. The duct eventually breaks up into a number of secondary divisions. Finally, a number of small ductules throughout most of the pancreas surrounding the common pancreatic and bile ducts (parabiliary). This last mentioned section is extensive (consisting of 10 to 20 ductules) extending into lobules of the pancreas surrounding the bile and common bile ducts throughout their course.

The largest of the four ducts is the splenic portion, which extends from the porta hepatis. It arises behind the stomach, near the pylorus, from a dorsal aspect of the common bile duct. It gives a few ductules off to the adjacent pancreas, proceeds cephalically and dorsally for a short distance in the retropyloric area and then bends sharply to descend caudally, ventrally and laterally into the pancreas along the splenic vessels and into the hilum of the spleen (Stevens and Lowe 1995).

The pancreatic ductal system begins in the acinus. The inner border of the acinar cells form the intra-acinar component of the intercalated duct, lined by a simple monolayer of cuboidal epithelium, designated centro-acinar cells by Junqueira and Carneiro (1980), eventually form a complex network of tubules. Intercalated ducts from individual acini fuse to form larger intralobular ducts. These run in the fibrocollagenous septa between the ill-defined pancreatic lobules. They are lined with columnar epithelium and join the main pancreatic ducts running longitudinally from tail to head of the pancreas.

The initial part of the intralobular duct is formed by the intercalated ductules, which are composed of flat cuboidal cells penetrating within the acinar unit. In sections through the centre of an acinus, cells in which are part of the ductal system, are recognised as centro-acinar cells (Argent, Githens, Kalser et al. 1992). No morphological differences have been found between intercalated and larger interlobular ducts except in their mean calibre in cross section.

Whereas the luminal surface of acinar cells reveals numerous microvilli, the corresponding surfaces of intercalated and intralobular duct cells are smooth. The cytoplasm is less dense and contains a large number of mitochondria, which are generally concentrated around a Golgi complex which usually consists of three to five flattened cisternae and numerous smooth vesicles. A characteristic feature of intralobular duct cells is elaborate interdigitations at the lateral and basal plasma membranes. In this respect intralobular duct cells of the pancreas resemble the cells of the striated ducts in salivary glands or the proximal convoluted tubules of the kidney.

The wall of the interlobular duct is formed by pyramid-shaped epithelial cells, which project short microvilli from their luminal surface and contain abundant secretory granules in their apical cytoplasm (Vay Liang, Gardner, Brooks et al. 1986).

The dorsal and ventral pancreas develops independently as a ramified system of tubules. The tubules join progressively larger tributaries and finally enter the major duct, which empties into the intestinal lumen. The two growing pancreatic glands merge at 16-17 days in rats and mice, but each pancreas retains its own duct. The principal pancreatic duct (of Wirsung) and the accessory duct (of Santorini) independently enter the intestinal lumen.

By the time the pancreatic buds fuse, secondary hollow branchings from the original endodermal outgrowths have appeared. These increase in length and extend into the surrounding mesenchyme. From their solid ends epithelial sprouts arise which become canalised to form the collecting ducts, and the acini arise by sprouting from the tips of these. During the fifth month of foetal life in humans (islet morphogenesis), secretory granules appear in the acinar cells and the release of pancreatic juice into the intestinal lumen occurs.

Throughout development the epithelial component exhibits all the characteristics of epithelia including apical microvilli projecting into the lumen, junctional complexes joining the cells to each other, and a basal lamina. The mesenchymal component is essential for further growth and normal differentiation of the embryonic epithelium in rats, mice and chickens (Githens 1988, Gittes 1994). It makes up as much as 55% of the volume of the pancreas on day 18 in the rat, but declines to less than 12% from 30 days postnatally onwards into adulthood.

2.2.3 The Acinar Pancreas

Although this study is primarily concerned with the endocrine portion of the pancreas, the exocrine pancreas, both acinar and ductular components need to be discussed as both are implicated in experimentally induced pancreas remodelling. Both components play a major role in the redefinition of the post pancreatic duct ligated components, which are presumed to bring about pancreatic duct proliferation in order to undergo neogenesis, and is also of importance in culturing of duct cells to induce the formation of endocrine cells.

Each acinus has an individual intra-acinar duct, which drains into progressively larger ducts. The acini are composed of protein-secreting cells, which have a broad base and narrow apical surface covered by a few short microvilli. The cells are rich in rough endoplasmic reticulum (ER), which is concentrated mainly in the lower half of the cell, and is responsible for their cytoplasmic basophilia. The upper half of the cell, close to the lumen, contains variable numbers of eosinophilic zymogen granules, which contain pre-enzymes synthesised by the cell. Some cells, which contain few or none, are thought to have recently disgorged their granules by exocytosis into the acinar lumen. It is thought that the pre-enzymes are synthesised by the rough endoplasmic reticulum and then transferred into the Golgi, which packages them into granules (Junqueira, Carnerio 1980).

2.2.4 The Endocrine Pancreas

In 1907, Lane published his findings on the structure of the cells of the pancreatic islets in the American Journal of Anatomy. It was he who designated a cell characterised by its content of cytoplasmic granules, which were soluble in alcohol but preserved in fixated chrome-sublimated tissue, giving it unique staining characteristics, as a beta (ß) cell (see Hellerström, 1984). Today with sophisticated methods of investigation, electron microscopy (EM), and immunocytochemical techniques, the structural and chemical aspects of the beta cell have been well characterised.

Stevens and Lowe (1995) have defined the endocrine pancreas in terms of a so-called neuroendocrine component existing in three forms. The first exists as Islets of

Langerhans, which are distinct structures. They account for most of the hormone-producing cells. The second exists as isolated nests or clumps of neuroendocrine cells, which form a minority population, gathered into small groups distinct from islets; and finally, there are the single cells, which are scattered within the exocrine and ductular components of the pancreas and are demonstrable by immunocytochemical methods.

The number of endocrine cells per islet also varies and individual endocrine cells may be interspersed among exocrine cells (Bouwens and Klöppel 1996). Functionally two types of islets may be distinguished. Those derived from the dorsal primordium are richer in alpha cells while those from the ventral primordium give rise to glucagon poor islets (Juncqueira and Carnerio 1980).

Species differences do exist and the islets of Langerhans in the laboratory rat are clearly defined largely spherical clusters of polygonal or rounded cells (Juncqueira, and Carneiro, 1980) scattered throughout the pancreatic tissue. Each islet is separated from surrounding pancreatic tissue by a fine capsule of reticular fibres embedded into the surrounding exocrine tissue. Islets are most numerous in the tail region and vary considerably in size and in the number of cells that they contain. Individual cells within the islets are smaller and paler than the exocrine cells and assume spherical or polygonal shapes from moulding by adjacent cells. Each islet has a capillary network, which is in contact with each cell. (For an in-depth discussion of this aspect please refer to paragraph 2.4.3)

Pancreatic islets develop as cellular buds from the same small ducts, which ultimately supply the exocrine pancreatic components. Occasionally, in man, the variably sized islets can be seen in association with a pancreatic duct in the supporting fibrous tissue. Each pancreatic islet contains a number of different neuroendocrine cells and is enveloped by a sheath of Schwann (peripheral nervous system glial) cells. These play a role (neurotrophin secretion) in the modulation of physiological and experimental conditions (Teitelman, Guz, Ivkovic, Ehrlich, 1998) see paragraph 2.2.7 for a discussion on islet innervation. Each different cell type is mainly concerned with the secretion of a single hormone. The nomenclature of these individual cell types was problematic until Stevens and Lowe (1995) categorised them according to the main hormone secreted by each cell.

Four main and two minor cell types were found to exist. Approximately 70% (60 - 80% Fitzgerald and Morrison 1980) insulin and amylin-secreting cells or B/ß/beta-cells; 20% (15 - 20% Fitzgerald and Morrison 1980) glucagon secreting or A/α/alpha-cells; a further 5 to 10% (5 – 15% Fitzgerald and Morrison 1980) somatostatin secreting cells or D/δ/delta cells (sometimes also referred to as type III) and finally 1-2% (0 – 2% Fitzgerald and Morrison 1980) pancreatic polypeptide (PP) secreting cells designated PP- or F-cells

The minor cell types referred to by Stevens and Lowe are designated vasoactive intestinal peptide (VIP) secreting cells also referred to as D_1 or type IV cells, and mixed secretion cells also designated as (EC above) or enterochromaffin cells. These minor cell types are present in small numbers in the islets and are scattered in the exocrine and ductular components and only make up about one percent of the total mass of the pancreas (Hellerström, 1984). Stevens and Lowe (1995) also report that the mixed secretion cells produce a number of active peptides, including serotonin (5HT), motilin and substance P (Bombesin). Fitzgerald, and Morrison (1980), further defines gastrin secreting (G-cells) and cells secreting biogenic amines (EC-cells - indicated as enterochromaffin cells above).

2.2.5 Embryonic Development

Most leading authorities on endocrine pancreas development and/or regeneration agree that the order of the events in regeneration mimics embryonic development. In more scientific terms phylogeny recapitulates ontogeny (Wang, Bouwens, Klöppel 1996; Scaglia, Cahill, Finegood, Bonner-Weir 1997; Githens 1988; Wolfe-Coote, Louw, Woodroof, Heydenrych, Du Toit 1997; Rosenberg, Vinik, Pittenger, Rafaeloff, Duguid 1996b; Teitelman and Lee 1987b; Teitelman, Alpert, Polak et al. 1993

The bulk of the human (Moore 1988) and avian (Andrew, Rawdon, Kramer 1988) pancreas (pancreatic parenchyma) develops from a dorsal evagination, (also called a pancreatic bud) of endodermal cells from the foregut splanchnopleure at the level of the liver diverticulum. In the rat the dorsal evagination appears on day 11 of gestation (20 somites) and a ventral evagination, appears approximately twelve hours later (28 - 30

somites – Githens 1993; Fitzgerald and Morrison 1980). The larger dorsal bud develops a slight distance cranial to the ventral bud which develops near the entry of the bile duct into the duodenum (Moore, 1988). In the rat the ventral and dorsal rudiments grow together on days 16 and 17 to become a single pancreatic mass (Githens 1988).

The human embryonic pancreas consists of an epithelial component of endodermal origin and a mesenchymal component of splanchnic origin. As the duodenum rotates to the right (clockwise) and becomes C-shaped, the ventral bud is carried dorsally with the bile duct and later comes to lie posterior to the dorsal bud with which it later fuses. The ventral bud forms the uncinate process and part of the head of the pancreas (Moore 1988, Sadler 1995).

It is well documented (Scaglia et al. 1997; Kaung 1994; McEvoy 1980; McEvoy, Madson 1980) that the most rapid increase in population size, for all four islet cell types, occurred during the last four days of gestation in rats, i.e. day 16 post-coitum to 10 days postnatally (McEvoy 1980). This population increase is brought about by an increase in proliferative activity, (E20-22), and a large influx of undifferentiated epithelial cells differentiating into islet populations.

Beta cells were found to increase more rapidly and comprised the major portion of the islets at E20. After birth the rate of increase of α- and ß-cells decreased. Delta-cells (δ- or D-cells) however did not increase as rapidly as α- and ß-cells before birth but increased in volume and density after birth (McEvoy 1980; McEvoy and Madson 1980).

Although the ß-cell capacity for regeneration decreased after birth, Wang et al. (1996) found that the ß-cell compartment increased in number up to six weeks postnatally and thereafter only in size. This finding is in line with that of Pang, Mukonoweshino and Wong (1994) in their study on endocrine pancreas development. They specifically identified the source of accelerated growth in endocrine mass (specifically ß-cell mass) in the latter stages of gestation using glut-2 expression as an indicator of epithelial cells and endocrine cells having the same progenitors.

By about the twelfth week in the human embryo, (rat E11) insulin could be found in the

ß-cells, and soon thereafter, granules appeared in both alpha and beta cells. In the ß-cell the secretory granules were usually concentrated at the cellular pole adjacent to the blood capillary while in the alpha cell the granules tended to be more uniformly scattered. By month four (rat E16) the islet tissue comprised approximately a third (Hamilton, Boyd, Mossman 1972) of the total pancreatic mass and the development of the vascular pattern was well on its way. In the laboratory rat (E20 - 22) the ß-cell mass doubled within 48 hours. Although only 20% of the mass increase could be attributed to the formation of new ß-cells, the remaining 80% were suggested to be due to mechanisms (neoformation from rapidly proliferating morphologically non-differentiated precursors) other than cell division (Sweene and Eriksson 1982; Sweene 1992).

As mentioned above, rat pancreatic development did not cease at birth. Scaglia et al. (1997) have shown that the endocrine pancreas undergoes significant modification during neonatal life and that apoptosis is an important mechanism in this remodelling of the ß-cell mass. Integral to the islet (structure and functioning) was the presence of a glomerular-like structure within the ß-cell core, referred to by Hellerström (1984) as an intra-islet microcirculation. The factors contributing to, or the reasons for, the presence of this type of arrangement is beyond the scope of this study. It is however of interest to note that the islets receive 15-20% of the total pancreatic blood flow while they only comprise 1% of the pancreas volume (Lifson, Kramlinger, Mayrand, Lender 1980; Jansson, Hellerström 1983 as quoted by Hellerström 1984).

Pang, Mukonoweshuro, Wong (1994) report glucose transporter type 2 (glut-2-protein) expression by the endodermal domain containing the pancreatic primordium at gestational age of eleven days (E11) in the rat, which continued as the endodermal epithelium, evaginated into the surrounding mesenchyme to form the pancreatic buds. In their study, cells of the dorsal and ventral bud were shown to maintain glut-2 expression as the epithelia grew to form ducts. As acini arose from the ends of the ducts, acinar cells ceased glut-2 expression. As cell differentiation, however, reached a more advanced stage, cells that were destined to become endocrine cells continued to express glut-2 and co-expressed it with insulin and glucagon. Insulin and Glut-2 co-expressing cells then segregated into large aggregates to form the ß-cells of the islets of Langerhans.

Pang et al. (1994) argues that two distinct populations of insulin expressing cells arose during pancreas formation. The first described as a relatively minor population associated with glucagon-positive cells, from the initial stages of embryonic development (E13). The second (E17-19), a larger population of insulin expressing cells closely associated with the Glut-2 expressing ductal network, which leave the ductal network to become islets.

The view is however not supported by Corbett, Serup, Bonner-Weir, Nielsen (1997), who maintain that during embryonal development (E10-12 - the first transition in the rat) "immature" cells, co-expressing insulin and glucagon, but not glut-2, were shown to exist, and these failed to associate into islets. During the E13-20 (second transition) stage, there was a rapid increase in endocrine cells secreting insulin co-expressing Glu-2, but negative for glucagon, and these developed into islets. The second transition occurred in the absence of NGF, (neuronal growth factor) from mesenchyme surrounding the islets, suggesting that this phase of development was due to mesenchyme independent mechanisms prompting the assumption that pancreatic islets developed by a default mechanism as proposed by Gittes, Galante, Hanahan et al. (1996).

In other parallel studies on postnatal rat pancreas development (Scaglia et al. 1997; Watanabe, Yonemura, Yonekura et al. 1993) ß-cell mass was found to remain stable between day two and twenty followed by a rapid increase until day 31. The increase was not ascribed to ß-cell replication (which decreased in this period) but increased neogenesis from day 17, as shown by an increase in BrdU labelling of cells in the duct region from day 13 to 31, i.e. duct cell replication and differentiation.

Different ß-cell lines expressed functional NGF receptors that responded to NGF by extending neurite-like outgrowths. NGF receptors were also expressed *in vivo* in mature rat islets and early development in pancreatic ductular cells, which represent putative stem cells. (NGF is produced and secreted by non-endocrine cells surrounding islets, Kanaka-Gautenbein, Dicou, Czerinchow et al. 1995a).

Fitzgerald (1980) suggests that islets of Langerhans may be formed from the non-endocrine cells surrounding the islets by a modified cell division such that the daughter cells escaped linkage to neighbouring epithelial cells. They underwent inversion of the

usual polarity axis of cell division so that one of the daughter cells was no longer joined to its neighbour by junctional complexes. Repetition of this process led to the accumulation of cells free from the acinar tubular structure but still surrounded by the basal lamina.

The species variation in the sequence of cellular development in organogenesis has been illustrated in studies on a variety of experimental animals (Wolfe-Coote, Louw, Woodroof et al. (1997) in primates; Wang et al. (1996), Scaglia et al. (1997) in rats, Githens (1988) citing various sources using a variety of animals, Rosenberg et al. (1996) on hamsters).

Two parallel mainstreams of thought/opinion exist on pancreatic organogenesis. The most popular maintains that during embryonic development, islet cells originate from differentiating stem cells. These stem cells are situated in the pancreatic ducts, which at E13-E15 in rat embryogenesis express cytokeratin 20 (CK20), similar to mature duct epithelium. During islet morphogenesis (E17-22) large aggregates of CK20 positive cells are formed which differentiate into endocrine cells. CK 20 positive cells within islets were not however observed in postnatal animals (Bouwens and de Blay 1996).

Cytokeratins (CK's) comprise two groups of cellular proteins present in all cells. They are usually expressed in pairs comprising a type I and II. The latter is usually 8kD larger than its counterpart. Primary CK's (CK 5, 8, 14, 18) are present in all cell types with 8/18 in simple epithelia and 5/14 in stratified epithelia. In complex epithelia (prostate) the inner luminal cell layer express simple primary CK's (8/18) and the outer layer expresses squamous primary CK's (5/14). All other CK's (1-4, 6, 9-13, 16) are secondary CK's. In squamous epithelium, as cells mature and migrate away from the basal layer the relative contribution of the secondary, differentiated cytokeratins increases at the expense of the primary simple CK's. Suprabasal layers express specific types of secondary CK depending upon the site. CK19 is a simple epithelial CK, which may also be expressed, in the squamous basal layer (Novocastra Laboratories Ltd, 1996 Product Catalogue, p39-41).

The second opinion on pancreatic organogenesis held by Gittes and Rutter (1992) working on chick embryo's, in essence agrees with the former, but asks the question "...what happens before evagination?" Their outstanding study showed that the onset

of expression in a number of pancreatic genes preceded evagination in those cells which were probably the precursor cells involved in the evagination of the duodenum to form the pancreas.

In the same study somatostatin messenger ribonucleic acid (mRNA) was first detected at eight somites (E7.5) while glucagon and insulin mRNA only appeared at the 20 somite stage (E8.5 - 9). The latter two hormones were localised to the "dorsal wall of the duodenal anlage, in the precise area that gave rise to the pancreas" while immediately adjacent cells did not contain any transcripts at the time of pancreatic evagination. Pancreatic polypeptide was detected at the 30-somite stage (E10.5) 8 to 10 hours after evagination.

While Bouwens and deBlay (1996) suggested an epithelial stem cell origin for both endocrine and exocrine pancreas tissue based on cytokeratin expression during embryogenesis, Gittes and Rutter (1992) suggested a mesenchymal origin based on endocrine hormone expression early in embryogenesis.

To summarise then, it is evident that the ductular system appears to be the first embryological structure to develop in the pancreas. All other pancreatic tissue initially (during the embryonic stage) then develops by differentiating, from duct epithelial cells (DEC's) hence the presence of the epithelial cytokeratin markers CK 7, 19 and 20 (Bouwens, Wang, De-Blay, Pipeleers, Klöppel 1994). Islets of the neonatal rat pancreas strongly expressed CK within the peripheral mantle zone that was continuous with the epithelium of the adjacent ductule, and was still present two weeks postnatally (Bouwens, Lu, De Krijger 1997). As *post partum* pancreatic remodelling occurs, acinar cells have been suggested to de- and/or transdifferentiate into endocrine cells (Melmed, Benitez, Holt 1972; Melmed 1978).

Finally, there are a number of signals that trigger and/or influence the growth and development of pancreatic tissue. The direction of development (exocrine or endocrine) is determined by the signal (Scaglia et al. 1997; Peers, Leonard, Sharma et al. 1994; Kanaka-Gautenbein, Tazi, Czernichow et al. 1995; Gittes et al. 1996; Rawdon 1984; Rawdon and Andrew 1991, 1993, 1997; Barro, Zaomi, Morel, Benhamou 1998).

A host of substances are claimed to be involved in ß-cell dynamics. Insulin is

important for optimal foetal and neonatal growth and development, and, insulin availability, ensured by ongoing islet cell growth within the pancreas. Insulin growth factor (IGF) and IGF binding proteins (IGFBP's) have been implicated as paracrine regulators of islet growth within the developing pancreas (Hogg, Hill, Han, 1994; Hill, Hogg, 1992) together with other mitogens, platelet derived growth factor (PDGF), Epithelial Growth Factor (EGF), Thyrotropin Releasing Hormone (TRH) (Hill, Hogg, 1991), mesenchymal factors (Gittes et al. 1996; see also chapter 5 paragraph 5.5.5).

The list of substances as well as their origin is varied. Kanaka-Gautenbein, et al. (1995a) are of the opinion that differentiation of pancreatic stem cells depends on factors produced by the non-endocrine cells surrounding the cells. Islet cell phenotype is not stable, and different environmental stimuli are able to induce the reappearance of cells with embryonic traits (Teitelman, 1993).

2.2.6 Islet Vascularisation

Islets contain a complex network of capillaries with fenestrated endothelium (much like the glomerular structures in the Malpighian bodies of renal units) which arise from small arterioles outside the islet and, after penetrating the islets, merge with capillaries supplying exocrine components of the pancreas (Hellerström 1984). This arrangement enables the islets (1% of pancreatic tissue) to receive a staggering 10-15% (Lifson, Lassa, Dixit 1985 – 6%) of the total pancreatic blood flow. Differential blood flow was also shown to be in effect for islets of varying size.

Lifson and co-workers showed that large islets (diameter of 140 to 300 μm) making up 72% of islet volume received 64% of islet blood flow; islets with a diameter of between 97 and 140 μm (18% of islet volume) received 21% of islet blood flow. Islets with a diameter of 77 to 97 μm (6% of islet volume) received 7% of islet blood flow. Islets with a diameter of 57 to 77 μm (3% of islet volume) received 5% of blood flow, and small islets (25 to 57 μm) making up 1.5% of islet volume received 3% of total islet blood flow.

Fenestrated capillary endothelium is a common feature of endocrine tissue. Indications (Stagner, Samols, Bonner-Weir, 1988) from islet cell perfusion studies on

dogs, are that the intra-islet micro-circulation is such that afferent arterioles crossing into islets do so through discontinuities of the mantle of non ß-cells, forming a glomerulus-like network within the ß-cell core of the islet. This arrangement makes down-stream effects from intra-islet hormones other than insulin on the ß-cells appear unlikely, rather, that the insulin content of the efferent capillaries has a modulating effect on glucagon release from alpha-cells as well as hormonal release by the remaining cell components within islets.

Bonner-Weir (1988) also showed from reconstructions from serial paraffin sections of pancreatic islet cells and the mircovasculature of the rat, that the afferent vessel to an islet, an arteriole, enters the islet directly in the ß-cell core. Immediately after entering the islet, it branches into capillaries. The ß-cells form a tube-like structure around a central capillary while the outer side of each ß-cell also abuts a capillary. A clear polarity of secretory granules was seen on the ultrastructural level providing morphological evidence of in situ ß-cell polarity that could be an anatomical basis for functional compartmentalisation.

2.2.7 Islet Innervation

Autonomic nervous system innervation of the pancreas seems to be from both sympathetic and parasympathetic components. Stevens and Lowe (1995) report innervatory twigs in contact with roughly 10% of all cells, with well developed gap junctions between adjacent cells, providing a mechanism for neural transmission from cell to cell. Blood vessels in the pancreas also have autonomic innervation, possibly affecting perfusion and blood flow. Parasympathetic stimulation has been shown to increase insulin while decreasing glucagon secretion, and the converse is true of the sympathetic divisions. Although islets present cells of differing size, Colella, Bonner-Weir, Braunstein, Schwalke, Weir (1985) found that both insulin and glucagon secretion was independent of islet size although larger islets utilised more glucose.

Koevary, McEvoy and Asmitia (1980) found that pancreatic tissue (both endocrine and exocrine) were innervated by serotonic fibres which was possibly involved in the secretory regulation of the pancreas. Myrsen, Keymeulen, Pipeleers and Sundler

(1996) report that pancreatic islets become innervated by sympathetic non-adrenergic nerve fibres (also storing NPY) and to a lesser extent by parasympathetic fibres, in renal subcapsular transplanted islet grafts. Neuropeptide Y has also been implicated in neuronal function in other areas of the body (Han, Yang, Chen et al. 1998).

The last mentioned authors found, using immunocytochemical (ICC) techniques, that the density of nor-adrenergic nerve fibres to islet grafts was often higher than in the graft bearing organ (kidney) suggesting that grafted islets contained factors that promoted the ingrowth of nerve fibres while non-ß-cell grafts remained devoid of nerve fibres. Neurons and ß-cells were shown to co-express neuron restrictive silencing factor (NRSF)/repressor element silencing transcription factor (REST). NRSF/REST is a nerve regulator of neuronal fate known to silence neuronal-specific genes in non-neuronal cells (Atouf, Czernichow, Scharfmann 1997).

Prior to this finding, Atouf, Scharfmann, Lasmezas and Czernichow (1994) found that various other neuronal factors (Prion-related Protein (PrP) mRNA) were expressed in pancreatic endocrine cells, while growth hormone (GH) and prolactin (PRL) were important regulators in the sensitivity of ß-cells to neuronal growth factor (NGF – Scharfmann et al. 1994)

2.2.8 Intermediate Cells

Cells with intermediate/transitionary character are not only found during the prenatal embryonic development of organs/organisms, neogenesis and/or anagenesis, also found to be present during experimentally induced tissue remodelling. The mature pancreas seems to possess multiple mechanisms for replacement of tissue, which includes retrodifferentiation (Scharpelli, Rao 1980) redifferentiation and transdifferentiation (see paragraph 3.1 and definition of terms on page xxxi). For example, pancreatic acinar cells have been shown to transdifferentiate toward ductal cells (Bouwens et al. 1994), hepatocytes (Wolfe-Coote, Louw, Woodroof, du Toit 1996) and so-called "acinar-islet cells" (Melmed, 1978).

Each of the sub-types in both the exocrine (acinar- and duct) and endocrine (α-, β-, δ-, and PP- as per paragraph 2.2.4) pancreatic cells, possesses intermediary transitional

cells with morphologic characteristics of more than one cell type. This is based on the immunogenicity of their cell membrane proteins and/or secretagogues, hormones or cellular matrix proteins. All of these are common to cells with a specific phenotype, but co-expressed with cells of differing phenotype (Melmed 1978; Gu, Lee, Krahl, Sarvetnick 1997; Ferrand, Astesano, Phan, Lelong, Rosselin 1995).

Dual and/or multiple immunogenicitic cells were first identified and categorised by Melmed et al. (1972) and confirmed, based on ultrastructural criteria in 1978 (Melmed 1978). Thus acinar cells co-expressing insulin were termed acinar-β-intermediates, α-cells co-expressing zymogen granules designated α (referred to forthwith without the suffix "-intermediates"). Melmed described acinar cells with endocrine and endocrine cells with acinar characteristics in a number of species. In his standard laboratory rat model however he was only able to distinguish five cell types of intermediates, namely acinar-α; acinar-β; acinar-δ; acinar-α, β; and acinar-β, δ. Acinar-β cells were found to be dominant while acinar-α and acinar-δ occurred less frequently.

Acinar-α, β were reported to appear in two forms. The first was to be found between islet and acinar cells, and the other clearly associated with the exocrine tissue. In animal species other than the rat, zymogen granules were occasionally found in β-cells within islets, duct-δ cells have been reported in *Amphisbaena*. In 1978 (Melmed 1978) only limited evidence existed of morphological endocrine cells with dual hormonal expression described by the author as α-β-intermediates (Abstract: Winborn 1963, Anat Rec 147:65-95 as quoted by Melmed 1979) in Rhesus monkeys. Subsequent research by Bouwens, Braet, Heimberg (1995) have reported cells with ductular/endocrine character and cells with dual endocrine character (Wolfe-Coote et al. 1992; Rosenberg 1998).

Ductular and islet cells with dual insulin-glucagon, insulin-somatostatin and insulin-PP immunoreactivity, ductal-exocrine and exocrine-endocrine intermediates were found to be present in foetal (Weir, Bonner-Weir 1985) and neonatal and normal adult transgenic mice (Gu, Lee, Krahl and Sarvetnick 1994). Islet growth continued throughout adulthood unlike normal animals whose islet formations ceased early in life.

In experimentally treated duct ligated (Bretelli, Bendayan 1997) or pancreatectomised laboratory animals, transitional cells have also been shown to occur. In addition to those mentioned by Melmed above, there seems to be three broad categories of

transitionary cells in the pancreas. The first are cells displaying acinar and endocrine characteristics, the second, those displaying ductal and endocrine characteristics and finally cells with dual and/or triple endocrine characteristics.

2.2.9 Endocrine Function

Islet function is directly related to the microvascular circulation within the islet. Stagner et al. (1988) proposed an order of cellular vascular perfusion of ß-cell to α-cell to δ-cell. This proposal is borne out by a paper elucidating the microvascular arrangement following pancreas perfusion by india ink (Bonner-Weir 1988). The author suggests than an afferent vessel to an islet, an arteriole, enters the islet directly in the ß-cell core and immediately branches into capillaries. Any ß-cell secretions are picked up before passing to the non-ß-cell endocrine components where their presence (ß-cell secretions) stimulates, inhibits and/or modulates secretions from the latter cell types (Samols, Bonner-Weir, Weir 1986).

Islet secretory response is mainly initiated and controlled by two mechanisms. The first is a nervous/neuronal mechanism, which has been shown to exert a strong influence under a variety of conditions. Secretion modulatory control was exerted by a variety of chemical/hormonal mechanisms. In 1981, McEvoy, Leung and Goggins reported that the secretions, by sub-populations of islet cells (non-ß-cells), were affected by corticosterone and cellular responsiveness to glucose. Subsequent studies have focused on a variety of substances, all of which affect the secretion of endocrine hormones by pancreatic islets in one way or another. Glucose was the most obvious regulator/initiator of endocrine response (Reaven, Gold, Walker, Reaven 1981).

Studies have shown that ß-cells in particular responded in a variety of ways to glucose infusion. These include an increase in mitotic index, ß-cell hyperplasia and ß-cell hypertrophy (Bonner-Weir, Deery, Leahy, Weir 1989). An increase in Islet size (due to ß-cell hyperplasia and/or hypertrophy) was not necessarily indicative of glucose utilisation or insulin release. Although larger islets (with 140 - 210 ng DNA/islet) utilised more glucose than smaller islets (60 - 120 ng DNA/islet), the

insulin secreted (per ng of DNA) in response to a given glucose concentration was the same in islets of all sizes. The insulin and glucagon content when expressed in terms of DNA does not depend on islet size.

ß-cell proliferative and/or secretory response to glucose infusion seems to have an upper and lower limit beyond which islets do not, or can not, respond. Human ß-cells for instance, when exposed to prolonged increased glucose levels lose their glucose regulation ability as a consequence of sustained cellular activation (Ling, Pipeleers 1996). On the lower end of the scale, only 50% of ß-cells exposed to 5 mmol/l glucose went into a "glucose response" state compared to in more than 90% of cells at 10 mmol/l (Ling, Heimberg, Foriers, Schuit, Pipeleers, 1998)

Beta cell function has also been shown to be positively influenced by glucagon, which induced ß-cells to produce factors mediating islet neurotrophism (Hellerström, Eizirik, Welsh et al. 1991; Keymeulen, Aselmo, Pipeleers 1997). The ability of the ß-cell to detect glucagon in the circulation when concentrated as a local signal in the islet interstitium (Moens, Flamez, van Schnawendijk, Ling, Pipeleers 1998) was also found to be a regulatory factor.

2.2.10 The Pancreatic Polypeptide Family

2.2.10.1 Introductory Comments

The three (PP, NPY, PYY) are structurally related peptides which have wide ranging effects on an organism (Goumain, Voisin, Lorinet, Laburthe 1998; Jackerott, Øster, Larsson 1996). All three peptides could be found in the circulation (Gehlert 1998) with PP found primarily in the pancreas and PYY found principally in the gut. NPY was also released into the circulation from neuronal stores in response to stress.

Organs that responded to NPY and/or PYY included the brain (St-Pierre, Dumont, Novel, Herzog, Hamel, Quirion 1998), placenta and amniotic fluid (Robidoux, Simoneau, St-Pierre, Chadli, Lafond 1998), intestine (Mulder, Ekelund, Ekblad, Sundler 1997), kidneys and blood vessels (Bischoff, Michel 1998), nerves of endocrine cells in the pancreas (Ding, Kimura, Fujimura, Fujimiya 1997). Peptide tyrosine

tyrosine (PYY) has further been shown to occur in murine pancreas, ileum and colon and was co-expressed in all four islet types when they first appeared. Colonic PYY has been identified in glucagon-expressing α-type endocrine cells, and is thought to be an early event in colonic endocrine differentiation which lends support to the view that a common progenitor to all entero-endocrine cells exists (Upchurch, Fung, Rindi et al. 1996). Pancreatic endocrine cells undergoing neogenesis exhibit/share hormone expression characteristics of neuronal cells in that both exhibit dual NPY and PYY immunoreactivity (Upchurch, Aponte, Leiter 1994).

2.2.10.2 The PP Family in the Adult

Substantial differences have been reported (Jackerott et al. 1996) in the NPY/PYY/PP secretory pattern in adult animals and neonates. NPY and PYY occurred in both the splenic and duodenal parts of the adult rat pancreas and PYY was localised together with PP to the peripheral mantle region of islets. Although most PP cells only exhibited IR for PP, many (50%) ST-IR cells displayed dual immunoreactivity for both ST and PYY, as did cells with GIR, although to a lesser extent. Cells with IIR showed no evidence of PYY-IR (Jackerott et al. 1996).

2.2.10.3 The PP Family during Embryonic Development

2.2.10.3.1 Pancreatic Polypeptide

The appearance of the PP-family, peptide tyrosine tyrosine (PYY), neuropeptide Y (NPY) and pancreatic polypeptide (PP) during embryonic development has been an issue of dispute between various researchers for more than ten years. The dispute centres around firstly the cross reactivity of anti sera used to tag the various peptides cross-reacting with one another, and secondly, microscopic sections that were either too thick likely comprised more than one cell, giving the impression of dual immunoreactivity (Herrera, Huarte, Sanvito et al. 1991).

Herrera et al. (1991) contends that PP/glucagon secreting cells are more likely to represent pluripotent stem cells than glucagon/insulin secreting cells. To substantiate

their finding Herrera et al. (1991), used three PP-antisera from different suppliers to detected PP in murine (NMRI, BALB/C and CD-1 mice) embryonic pancreas at E10.5. mRNA analysis of their finding revealed the presence of PP mRNA co-localised with glucagon in pancreatic buds,. This was in contention with the finding of glucagon and PYY dual immunoreactivity by Alpert, Hanahan, Teitelman (1988) and reported in adult rat pancreas by Polak et al (1994). Teitelman et al. (1993) in turn contended that the findings of Herrera et al. (1991) could be ascribed to cross reactivity of the primary anti-sera, especially to bovine PP, to either NPY and/or PYY. The study once again reaffirmed their previous finding of the presence of insulin and glucagon in mouse embryonic foregut on day 9.5.

Shortly after this, Wolfe-Coote et al. (1996) published the findings of their investigation into the embryonic lineage development of the Vervet monkey (*Cercopithecus aethiops*). The group found co-localisation of glucagon and PP preceded by somatostatin and PP in the primitive gut at five weeks gestation. They also found evidence to suggest that both primordial buds had the potential to form all pancreatic endocrine cells (Wolfe-Coote, Louw, Woodroof, du Toit 1990b).

In the pig, between days 50 – 70, foetal pancreatic cells contained secretory granules with all four major pancreatic hormones. The first true mono-hormonal cell was found to contain PP followed by ST. Mature insulin and glucagon containing cells were only found to be present after birth (Lukinius, Ericsson, Grimelius, Korsgren 1992).

On this aspect Herrera, Huarte, Zuffery et al. (1994) suggest that there was an ontogenic relationship between α- and PP-cells with PP occupying an unexpected place in the lineage of endocrine cells. The study also found that the removal of glucagon or insulin gene-expressing cells did not affect the development of other endocrine cells, while embryo's lacking PP gene expressing cells did, however, not lack glucagon cells.

The co-localisation of glucagon and PP was found in foetuses in which duct in pancreatic buds had budded to form early islets displaying dual immunoreactivity for glucagon in one clearly defined area, expressed glucagon predominantly whilst in a different area, (often adjacent areas) they expressed predominantly PP (Wolfe-Coote et al. 1990a, 1990b).

In 1996, Wolfe-Coote et al. determined, by immunocytochemical staining of cellophane wrapped vervet monkey pancreas, that a number of proliferating ductular epithelial cells separated from the ducts and differentiated to become predominantly PP or somatostatin secreting cells. The paper reported that PP and δ-cells had been found earlier than glucagon or insulin in pancreatic ducts during organogenesis.

2.2.10.3.2 NPY and PYY

Various studies have also highlighted the difference of opinion regarding NPY and PYY. In the developing murine pancreas Jackerott et al. (1996) reported PYY expression first observed at E11.0 and occurred/appeared before either PP or NPY while PP was totally absent from the pancreas until E20.5. NPY was only detectable at E16.5. The PYY cells at E11.0 also expressed glucagon although some expressed only PYY. By E11.5 a number of cells co-expressing glucagon and PYY were also insulin positive.

This triple expression however declined from E14.5 as cells with IIR gradually lost their dual GIR and PYY-IR while cells co-storing insulin and PYY (but not glucagon) as well as cells with IIR only appeared. Between E14.5 and E18 the number of cells with glucagon/PYY-IR declined and cells with insulin/PYY-IR disappeared totally by birth.

Somatostatin was first detected at E16.5 and PP at E20.5. These cells were also PYY immunoreactive but declined from birth. PYY was found to be the earliest detectable and most widely spread hormone of the PP-family in the developing pancreas of the rat. All early endocrine cells contained PYY-IR of which the majority were also glucagon positive. Cells with dual PYY/IR included ST and PP cells as well as insulin cells (Jackerott et al. 1996).

Teitelman et al. (1993) reported that early endocrine progenitor cells to pancreatic islets (mouse embryo's E9.5) first expressed insulin and glucagon, with many cells co-expressing both hormones (compare with Gittes and Rutter 1992). Neuropeptide Tyrosine (NPY) also first appeared with insulin and glucagon (at E9.5) and was co-expressed with glucagon in the majority of mature (adult) α-cells.

This finding is however contested by Upchurch, Aponte, Leiter (1994) and Jackerott et al. (1996) who maintain that Peptide Tyrosine Tyrosine (PYY) could be identified in the earliest endocrine cells in the foetal pancreas. PYY was co-expressed by each islet cell type during development with a high degree of co-localisation with insulin and glucagon producing cells.

PYY secretory cells were however found to be absent in adult rat cells. Upchurch and co-workers did however also concede that PYY was expressed in less than half of α-cells and not at all in ß-cells and was also co-expressed with somatostatin and pancreatic polypeptide when these cells first appeared and most δ- and PP-cells continued to express PYY throughout development.

2.3. EXPERIMENTAL MODELS IN PANCREATIC CELL LINEAGE RESEARCH

2.3.1 Historical Overview

Work done prior to 1988 on especially pancreas regeneration has focused on developing a viable functioning model in which transplanted tissue was able to grow and develop with a view to restoring and maintaining normoglycaemia after pancreatectomy. Du Toit, Heydenrych, Smit et al. (1987b) showed that pancreatectomised baboons produced a reliable diabetic model, which was uniformly lethal if left, untreated. This model then served as a basis for a series of transplantation studies starting with autotransplantations (Du Toit, Heydenrych, Louw et al. 1987b) into pancreatectomised baboons, resulting normoglycaemic experimental animals.

Transplanted tissue at six weeks consisted of atrophied exocrine pancreas, graft fibrosis and mononuclear infiltration while endocrine tissue (α-, ß-cells and islets) remained unaffected.

Prior to establishing this viability, Du Toit and his co-workers conducted a series of segmental allograft transplantation experiments on baboons with (Du Toit, Heydenrych, Louw et al. 1983b, 1984b) and without (Du Toit, Heydenrych, Louw et al. 1983a, 1984a) Cyclosporin A (CsA). Although normoglycaemia was established, transplant recipients were however glucose intolerant, exhibited low K-values and were hypoinsulinaemic.

Segmental pancreatic allograft recipients were then treated with total body lymphoid irradiation (TLI) and pre-operative blood transfusion (Du Toit, Heydenrych, Smit et al. 1984c) and later combined with CsA (Du Toit, Heydenrych, Smit et al. 1988a) which in combination (CsA & TLI) gave significant segmental allograft survival compared with only modest allograft survival observed in dogs on CsA only (Du Toit, Heydenrych, Smit et al. 1985 & 1988b; Du Toit, Heydenrych 1988). It is perhaps of note to mention at this stage that a very low success rate was obtained in studies involving heterotopic intraperitoneal segmental pancreatic allotransplantation into pancreatectomised primates treated with TLI only (Du Toit et al. 1984c, 1987). Having established a working transplantation model the time had come to evaluate

transplantation success in terms of metabolic function as well. In 1987 Du Toit & co-workers (Du Toit et al. 1987a, 1987b, 1987c, 1987d) published a series of articles on their work in which they showed that segmental and whole pancreatic allotransplantations did not improve the general metabolic profile of hemipancreatectomised baboons. They also found that glucose tolerance was not directly related to insulin or glucagon release as pancreato-duodenal-splenic allotransplantation recipients displayed normal glucose tolerance in the presence of hypoinsulinaemia, hyperglucagonaemia and reduced C-peptide values.

Insulin release was found to be affected by TLI resulting in glucose intolerance and impaired K-values although remaining normoglycaemic. This finding has been consistently reaffirmed by researchers worldwide (Tyrberg, Eizirik, Hellerström et al. 1996).

Although it has been possible then, to ensure graft survival, these studies have shown that segmental transplantation did not restore metabolic function. Glucose, insulin, glucagon and C-peptide responses were tested under GTT conditions (Du Toit et al. 1988a) at 100 days post transplantation, without a favourable prognosis (Du Toit, Heydenrych, Smit et al. 1988c). Having exhausted this avenue of endeavour various subsequent studies conducted have focused on alternative experimental models with feasible application and transferral to the clinical setting.

In the last five years, great advances have been made in immunesuppressants enhancing post-transplantation graft survival. FK506, Mycophenolate Mofetil (MMF) and CsA (Du Toit, Muller, Page et al. 1998) have been used in conjunction with one another and this has brought about a significant improvement in especially long-term graft survival which appears to be related to post-transplantation protection offered by new agents. As surgical technique became less traumatic and post-operative care improved, renal-pancreas transplantation recipients exhibited improved metabolic function and prolonged organ retention, currently in excess of ten years (Najarian, Greussner, Drangsteveit et al. 1998). Not only was there a reversal of IDDM in that patients no longer required exogenous insulin but the secondary complications induced by DM were negated by persistent euglycaemia (Sutherland, Greussner, Greussner 1998)

The isolation and transplantation of foetal and adult islet tissue into a number of sites in

the body (renalsubcapsular, testicular sac, intraportal, interventicular, intraocular, to mention a few) in rats, mice, hamsters, dogs, pigs and primates has been going of for some time. Currently studies are focused on whole foetal pancreas transplantation and mechanisms involved in inducing pancreatic endo- and exocrine regeneration. Hence the use of Streptozotocin, Alloxan and other similar ß-cell toxins (Weir, Bonner-Weir 1997) to pancreatic duct ligation, cellophane wrapping, squeezing and stroking.

Researchers are aware that the pancreas had the capacity for postnatal growth and development (Cantenys, Portha, Dutrilluaz et al 1981, Wang et al. 1996) although studies have shown that species differences do exist in susceptability to ß-cell damage (Eizirik, Pipeleers, Ling et al. 1994). Neogenesis depended on the presence of active though dormant stem cells while replication depended on the replicative ability of cells, which has been shown to be limited (Bouwens and Klöppel 1996, Pipeleers 1992).

Studies conducted on the regeneration of the liver and transdifferentiation of hepatic oval cells led to the discovery that it was possible to chemically induce existing pancreatic cells to transdifferentiate into pancreatic oval cells (Ide, Subbarao, Reddy, Rao 1993). Pancreatic carcinogenic cells were shown to develop from cells that exhibited increased mitotic indices (Rao, Subbarao, Scarpelli 1987). This led to an investigation into the triggers and mechanisms involved in the differentiation of pancreatic acinar cells (Rao, Divivedi, Subbarao et al. 1988) and the discovery that hepatocyte development could be induced in animals. (Rats fed a copper deficient diet, intraperitoneal administration of TCDD (2,3,7,8 tetrachlorobenzo-P-dioxin) and hamsters (Rao, Subbarao, Scarpelli 1988).

Cells in the homeostatic environment of a complex organism (animal) are subjected and respond to influences from the cellular environment (Keymeulen, Korbutt, de Paepe et al. 1996; Rosenberg, Duguid 1990) which include hormones (Wang, Bonner-Weir, Oates et al. 1993), cytokines (Gittes, Rutter 1992), growth factors (Hu, Evarts, Fujio, Mardsen, Thorgeirsson 1995; Jeffers, Rao, Rulong et al. 1996; Rosenberg, Vinik 1993; Zhang, Siegel, Odenthal et al. 1997; Hill, Hogg 1992) and interaction with the intercellular matrix (Gittes et al. 1996).

Different types of cells respond in different ways to these changes by either differentiating (Elässer, Lütcke, Kern, 1986; Githens 1993), proliferating (Githens

1988; Teitelman 1993), functioning and/or undergoing apoptosis (Doi, Wada, Hosotani et al. 1997; Paraskevas, Duguid, Maysinger et al. 1997; Scaglia et al. 1997; Wada, Doi, Hosotani, Lee, Imamura 1995; Walker 1987), or maintaining the status quo.

Certain types of cells, however, are capable of undergoing transdifferentiation (Scarpelli, Rao 1980) and/or neoplastic transformation (Teitelman 1993). These often involve several stages including dedifferentiation, proliferation, redifferentiation and/or direct transdifferentiation without cell division, both *in vivo* and *in vitro* (Bertelli, Bendayan 1997; Teitelman, Lee, Reis 1987).

The presence of cells with intermediate phenotypes under a variety of conditions have been reported by Alison, Golding, Lakins et al. (1997); Blakomer, Jaskeiwicz, Dunsford, Robson (1995); Bouwens et al. (1995); Bouwens et al. (1994); Lopez, Upchurch, Rindi, Leiter, (1995); Pang et al. (1994). Available evidence would seem to suggest that pancreatic acinar cells are able to transdifferentiate to ductal cells (Bouwens et al. 1994) and so-called "acinar-islet cells" (Melmed 1978), while the presence of hepatocytes has been reported in cellophane wrapped vervet monkey pancreas (Wolfe-Coote et al. 1996).

The organs of the hepato-pancreatic-biliary tract (HPBT) have been known to have the dormant capacity to undergo regeneration after partial removal (hepatectomy, pancreatectomy) and/or damage (cirrhosis, pancreatitis). This is thought to occur via a population of pluripotential stem cells, residing within the organ, which, when exposed to the correct stimulus, could develop into hepatocytes, acinar cells or pancreatic endocrine tissue (also see section on Oval Cells).

Ide et al. (1993) have suggested that rat pancreatic oval cell cells could be precursors for the pancreatic hepatocytes which have been found to appear in experimentally manipulated pancreas undergoing regeneration. Wolfe-Coote et al. (1996) however found this to be the exception rather than the rule. Rosenberg and Vinik (1989) were able to induce a trophic stimulus, by placing a cellophane wrap around the head portion of the pancreas, which led to ductular proliferation which was associated with neogenesis (Rafaeloff, Qin, Barlow, Rosenberg, Vinik 1996). Areas of neogenesis contained a predominance of ß-cells and smaller numbers of α- and δ-cells. Cellophane wrapping also brought about a 2.5X increase in the number of islets present

in each square millimetre of tissue.

2.3.2 Pancreas replication, regeneration and proliferation

2.3.2.1 Overview

Scientists generally agree that the pancreatic ß-cell has a limited replicative ability, hence the inability of the diabetic patient to maintain normoglycaemia. Evidence suggests that any source of ß-cells, other than by replication, must originate from an alternative site to the pancreatic islet.

In the late 1980's and early 1990's the general consensus was that isolated and/or individual ß-cells existed within the duct epithelium (Githens 1988) which replicated to give a continual source of new ß-cells. Sarvetnick and Gu (1992) and Gu and Sarvetnick (1993) modified this idea. They proposed that new islet cells were continuously forming from duct cells, which was evidenced by (a) proliferation of duct-cells, (b) the appearance of primitive cells (oval cells) and (c) their subsequent differentiation into islet (endocrine) cells under experimental conditions (also Bouwens and Klöppel 1996) with the induction of interferon-g (IFH-g) gene activity which stimulated islet neogenesis similar to embryonic islet morphogenesis (Gu and Sarvetnick, 1993).

Bouwens and Kloppel (1996) claim that beta cell population of the endocrine pancreas may expand by either of two processes, neogenesis or replication. While replication infers the division of already differentiated ß-cell that is, in humans, very limited (Bouwens, et al. 1997), neogenesis depends on the presence of active stem cells. Since replicative activity of highly specialised cells such as ß-cells is limited, the potential for the endocrine pancreas is at present a topic of exhaustive study. Factors affecting the morphogenesis of both endocrine and exocrine pancreatic tissue from ductal epithelium is however not clearly understood (Rooman, Schuit, Bouwens 1997).

Recent developments indicate vascular epithelial growth factor (VEGF) and hepatocyte growth factor (HGF - Lefebvre, Otonkoski, Ustinov et al. 1998) at the site of duct cell proliferation but not of ß-cells.

During embryonic development and also in early neonatal life (Scaglia et al. 1997) pancreatic islets of Langerhans originate from differentiating epithelial stem cells. These stem cells are situated in the pancreatic ducts, but are otherwise poorly characterised. Immunohistochemical staining has shown (Bouwens, De Blay 1996) that protodifferentiating pancreatic epithelial cells from rat embryos of day 13 to day 15 expressed the cytoskeletal protein keratin 20 (CK20), similar to mature duct epithelium. During the period of islet morphogenesis, which occurs between day 17 and birth, large aggregates of CK20-positive duct cells are formed which gradually differentiated into endocrine cells. Various studies (Wang et al. 1996) have also shown that the neonatal pancreas undergoes further growth and development even in the face of subtotal ß-cell destruction by streptozotocin (STZ), but that growth ceases between ten and twenty weeks later.

2.3.2.2 Normal (in vivo) endocrine replacement

The extent to which adult pancreatic ß-cell mass can respond to in vivo glucose stimulus by increasing their mass through either hyperplasia or hypertrophy remains unanswered. Bonner-Weir et al. (1989) showed that short-term (96 hr) hyperglycaemia, by glucose infusion, enhanced replication in rats as evidenced by a five-fold increase in the mitotic index of ß-cells. This enhanced replication of ß-cells led to an increase in cell number, hyperplasia and hypertrophy of ß-cells. Mean cell volumes were determined from the mean cell cross-sectional area measured planimetrically from low-magnification electron micrographs, increased to 150 percent of normal controls after 96 hours of 50% glucose infusion. ß-Cell masses had not returned to normal after a further 96 hours, although the mitotic index had declined significantly, although not to those of normal non-infused rats or of saline infused controls.

Bouwens, et al. (1997) made an exhaustive study of embryonic pancreas development in humans. Examining the foetal pancreas at 12 to 41 weeks for cytokeratin 19 (CK 19); (CK 19 and 20 are only expressed in ductal cells from centro acinar to main ducts; CK 7 by islets and duct cells from main-; inter- and intralobular ducts; CK 7, 19 & 20 in proliferating duct cells during tissue regeneration and/or different periods of culture,

Bouwens et al. 1995) synaptophysin, insulin, glucagon, somatostatin and pancreatic polypeptide and a cell proliferation marker (Ki-67), they showed that the most actively dividing cells were ductal cells, although other cell types also bound Ki-67 but to a lesser extent. The study furthermore reports the existence of an intermediary cell population expressing synaptophysin but no islet hormones. As gestational age increased, the Ki-67 labelling index (LI) decreased (17% to 4%) for duct cells and (9% to 1%) for synaptophysin positive cells. From weeks 12 to 19 all cells expressed CK19 after which it disappeared from most islet cells but remained in duct cells. The study showed that transitional differentiation occurred in duct cells on their way to endocrine cells.

In rats at gestational age E13 to E15 pancreatic epithelial stem cells also expressed CK20 (similar to mature duct epithelial cells). Bouwens, De Blay (1996) and Bouwens (1998a) further found the E17-20 gestational period to be one of islet morphogenesis in which large clusters of CK 20 positive duct cells were present which gradually differentiated into endocrine cells. These CK 20 positive cells were identified as pancreatic islet stem cells. Regeneration of neonatal ß-cells in laboratory rodents was due to replication of pre-existing intra-islet ß-cells and/or extra islet precursor cells and has been shown to not occur in primates. In normal rats Wang, Bouwens, Klöppel (1994) found a three-fold increase in total ß-cell volume during the first five days of life with no further expansion until day 20. 5-Bromo-2-deoxy Uridine (BrdU) labelling indicated 2-3% intra-islet and 15-20% extra-islet ß-cells proliferating. Scaglia, Smith, Bonner-Weir (1995) and Githens, Pictet, Phelps, Rutter (1976) reported that ß-cell mass and size declined during a four-day period in post partum female rats which returned to normal by ten days post partum.

The authors mentioned above found the mechanism of ß-cell loss to be apoptosis (programmed cell death) leaving them to conclude that ß-cells have the ability to down-regulate their mass using mechanisms of changes in rates of cell replication and death and changes in ß-cell size to achieve homeostasis of functional endocrine tissue.

The development of endocrine cell types within the pancreas is thought to involve the "progressive restriction of pluripotential stem cells, which gives rise to the four different types of endocrine cells" (Peers et al. 1994). The development of pancreatic endocrine growth involves what Rosenberg et al. (1996) referred to as paracrine and/or

autocrine regulatory mechanisms.

One such factor, a putative insulin gene transcription factor in ß-cells (STF-1, Guz, Montiminy, Stein et al. 1995) was found to be expressed by most ß-cells, subsets of other islet cell types as well as mucosal epithelial cells of the duodenum, pancreatic duct epithelial cells and pancreatic exocrine cells. BrdU labelling indicated glucagon containing cells within proliferating ducts, duct epithelial cells expressing the neuroendocrine markers NPY and PYY; the home-box gene product insulin promoter factor-1 (IPF-1, O'Reilly, Gu, Sarvetnick et al. 1997).

On the one hand, ß-Cell regeneration is thought to occur by budding of new islets from ducts with acinar cells (Gu et al. 1997, Smith, Bonner-Weir, Leahy et al. 1994) as possible precursors of islet cells with transitional cells harbouring both exocrine and endocrine granules, and new ß-cells being produced by the formation of new islets from small pancreatic ducts. This experimentally induced process is thought to recaptulate ontogeny (O'Reilly et al. 1997). On the other hand, ß-cell birth can also be achieved by firstly, differentiation from undifferentiated precursor cells (neogenesis) and secondly, replication (enhanced replication) Corbett et al. (1997) of ß-cells. Precursors have already been identified, and briefly include, duct epithelial cells, acinar cells (which are able to transdifferentiate) and pancreatic oval cells, all of which respond to islet neogenesis associated protein (INGAP – Rafaeloff, Pittenger, Barlow et al. 1996); prolactin (Brelje, Parsons, Sorensen 1994); insulin like growth factor (IGF - Hill, Hogg 1992); hepatocyte growth factor (HGF - Jeffers et al. 1996); nerve growth factor (NGF - Kanaka-Gautenbein et al. 1995a; Kanaka-Gautenbein et al. 1995b); glucose (Ling, Kiekeus, Mahler, Schuit et al. 1996a; Ling, Pipeleers 1996b); endothelial growth factor (Rooman et al. 1997); pancreatic gastrin (Wang et al. 1993); clusterin (Min, Jeong, Kang, Crabo et al. 1998); matrix metalloproteinase (Barro et al. 1998). See also figure 5.6 on page 187.

Under normal conditions, then, there is a steady turnover of ß-cells in response to the changes in body metabolic requirements. ß-Cells are not terminally differentiated but in an ongoing dynamic state of change (replication, hyperplasia, apoptosis, differentiation) in order to compensate/adapt to the insulin demands of the body. The slow but steady turnover is maintained by balancing ß-cell birth with ß-cell death (Rosenberg 1998).

2.3.2.3 Pancreatectomy

As in the liver, where hepatectomised animals and patients experienced hepatic regeneration (Kahn, Hickman, Terblanche 1988; Blakomer et al. 1995; Factor, Radava, Thorgeirsson 1994; Hu et al. 1995; Novickoff, Yam, Oikava 1996; Steinberg, Frank, Odenthal et al. 1997) the pancreas underwent regeneration after pancreatectomy (Brockenborough, Weir, Bonner-Weir 1988; Elässer et al. 1986, Zhang, Bonner-Weir, Lee et al. 1997a).

Regeneration in the liver is commonly thought to originate from a dormant population of hepatic oval cells (see section 3.3, page 49, on oval cells) which undergo differentiation and proliferation (Kahn et al. 1988) under the influence of a variety of chemical stimuli (Radaeva, Steinberg 1995; Richards, Yoder, Isfort et al. 1997; Tain, Smith, Yeoh 1997; Tee, Kirilak, Huang et al. 1994; Yang, Faris, Hixson 1993; Yasui, Miuri, Terada et al. 1997; Zang et al. 1997b). As is the case in the liver, the pancreas also is capable of both endocrine and exocrine regeneration from duct epithelial cells, single ß-cells in ducts and pancreatic oval cells as already mentioned in the preceding section.

In a paper published in 1988, Brockenborough et al. reported a dramatic increase, within 7 days, in pancreatic remnant mass in 90% pancreatectomised experimental animals (Sprague-Dawley rats) over sham operated controls. The increase in tissue mass occurred between days three and seven, and endocrine tissue which showed a three-to-fourfold increase in mitotic activity of cells (mitotic index, MI) over controls. After fourteen days exocrine cells had only a slightly elevated mitotic index, while that ß-cells was double that of controls. By day 21 there was no difference in MI between endo- or exocrine tissue but endocrine tissue still had an MI double that of controls.

The trophic factor involved in this cellular proliferation was found to be insulin-like growth factor-1 (IGF-1, Smith, Rosen, Villa-Komaroff et al. 1991) which was identified as early as day three in rats that had undergone 90% pancreatectomy. IGF-1 levels showed a fourfold increase by day three and gradually returned to normal by day 14. This IGF-1 increase then explains the increase in MI observed by Brockenborough et al. (1988) by day seven post pancreatectomy. The study found that IGF-1 was localised to capillary endothelial cells in normal rats but was localised to areas of

regeneration, epithelial and connective tissue cells, in pancreatectomised animals suggesting that IGF-1 may play an important role in growth and differentiation of pancreatic tissue.

Bouwens et al. (1995) found cytokeratin 7, 19 and 20 expression in islet and duct cells from main, inter- and intralobular ducts but not centroacinar or terminal duct cells in pancreatectomised rats indicating that surgical interference (pancreatectomy) brought about both exocrine and endocrine element regeneration from cells with a dominant predisposition to transdifferentiate to give rise to tissue lost by pancreatectomy. As has already been indicated (Paragraph 2.2.9 and 2.3.2) any form of chemical or mechanical stimulus can induce this process.

The microscopic events that occur in both 90% pancreatectomy and duct ligation have been shown to be similar. The study reports that all proliferation can first be observed in the common pancreatic duct and rapidly spreads to main ducts to progressively smaller ducts to finally small ductules (Walker, Winterford, Kerr 1992). The morphological character of the ducts are described as firstly, the common hepatic duct, described morphologically in terms of exhibiting more than a hundred cells in circumference. The duct is characterised by small evaginations, which lead to small blind outpockets or junctions with main ducts lined with columnar epithelium accompanied by large amounts of connective tissue. The second occurs in the main and large interlobular ducts with a cell circumference of between twenty and a hundred cells possessing low columnar or cuboidal epithelium with moderate amounts of surrounding tissue. Thirdly, small interlobular and intralobular ducts with five to ten cuboid cells in circumference associated with small amounts of connective tissue; and lastly, small ductules found clumped together in loose connective tissue having five to ten cuboidal cells in circumference.

Ninety- percent pancreatectomies were performed on laboratory rats by Brockenbrough et al. (1988). The results showed that extensive regeneration of both exocrine and endocrine components occurred within a relatively short period of time. By day seven the pancreatic remnant weighed more than the anatomically equivalent tissue in controls. At three and seven days post-operatively, both exocrine and ß-cells had mitotic indices (MI's) three to four times that of sham-operated controls. At two weeks the exocrine cells had a slightly elevated mitotic index while that of ß-cells was still

double the control group. By three weeks MI's for exocrine tissue was similar between experimental and control groups while that for ß-cells persisted.

2.3.2.4 Pancreatic Duct Ligation (PDL)

A number of variations on the duct ligation model first described by Hulquist and Johnsson in 1965 have produced a variety of results. The first variation on their occlusive ligation model involved pancreatic bisection (Idezuki et al. 1968). This resulted in severe atrophy and fibrosis of exocrine tissue secondary to ductal congestion finally leading to endocrine insufficiency by 28 weeks. In 1987 Walker published his findings on a ligation model where two silk ligatures were tied about the pancreas at the level of the superior dorsal pole of the spleen, and the parenchyma between the ligatures divided. The procedure resulted in acinar deletion by apoptosis over a five day period following initial mononuclear infiltration, cytoplasmic vacuolation and necrosis which disappeared after 24 hours. No change in the endocrine compartment was described.

Ligation of the pancreatic ducts of the duodenal segment, one to two millimetres distal to the choledochoduodenal junction, along the entire length of the common bile duct was the next model, described by Yamaguchi et al. 1993. Their results include acute pancreatitis, an absence of biliary stasis, interstitial oedema with ductal dilation after 12 hours, focal parenchymal necrosis and polymorphonuclear infiltration that peaked at day four and then receded. Acinar deletion occurred from days three through 28 while islets were well preserved and did not show any degenerative or necrotic changes.

The work of Yamaguchi et al. (1993) was followed by a non-occlusive 2-0 silk tie placed around the splenic lobe at the junction of the splenic and gastric lobes of the pancreas by Zenilman et al. in 1995. This non-occlusive wrap resulted in mild pancreatitis in less than one half of the experimental animals, ductular proliferation accompanied by mild limited acinar deletion. Islets underwent hyperplasia and the formation of new endocrine tissue (insulin producing) occurred as single cells extending over a 56-day period resulting in a one percentage increase in endocrine tissue.

Concurrently to this study, was the ligation of the pancreatic ducts of the gastric and splenic segments (as described by Hulquist and Jonsson in 1965) performed by Wang et al. (1995). Their results indicate acinar replacement by ductal tissue, a significant increase in β-cell volume due mainly to hyperplasia and an increase in small islets, islet clusters and scattered single islet cells suggestive of neogenesis.

Two years ago (1997) Bertelli and Bendayan reported the appearance of cells with transitional characteristics resulting from duct ligation studies on SD rats. As is the case of this thesis study, the ligation was performed as reported by Hulquist and Jonsson (1965). Bertelli and Bendayan showed that acinar cells that were undergoing involution displayed signs of apoptosis, cellular disruption, accumulation of zymogen granules, swollen endoplasmic reticulum and large autophagic vacuoles. Some endocrine β-cells displayed some zymogen-like granules and were located either at the periphery of the islets or within their core.

Rosenberg described a further variation on the original duct ligation model of Hulquist and Jonsson (1965) which involved partial duct obstruction, in 1998. These techniques was similar to that set out by Yamaguchi et al. (1993) and involved a cellophane wrap rather than silk placed around the duct and surrounding parenchyma at the point of the confluence of the pancreatic and common bile ducts. Rosenberg et al. (1998) found evidence of transdifferentiation of endocrine cells with three patterns of endocrine differentiation. The first consisted of mature islets in which glucagon, insulin and somatostatin were expressed; the second of foci of new islet formation in which only one islet hormone was expressed, usually either insulin or glucagon; and finally individual cells in the ductular epithelium staining for glucagon or insulin.

Ligation (a tight surgical stitch) placed over the main pancreatic duct before it joins the bile duct (see Materials & Methods Chapter 3) acts as a trigger to initiate a series of changes (Merriam, Webster, Joehl 1997) which will bring about the development of both endocrine and exocrine cells from duct epithelial cells which is said to recapitulate embryological development.

Apart from the microscopic changes occurring at a cellular level discussed in the previous section, a number of studies have focussed on the factors that induced neogenesis as well as the stages through which duct cells go in order to become either

endocrine cells and/or new acinar cells (Edström and Falkmer 1967). These factors will be discussed later.

Although cell replication is recognised to be one mechanism by which cells, especially endocrine cells, can increase in number, it did not account for the substantial transformation and one sided increase of islet tissue found to be the case in duct ligated animals. PDL is followed by total acinar cell deletion (Abe, Watanabe 1995; Wada, Doi, Hosotani, et al. 1997) due to pancreatitis (Yamaguchi, Matsumo, Goto et al. 1993; Merriam, Wilcockson, Samuel et al. 1996) characterised by mononuclear and macrophage infiltration (inflammation). The resulting tissue oedema causes the pancreatic volume to increase (Watanabe, Abe, Anbo, Katoh 1995). Acinar cells showed an accumulation of zymogen granules, associated with hyperamylaseaemia (Merriam, et al. 1996) and the cessation of secretory function associated with endoplasmic reticulum disorganisation and the suspension of granule formation (Abe, Watanabe 1995; Isaksson, Ihese, Lundquist 1983).

Cytoplasmic condensation and pyknotic figured nuclei, which were thoroughly deleted by macrophages (Watanabe et al. 1995), were described as acute necrotising pancreatitis by Samuel, Wilcockson, Regan et al. (1995). These changes before apoptosis are described as being irreversible (Abe, Watanabe 1995).

Near total deletion of the acinar pool is thought to create severe expansion pressure on oval and ductular cells to fill the vacuity (Rao, Reddy 1995). Ligation induced acute pancreatitis is ascribed (Merriam et al. 1996) to intra pancreatic protease activation. Disappearance of the acinar tissue took about five days (Doi et al. 1997; Abe, Watanabe 1995 report seven days) but is preceded by an increase in mitotic index from twelve hours post-PDL but succumbed to apoptosis (Wada et al. 1995; Wada et al. 1997). Gukovskaya, Perkins, Zaninovic et al. (1996) who found that the chief mechanism of acinar cell deletion in opossums was predominately necrosis and not apoptosis as in the case in rats (Doi et al. 1997) reported a species difference.

While acinar deletion was progressing, factors (apoptosis of acinar cells - Abe, Watanabe 1995) to bring about the proliferation of duct cells (duct epithelial cells, Rao, Reddy 1995) and pancreatic oval cells were already set in motion. BrdU pulse labelling (Yamaguchi et al. 1993) indicated a proliferation of duct epithelial and "wall

cells" of interlobular pancreatic ducts by days one and two post-PDL. The void left by the deleted acinar cells was found to fill with ductal complexes (Wang, Klöppel, Bouwens 1995) who also reported a significant growth of islets. They confirmed, with ICC, that the ß-cell population doubled within seven days. These cells, called intermediate pancreatic cells (which appeared within 24 hours) by Bertelli and Bendayan (1997), simultaneously displayed endocrine and exocrine phenotypes expressing insulin and amylase, suggesting that acinar cell apoptosis was not the only option for cells to take but that transdifferentiation (with acinar cells becoming duct/oval cells could also be a route cells followed, although these cells were present in the acinar parenchyma and islets.

Wada et al. (1997) is however of the opinion that duct proliferation and interstitial fibrosis followed total acinar deletion by apoptosis. The mechanism of duct proliferation post-PDL, they reported to be via Bcl-2 (anti-apoptosis protein) and PCNA (cell cycle related protein). PCNA expression appeared in ductules and centroacinar cells very soon after PDL while Bcl-2 expression increased in duct cells as acinar cells were disappearing.

It would seem as if there could be four sources for the formation of new islets. The first, the existing islets themselves as reported by Rosenberg et al. (1996), Rosenberg, Rafaeloff, Clas al. 1996a; 1996b. They report an increase in the mass of existing islets, (described as "increased mitogenic activity causing hyperplasia") and single cells with ductular characteristics, expressing the duct cell markers CK19 and 20. The single cells seemed to evaginate out from small ducts to establish single cells, nests of cells and new islets all staining (ICC) for both insulin and glucagon.

Further sources are said to be duct epithelial cells, transdifferentiated acinar cells and dormant pancreatic oval cells. Wang et al. (1995) confirms the finding of an increase in islet size reported by Rosenberg et al. (1996) and the dramatic increase in ß-cell population, in the form of small islets and islet clusters, (doubles within seven days) confirmed with ICC techniques. The aforementioned authors also report an increase in the ß-cell population especially in the ligated tail portion of the pancreas.

Wang, Rehfeld, Nielsen, Kloppel (1997) found gastrin and transforming growth factor-α (TGF-α) expression by ß-cells in the first days post-PDL. The study speculates on

the role that they might play as growth factors in islet neogenesis. The induction of gastrin and TGF-α expression preceded a peak in the BrdU pulse labelling index of ß-cells known to occur on day seven. A year earlier Rosenberg et al. (1996b) reported that cytosolic extracts from duct ligated pancreas administered intraperitoneally to laboratory animals induced an increase in cell mitogenesis of ductular and islet cells which brought about an increase in islet mass. Not only do ductular complexes replace acinar tissue but also there is an increase (from mitosis) in the number of duct lining cells. PCNA expressing was found in duct tubular complexes, ductules and centro-acinar cells (Wada et al. 1997).

Wang et al. (1995) reported the existence of transitional cytodifferentiated forms between duct cells expressing CK20 and ß-cells expressing insulin or α-cells expressing glucagon and PDL induced expression of ß-cell specific glucose transporter type-2 (Glut-2) in duct cells.

It would appear as if there are cells (oval cells) present in the HPBT, which under normal circumstances, are in a slow mode of differentiation. Together with existing endocrine cells they are thought to be responsible for a continual replacement of, or addition to, cells in the endocrine compartment of the pancreas.

When the pancreas is however disturbed (mechanically by PDL, cellophane wrapping or handling and/or chemically by a change in circulating glucose, insulin deficiency, glucagon excess, growth hormone (or any one of a host of substances already mentioned) a new generation of endocrine tissue, in particular develops. These are derived from the transdifferentiation of pancreatic duct epithelial cells, acinar cells, endothelial cells and islet capsular mesenchymal cells (Gittes et al. 1996).

The current dispute over the sequence of cell lineage in regeneration should perhaps be reconsidered from the angle that not all cells involved in regeneration necessarily go through the same developmental sequence to reach the ß-cell stage. The sequence depends on the origin of the cell involved, and factors that influence it along its pathway of development.

Richards et al. (1964) described ligation of the splenic segment of the pancreatic duct, proximal or distal to its junction with the gastric duct. It was shown to induce the

disappearance of most of the acinar tissue, hyperplasia of the duct and ductular cells of the splenic segment within a week of ligation without any effect on any of the other segments (Hulquist, Jonsson 1965). Wang et al. (1995) performed PDL on large numbers of male Wistar rats and described the happenings over a two-week period under two categories. Firstly, those pertaining to the general post-PDL events and those more specific to the histological findings. The general comments are:

1. PDL brought about acinar apoptosis,

2. The beta-cell population was doubled within a week and a significant growth in the number of alpha cells recorded,

3. Small islet clusters were more numerous in the tail, indicating islet neogenesis,

4. ß-Cell hyperplasia took place from a proliferating stem cell compartment and that PDL induced the expression of a ß-cell specific glucose transporter type 2 (Glut-2) in ductal cells indicating their metaplastic (change in the type of adult cells in a tissue to a form which is not normal for that tissue) state,

5. Normal adult rat islet cell neogenesis could be reactivated by the stimulation of duct cells.

6. Their histological findings are:

    6.1 Acinar cells disappeared within three days and were replaced by ductal complexes (Wang, Klöppel, Bouwens 1995);

    6.2 Small ducts increased in number and formed ductal complexes, possibly due to VEGF (vascular epithelial growth factor) derived from infiltrating inflammatory leukocytes (Rooman, Schuit, Bouwens 1997).

    6.3 Changes associated with interstitial fibrotic and inflammatory reaction involved macrophages,

    6.4 On day five post-PDL, the pancreatic tail consisted of ductal complexes embedded in connective tissue and of islets. Many of the connective tissue cells and duct epithelial cells incorporated BrdU,

6.5 Small islets, islet cell clusters and single islet cells in the ligated pancreas were a frequent occurrence after day five,

6.6 Duct association cells expressing CK20 and insulin or glucagon were observed most frequently on days three and five but later disappeared,

6.7 During the first week post-PDL, a doubling of the ß-cell population occurred (Wang, Klöppel, Bouwens 1995) while the head of the pancreas remained unchanged. α-Cell volume increased 20 to 30 percent above normal while the area of ß-cells remained stable,

6.8 By day seven the tail portion (ligated area) showed significantly more islets and islet aggregates (islet areas < 1000 μm^2 as well as large numbers of single ß-cells; and

6.9 BrdU pulse labelling index (LI) generally increased (α = 3x; ß = 5x, duct cells = 10x) in the tail of the pancreas. However the LI of islet cells were less than one percent while ductal cell proliferation was up by eighteen percent.

2.3.2.5 In vitro duct cell culture

2.3.2.5.1 Interlobular ducts

Interlobular ducts are obtained by digestion of minced pancreatic tissue in a mainly collagenase medium supplemented with soybean trypsin inhibitor to remove surrounding, less resistant acinar cells, islets, intralobular ducts and small vessels. The larger fragments can be picked out under a dissection microscope or trapped on a sieve. Further digestion produces small clusters of cells with ductal properties from an unknown part of the duct system. These interlobular fragments consist of duct epithelium and connective tissue which can be removed by dissection or all the fragments trapped on the sieve may be cultured for a few days on a collagen gel to make duct fragments more easily recognisable due to their ability to form elongated cysts and distend through the secretion of fluid. The distended fragments are then harvested manually after dissolution of the collagen gel and further dissociation by

divalent cation chelation and collagenase digestion followed by separation by centrifugation through 4% bovine serum albumin.

2.3.2.5.2 Intralobular ducts

Due to their small size, intralobular ducts (ductules) pose a bigger challenge as it is not possible to isolate them under a microscope and currently no successful method for their isolation has been described. Although ductules represent ±80% of the pancreatic duct system in laboratory rats, separation by differential gradient and Ficoll centrifugation, which has been successfully applied to isolate ductular tissue from larger animals produces a very low yield in rats.

2.3.2.5.3 Cells of unknown origin

Duct cells from an unknown part of the duct system have been isolated and cultured after extensive digestion (Githens 1994). These cells seem to share features of the other duct cell cultures. Bisgaard and Thorgeirsson (as quoted by Githens 1994) have argued in favour of identifying them as "stem cells" (or "oval cells" - next section) due to them “consisting of cells with identical properties to cells isolated from other organs of shared embryological heritage as the pancreas”.

2.3.2.5.4 Islet Cells

Exhaustive studies have been conducted over the past fifteen years on pre- and post transplantation survival of pancreatic islets. Aspects considered were developments after segmental pancreatic transplantation (du Toit et al. 1988a), pancreato-duodenal-splenic allotransplantation (du Toit, Heydenrych, Smit, et al. 1987), with cyclosporine (du Toit et al. 1983a, 1984a) and irradiation immunesuppression (du Toit et al. 1988b) hetero-and ortho-topic segmental non-immunesuppressed transplantation (du Toit, et al. 1988a). All have lead to a better understanding of the factors that affect and influence islet survival in a wide range of scientific models and islet isolation and culture

2.3.2.5.6 Duct and Islet Cell Culture (in vitro)

Duct cells are minor cell types of the pancreas, comprising "10% of cells and "4% (Githens 1994) of pancreatic volume. They comprise a tubular epithelium through which the secretions of the acinar cells reach the duodenum. Intralobular ducts are the smallest of the pancreatic ducts, and are in direct epithelial continuity with the acinar cells, projecting into the acini as centroacinar cells. Ductular cells comprise "80% of the cells of the duct system (Githens 1994). As duct size increases the height of the epithelium and thickness of surrounding connective tissue increases. On a microscopic level, duct cells present an unspecialised cytoplasm with few mucin secretory granules in interlobular and main ducts, apical microvilli, a single apical cilium, and extensive interdigitations of lateral plasma membrane. Small numbers of goblet, brush and endocrine cells are interspersed between the principal cells of interlobular and main ducts.

Techniques for the isolation of main duct cells, interlobular duct cells and intralobular duct cells all follow a similar pattern with minor variations in isolation and culture procedure which vary from researcher to researcher (Gotoh, Maki, Satomi et al. 1987). The main strategy for duct cell isolation is a function of the diameter of the duct. Main pancreatic ducts in both large and small animals, and the interlobular ducts of large animals, are large enough to permit them to be isolated under a dissection microscope and the cells harvested by scraping or enzymatic digestion of the collagenous stroma. Interlobular ducts of small animals and all intralobular ducts cells require more sophisticated methods.

Duct cells are identified histologically by their ability to express cytokeratins 7, 19 and 20 (Bouwens et al. 1995) on their way to becoming ß-cells CK7 was expressed by islets and by duct cells of main, inter and intralobular ducts but not by centroacinar and terminal duct cells. CK 7, 19 and 20 were also expressed in proliferating duct cells during tissue regeneration and after isolation (Bouwens et al. 1994) and different periods of culture indicating the interrelationship between duct epithelial cells and islet tissue.

2.4 OVAL CELLS

A closer look at the whole issue of both hepatic and pancreatic stem cells or oval cells was prompted by findings by Rao et al. (1986, 1988a, 1990, 1995) and Uetsuka, Suzuki, Nakayama, Doi (1997). Their work describes pancreatic acinar cells, under experimental conditions, transdifferentiating into hepatocytes to occupy up to 60% of pancreas volume. The presence of hepatocytes in regenerating areas in cellophane wrapped primate pancreas was described by Wolfe-Coote et al. (1997), and the presence of oval cells in areas of hepatic regeneration (Radaeva & Steinberg 1995). Consultation with Professor D. Thomas (St. Andrews University, Scotland) prompted a further look into issue of cells, which appeared to have pluripotent stem cell ability.

Embryologically both the pancreas and the liver developed from outgrowths of the endodermal lining of the foregut (as the liver diverticulum, and dorsal and ventral pancreatic buds respectively) and are collectively referred to as the hepato-pancreatic-biliary-tract (HPBT). In both organs the ductal system first develops and undergoes budding and differentiation to form the two organs. Histologically there is very little difference between the ductal system of the pancreas and the liver, and are they continuous with one another.

Both organs have been found to be capable of regeneration (Radaeva and Steinberg 1995; Wolfe-Coote et al. 1996). Both are thought to possess dormant pluripotential stem cells capable of differentiating into hepatocytes (in the case of the liver) and both endocrine and exocrine tissue as well as hepatocytes (Radaeva, Steinberg 1995) in the case of the pancreas.

Partial hepatectomy (Novikov et al. 1996) has been found to give rise to extensive bile ductule proliferation (bile duct epithelial cells BDEC's - Radaeva and Steinberg 1995) in the portal triad-region. This proliferation extended for "considerable distances" from the triad-region to develop multiple branches extending from portal to midzonal regions into hepatic parenchyma, all within two to three days. These small non-epithelial oval cells, also referred to as "blast-like cells" by Novikov et al. (1996) were found to be identical to BDEC's (Radaeva, Steinberg 1995). They represented a heterogeneous population of bipotent stem cells (Ruck, Xiao, Kaiserliung 1996) which

could give rise to hepatocytes and bile duct cells (Tee, Kirilak, Huang et al. 1994; Yang et al. 1993). Under pathological conditions some also acted as progenitors for hepatocellular and cholangiocellular carcinomas (Radaeva, Steinberg 1995). Novikov et al. (1996); Sarraf, Lalaui, Golding et al. (1994) and Omori, Omori, Evarts et al. (1997) described their development (stem cells) to form oval cells (OC's) which were identical to bile duct cells (BDC's) while others only give rise to cholangiocellular carcinoma cells (Radaeva and Steinberg 1995; Hixson, Chapman, McBride et al. 1997).

Morphologically oval cells (both liver and pancreatic) fell into one of four categories. The first were described as small elongated cells with a very high cytoplasmic nuclear ratio. Cells generally possessed oval and/or round nuclei and had an organelle-poor cytoplasm with few mitochondria, rare endoplasmic reticulum (ER) and Golgi complexes (Radaeva and Steinberg 1995). Novikov et al. (1996) found them to be 3-5μm in size, of an undifferentiated phenotype with few surface features.

The second type of cell was described as being a larger cuboid cell (±10 μm) with a large complex cytoplasm characterised by abundant free polysomes, well developed Golgi, mitochondria, lysosomes, and other cytoplasmic organellae. These cells did not develop the polarised phenotype of bile duct epithelial cells (such as desmosomes, surface micvovilli, actin filaments), express PCNA (proliferation cell nuclear antigen) or undergo mitosis, and did not possess any of the other cellular characteristics of BDEC's, namely expression of CK 7 or 19 (Lapas, Sarose, Bosci, Thorgeirsson 1995), oval cell (OC) antigen, lymphocytic surface markers or have any of the properties of epithelial cells, oval cells, BDC's or lymphocytes. Thirdly, multi-nucleated giant cells, and finally, transitional cells displaying features of hepatocytes and BDC's.

Bile duct epithelial cells consisted of two types of polarised cells. The first having a clearly defined oval nucleus of a BDC and a "dense" cytoplasm (Novikov et al. 1996), and the second, defined as a transitional epithelial cell with a rounder nucleus and prominent nucleoli, only occasionally showing microvilli and peroxisomes.

To summarise then, oval cells share overlapping morphological and antigenic properties with BDEC's and appeared to be a heterogeneous collection of bile DEC's in various stages of maturation. Small periductal cells may be pluripotential stem cells,

hepatocytes and nodules (carcinogenic). Terminal BDC's behaved as stem cells and were progenitors of oval cells (Novikov et al. 1996).

The primary location sites for these cells, has long been thought to be at the base of ductules, under ductal epithelium but not in contact with duct lumen. A distinct "halo" was present around these small cells which separated them from surrounding duct epithelial cells.

A number of factors played a role in initiating oval cells to transdifferentiation into hepatocytes (Lee, Rim, Sell 1997), cholangiocytes, cholangio- and hepatocellular carcinomas, BDCE's (or pancreatic OC's into hepatocytes). They include stem cell factor (SCF - Rao, Yakawa, Omori, et al. 1996); bile duct antigen (Yang et al. 1993) and hepatocyte growth factor (Hgf - Alison, Poulsom, Jeffrey et al. 1993; Imai, Ichinose, Yanai et al. 1997). HGF induces proliferation, migration and morphogenesis of pancreatic oval cells (Jeffers et al. 1996). Other inducers are fibroblast growth factor (FGF - Hu et al. 1995), insulin growth factor II (IGF-II), responsible for malignant transformation of rat liver oval cells (Zhang et al. 1997b) and haemopoietic stem cell markers (Blakomer et al. 1995).

In short oval cells were able to change their differentiation pathway depending on environmental influence toward either a hepatocyte (or other) Radaeva and Steinberg (1995) or biliary lineage (Yang et al. 1993). Finally, the differentiation of hepatocytes in the pancreas (Rao et al. 1995, Wolfe-Coote et al. 1992) is preceded by 90% irreversible depletion of pancreatic acinar cells.

Acinar cell loss resulted in oval cell proliferation which served as precursor for either hepatocytes or pancreatic duct cells, or as put by Sarraff et al. (1994) proliferating ductules represent the oval cell compartment capable of producing pluripotential progenitor cells. A recent study has suggested that haemopoietic bone marrow cells could be a possible (and/or additional) alternative source of cells with pluripotent stem cell ability.

2.5 DIABETES MELLITUS

2.5.1 Introduction

Diabetes mellitus, it is generally accepted, is on the increase (King, Zimmet 1988). In the USA alone 30 000 new cases of IDDM are diagnosed each year (Najarian, et al. 1998), so also are the to be expected insidious effects on the general health of affected individuals (Weir, Bonner-Weir 1997). The disease impacts on the efficiency and productivity in the workplace, costs employers and the economy many thousands of dollars in absenteeism, lost production and healthcare expenditure (Murray, Lopez 1994). Patients experience a steady decline in general health, necessitating early retirement, frequent hospitalisation and expensive medication (Murray, Lopez 1994). Patients have a poor prognosis and many require sophisticated modes of treatment (kidney/pancreas transplantation – Sutherland et al. 1998) as organs fail amputations and dialysis become necessary, the incidence of cardio-vascular disease, treatment for strokes and loss of eyesight increase.

Researchers have for quite some time been aware of the potential value which experimental procedures could have in reversing diabetes and although some present a number of ethical problems, much valuable work is being done on a number of fronts to address the issue. Diabetic patients do not have many options open to them. The IDDM patient must use exogenous insulin and most NIDDM patients follow a strict dietary plan, more often/mostly supplemented by medication. More radical options, although acceptable to diabetics themselves, need to be discussed in public forums, as a number of them would cause considerable controversy. Of the milder alternatives could/would be pancreas xenotransplantation (Sutherland et al. 1998); pancreas/kidney transplantation (Najarian et al. 1998). The risks involved in poorly or uncontrolled DM however mostly outweigh the risks involved in the use of immune suppressants (Sutherland et al. 1998).

Among the remaining options are the intra-portal transplantation of isolated islets (du Toit et al. 1984); the insertion of a bio-artificial pancreas (Bouwens 1998b), the subcutaneous placement of a slow-release insulin mass, on a similar basis as currently in use for contraception, and foetal pancreas tissue transplantation (Du Toit

et al. 1998). The last of these presents perhaps the greatest challenge to our thinking and system of moral ethics. Abortion on request, although practised overseas for many years, is a relatively recent development in South African medical history; and has, on paper at least, a number of important restrictions as to timing and reason for procurement.

As more and more human foetal tissue becomes available, the distinct possibility exists that it may be used in diabetes mellitus cases in the foreseeable future. A peripheral spin-off from this concept is the highly contentious and somewhat problematic use of cloned material and/or intra-familial pregnancy for the express purpose of harvesting the foetal tissue for organ transplantation. The advantage of this is that it would eliminate many of the tissue rejection and immune suppression problems that are still experienced in transplantation.

To way-lay the possible misuse of scientific knowledge in the fashion mentioned above, it is vitally important for medical science to find viable alternatives to the bizarre scenario of using cloned individuals for tissue and organ transplantation or the pregnancy of a family member in order to replace the diseased organs, using tissue from the foetus.

For DM, specifically IDDM, which is the most widespread form, any mechanism that is able to induce islet cell proliferation (Mulder, Myrsen-Axerona, Gebre-Medhin et al. 1998) should be considered. Duct ligation (Bouwens 1998) presents an interesting alternative to conventional treatment regimens. PDL has a number of both positive and negative aspects that require resolution before it can be considered as a treatment option to any form of DM in humans.

Technically duct ligation is a relatively minor surgical procedure easily performed using keyhole surgical technique and a laparoscope. The resulting wound would be small; recuperation rapid and patients need not spend any time in a health care facility. The procedure, being simple, could be performed repeatedly.

Secondly, the proliferation of endocrine tissue shown to occur during duct ligation is independent of body insulin requirements, the number of islets present or glucose

imbalance that may exist from DM. Supplementation of existing endocrine tissue by ligation induced neogenesis may, for a time, lead to hyperinsulinaemia. This issue however is, as yet, unresolved as a homeostatic balance (Rosenberg 1998) is maintained (under normal circumstances) between blood glucose levels, insulin demand and supply and islet formation and regression.

The waxing and waning of pancreatic endocrine tissue, whether by islet and/or endocrine neogenesis, hyperplasia and replication, has been shown to be a life-long on-going process in laboratory animals other than primates. It responds in the short, medium and long term to insulin demand and glucose excess balanced by insulin oversupply and glucose insufficiency (Bonner-Weir et al. 1989).

If duct ligation was shown to be a mechanism of inducing islet tissue formation by neogenesis, the question arises as to whether it would eventually succumb to the dynamics of insulin and glucose supply and demand and leave enough new endocrine tissue in place to meet body requirements.

Thirdly, it is a distinctly possible future scenario that a DM patient could undergo PDL. Of the ligated tissue some could be removed and preserved for possible future transplantation in the eventuality of the re-occurrence of NIDDM. Of importance in this option is choosing the correct time at which to excise tissue for preservation and/or culture. This study has shown that insulin immunoreactivity increases on day 3½ post-PDL in the rat, while somewhat later in related species. Endocrine tissue isolation and culture prior to transplantation has been demonstrated to be a viable option (du Toit et al. 1984). Alternatively, the newly formed endocrine tissue could be stored (Charles, Harland, Opara 1998) and reintroduced into the patient at a later stage after isolation and culture of only the endocrine components.

The induction of neogenesis (duct to islet cell differentiation) by streptozotocin (STZ) induced ß-cell destruction (Rosenberg, Duguid, Brown et al. 1988; Wang, et al. 1994); cellophane wrapping of the pancreatic head (Wolfe-Coote et al. 1995); ligation of the pancreatic duct distally to its junction with the common bile duct (Gukovskaya et al. 1996; Wang et al. 1995; Zenilman, Perfetti, Swinson et al. 1996; Yamaguchi et al. 1993; Watanabe et al. 1995; Wang et al. 1997); dietary depravation

(Scharpelli, Rao 1980; Rao et al. 1988a, 1988b); pancreatectomy (Smith, Bonner-Weir, Leahy et al. 1994; Zang et al. 1997a); Kilham Rat Virus (KRV) infection (Stubbs, Guberski, Like 1997); ins-IFN-g-transgenic mouse model (Gu, Sarvetnick 1993) all bring about the release of hormones (Wang 1993; Brelje et al. 1994), growth factors (Wang et al. 1997; Alison et al. 1993; Atouf et al. 1997), chemical and neural signals (Ding et al. 1997), all of which modulate a variety of pancreatic cells (both endocrine and exocrine) to bring into existence a wide variety of cells, not usually seen in the pancreas (Bertelli, Bendayan 1997), all on their way to replace, compliment and/or augment the experimentally induced cellular deficiency.

Occasionally, cells receive and react to incorrect information bringing about the formation of cells, which are foreign to the parent organ (Rao et al. 1986; 1988a; 1990; Rosenberg et al. 1988, Scarpelli and Rao 1980; Wolfe-Coote et al. 1996).

Diabetes Mellitus is a complex syndrome characterised by a diminished availability or effectiveness of insulin and is "uniformly lethal" if left untreated (du Toit et al. 1987). Primary (idiopathic) diabetes presents itself in 50 000 new cases annually in the UK and another 500 000 go undiagnosed while in the United States the figures reported in 1994, were 30 000.

Secondary diabetes, being relatively uncommon, is due to pancreatic destruction caused by carcinoma of the pancreas, pancreatitis (Taniguchi, Yomota, Kume et al. 1997), pancreatectomy, haemochromatosis. Insulin antagonism induced by steroid therapy, Cushing's syndrome, acromegaly and haemochromocytoma, and finally, so-called "other causes of glucose intolerance" with thyrotoxicosis, pregnancy, thiazide diuretic and advanced liver disease as epitopes (Ling, Pipeleers 1996b).

2.5.2 An Overview of Diabetes Mellitus

Diabetes mellitus is defined as a random blood glucose level greater than 11 mmol/l, termed hyperglycaemia. Blood glucose levels in excess of this precipitate a multitude of symptoms that have a debilitating effect on the patient. Exhibited additional in

symptoms such as proteinuria, hypercalcuria, hyperphagia, polyuria, polydipsia, weight loss, glucosuria and dehydration. Less obvious symptoms include microvascular complications, retinopathy, nephropathy, vascular disease, neuropathy, complications during pregnancy, predisposition to infections, peripheral vascular degeneration, hyalinisation of capillary basement membranes; and metabolic changes leading to ketosis, ketoacidosis and coma which may culminate in death.

In humans, diabetes mellitus is classified to include two broad distinctive types: type 1 and type 2. The "juvenile-onset" type or insulin dependent diabetes mellitus (IDDM) occurs characteristically with an abrupt onset, in patients under 25 years of age who are usually underweight, and is associated with autoimmune disease. IDDM occurs in one to two percent of the population (USA), accounts for ten percent of all cases of, and is seventh on the list of disease-related fatalities in the developed world. These patients are prone to develop ketoacidosis and require daily insulin administration. Tailored regimen to individual requirements include firstly, a basal single dose of very long acting insulin (Monotard) plus three doses of soluble insulin 15-30 minutes before each main meal; secondly, a mixture of short and medium (Actrapid) acting insulin (e.g., soluble and *Lente*) at 7am and 6pm (half an hour before a meal). Two-thirds of the required morning dosage is taken (two-thirds as *Lente*). To improve control before breakfast, the evening dosage of *Lente* can be adjusted, so also with pre-lunch and pre-supper control. Thirdly, in elderly a single daily dose of the above mentioned mixture could sustain normoglycaemia, and lastly, continuous insulin infusion pumps are available.

The second, Type II diabetes is due to delayed insulin secretion or insulin insufficiency one to two hours after a meal, and exists in 90 percent of all cases of diabetes. Eighty percent of diabetics in this group are obese. These patients are less likely to develop ketoacidosis and although insulin may sometimes be needed it is not essential for survival, hence it is described as non-insulin dependent diabetes mellitus (NIDDM). Normoglycaemia can be maintained by a combination of dietary discipline and oral anti-diabetic agents such as Daonil, Minidials etc.

2.5.3 Clinical symptoms

The primary abnormality of diabetes mellitus Type I is hyperglycaemia. The World Health Organisation (WHO) diagnostic criteria for diabetes to be present requires two fasting venous plasma glucose levels of above 7,8 mmol/l. Levels between 6 and 7,8 mmol/l are diagnosed as impaired glucose. As soon as the blood glucose concentration exceeds the renal threshold i.e. approximately 9 mmol/l/min, and 11 mmol/l (Zilva, Pannall and Mayne 1991), the tubular reabsorptive capacity is significantly exceeded and glucose spills over into the urine. The presence of large amounts of osmotically active glucose disrupt the osmotic balance between the blood in the vasa recta and the fluid in the renal tubules resulting in the loss of large amounts of water (polyuria). Polyuria and the increased plasma osmolarity due to hyperglycaemia causes thirst, (polydipsia). A prolonged osmotic diuresis may cause excessive urinary electrolyte loss.

Because glucose is inaccessible to peripheral tissue as fuel, adipose tissue is mobilised and utilised, producing ketone bodies which disturb the acid base balance, and diabetics consequently run the risk of ketoacidosis, coma and death (Rubenstein, Wayne 1981).

2.5.4 Pathology of Diabetes Mellitus

2.5.4.1 Introduction

Although diabetes mellitus has hyperglycaemia as a primary symptom, it is simplistic to assume that beta cell insufficiency is the only cause; since research has shown that a proportion of diabetics have adequate secretory granules, but still suffer from hyperglycaemia and its complications. The presence of adequate secretory granules does not exclude the possibility of diabetes since there could still be defects in secretion, barriers to insulin transport or lack of insulin receptors on cell membranes of target organs (Jaffe, Hashida, Yunis 1980).

2.5.4.2 Islets of Langerhans

Beta cell degranulation seems to be a frequent finding in DM. Cellular definition is lost, cytoplasmic clumping is followed by necrosis and cell disintegration. This process can also be induced experimentally in laboratory animals, but is reversed by insulin treatment and in diabetic patients treated with insulin. Autopsies on longstanding IDDM patients (20-30 years) have shown the pancreas to be small and islets severely reduced and hard to find. Islets were reduced with small pyknotic-appearing cells; compressed or distorted in outline and did not stain like conventional beta cell granules.

Hyalinisation - the presence of hyalinisation of the basement membrane of islet tissue as a possible primary cause of islet pathology. Similar changes are found in ±10% of non-diabetic elderly patients and 30% of diabetics, which suggests that it may be a complication secondary to hyperglycaemia. The deposition of hyaline amyloid material seemed to occur between the islet cells and the capillaries in contact with the capillary basement membrane and is less "smudgy" than the material in small blood vessel walls of diabetic microangiopathy. Hyalinisation of islet tissue also appears to be rare in IDDM and is restricted to individuals with longstanding diabetes, and appears to be closely related to the age of the patient rather than duration or severity of diabetes mellitus. Hyalinisation tended to crowd out islet cells resulting in poorly granulated beta cells, although this seems to be the exception rather than the rule. Rubenstein and Wayne (1981) cite many instances where beta cells are well granulated in spite of hyalinisation, and there is evidence to indicate that hyaline material develops from degenerating beta cells.

Insulinitis seems to be prevalent and consistently present in acute juvenile diabetes and in patients with recently developed IDDM. It is characterised by lymphocytes, plasma cell and/or histiocyte infiltration of islet tissue, resulting in a significant decrease or total absence of beta cells with alpha and delta cells remaining normal. This infiltration of islet tissue by mononuclear inflammatory cells points toward a reaction similar to lymphocytic thyroiditis or immune reaction in transplant rejection. The however seems to be more complex as there is evidence to suggest that where certain endocrine tissues, e.g. ovaries and parathyroid, may be immunologically isolated, pancreatic islets

apparently do not share this status. Experimental evidence points to the rapid rejection of fresh, foetal and neonatal islet allografts in the absence of immunosuppression. Islets seem to be so strongly immunogenic that they are rejected at a faster rate than transplanted heart, kidney and skin.

2.5.4.3 Kidneys

Renal changes commonly encountered in diabetics include firstly, diffuse glomerulosclerosis This abnormality is a combination of more diffuse deposits in the mesangial matrix and is closely associated with proteinuria and renal failure. Hyperfiltration of the kidney seems to be linked to an increased renal (glomerular) blood flow, which stems from the absence of the normal insulin-induced regulation of glomerular blood flow by contractile mesangial cells.

Secondly, exudative lesions - These lesions are characterised by homogenous lipid containing deposits of brightly acidophilic, glossy material having a spherical or drop-like shape, which occur in glomerular tufts, over a glomerular loop or attached to Bowman's capsule. Fibrin and immunoglobulins are also present.

Thirdly, necrotising renal papillitis - This is particularly associated with DM and characterised by necrosis of ±2/3 of the renal pyramid, including the papillary projection of the pyramid into its respective calyx and may occur uni- or bilaterally.

Fourthly, nodular glomerulosclerosis, a degenerative complication prevalent in long term diabetics characterised by fibrosis and nodular-like scarring of the glomerulus manifested by albuminuria, nephrotic edema, hypertension, renal insufficiency and retinopathy. The outer wall of the glomerular capillaries is usually very thin. Nodules stain strongly with Periodic acid-Schiff (PAS), and are composed of glycoprotein, carbohydrate and lipids, all of which appear as basement membrane-like dense material in and around mesangial cells.

Fifthly, pyelonephritis is inflammation of the kidney and its pelvis caused by bacterial

infection. It begins in the interstitium and rapidly extends to involve the tubules, glomeruli and blood vessels. The ailment shows an increased frequency in diabetics and is often associated with diabetic nephropathy.

Sixth, renal hypertrophy has been observed within 48 hours after the onset of glycosuria as in experimental diabetes, and consistently in patients shortly after the onset of diabetes.

Penultimately, renal vascular changes are characterised by thickening of arteriolar walls leading to vascular occlusion. Nodular sclerosis and arteriolosclerosis (atherosclerosis) are characterised by lipid deposits in the intima, irregularly distributed in large and medium sized arteries. The lipid deposits are associated with fibrosis and calcification, and are always present to some degree in middle-aged and elderly patients. Renal arteriolar (both afferent and efferent) occlusion, atherosclerosis and arteriosclerosis also occur with greater severity in diabetics than in non-diabetics.

Finally, basement membrane thickening occurs in conjunction with diabetic glomerular lesions, especially in the cells of the straight descending segment of the proximal convoluted tubule and the loop of Henle. It is reversible and of no apparent clinical significance except for forensic purposes. Vacuolisation is due to glycogen deposition of the tubular epithelium (Armanni-Ebstein lesion) occurs, especially during poor diabetic control and in acidosis.

2.5.4.4 Eyes

The most dramatic effect of diabetes mellitus on the eyes is resulting from microaneurysms of retinal capillary or arteriolar walls, usually in the posterior region in the inner nuclear layer. The aneurysms progress and develop a thick wall. Thrombosis often occurs, as well as rupture or leakage, resulting in haemorrhage into the vitreous body; with exudates, oedema and other degenerative changes. Aneurysms are secondary to venous stasis, hyaline and proliferative lesions of retinal veins, kinks in the capillary wall, abortive myovascular changes, intervascular fibrous bands or

damage of the capillary wall due to disturbed carbohydrate metabolism. There is a loss of mural cells resulting in weakening or loss of tone of the capillary wall with subsequent development of microaneurysm formation.

Simultaneously, endothelial cells proliferate to form aneurysms and thick arteriovenous shunts, bypassing segments of the cellular capillary network. As haemorrhage progresses into the vitreous body neovascularisation develops, organisation occurs and retinal detachment may develop. The end stage being retinitis proliferans, with a scarred, vascularised humor. Vision deteriorates and is finally lost.

2.5.4.5 Nervous system

Most authorities on diabetes mellitus agree, and there is ample evidence to support them, that nearly all diabetics exhibit neural dysfunction during some stage of the illness. Diabetic peripheral neuropathy is a frequent finding of long-standing diabetes presenting with pain, paraesthesia, loss of vibrational sense, and in more severe cases of diabetes, anaesthesia of the entire limb may be encountered. Not only sensory nerves, but also motor nerves, may be affected, culminating in muscle atrophy.

Diabetic amyotrophy is the progressive weakening and wasting of muscles accompanied by aching or stabbing pain most often seen in elderly patients with uncontrolled diabetes.

Symptoms include vasomotor and sudomotor stimulation of sweat glands, postural hypotension, pupillary abnormality, paresis of urinary bladder, gastroparesis and diabetic diarrhoea. A prominent component of nephropathy concerns concentric hyalinisation of arteriolar, capillary and sometimes venous walls. Prominent sites for micro-angiopathy are the pancreas, retina and pericapsular zone of the adrenal glands, resulting in tissue degeneration following ischemia. Basement thickening varying from 5900Å in non-diabetics to 13300Å in diabetics. (E.M.) may occur.

2.5.4.6 Vascular Disease

Diabetics show an increased occurrence of arteriosclerosis of muscular and larger arteries, which accounts for the majority - between 75% and 80% of diabetic related fatalities. Atheroma is widespread and early in onset. Ischaemic heart disease produces angina and myocardial infarction (five times more common in middle-aged diabetics than in general population). Atheromatous occlusion of large vessels is forty times more common and may produce gangrene of the feet. Arteriosclerosis also includes atherosclerosis, which seems to occur at an earlier age in diabetics and with greater severity than in non-diabetics. Arteriosclerosis of muscle arteries of the legs and feet may cause peripheral tissue to die and become gangrenous due to gaseous and nutrient starvation as a consequence of impaired blood-flow, Rubenstein and Wayne (1981).

2.6 HISTOLOGICAL PROCEDURE

2.6.1 Fixation

The aim of a good histopathological technique is to produce microscopic preparations of tissue, usually stained, that represent as closely as possible their structure in life (Raphael 1983). Although the aim of ideal fixation is to preserve the tissue in as lifelike a manner as possible, only the very rapid freezing of small pieces of tissue in isopentane in liquid nitrogen will actually achieve this end, and then only while it is maintained in a frozen state without any loss of water. It may then be dried in a special freeze-drying apparatus and immediately imbedded in wax. Sections of such blocks may be used for enzyme, carbohydrate, and lipid demonstrations but usually must be fixed before being mounted. Chemical fixation represents a compromise in that, a reactive state with a single fixative, by a suitable combination of the latter all constituents can be preserved and demonstrated.

Fixation is the process by which the constituents of the cells, and therefore of the tissues, are fixed in physical, and partly also in a chemical state so that they will withstand subsequent treatment with various reagents with a minimum of loss, significant distortion, or decomposition. Most fixatives act by denaturing or precipitation of proteins, of which then form a sponge or meshwork, tending to hold the other cell constituents. Ideally, a fixative should penetrate a tissue quickly, be rapid in action, be isotonic, cause a minimum loss and minimum physical and chemical alteration of the tissue and its components, be cheap, stable and safe to handle. It should not bind with those reactive groups upon which specific staining of the tissue elements will depend.

Most fixatives produce some tissue hardening, which helps in the cutting of sections, but this hardening effect will be reinforced by the action of the alcohols used during the process of dehydration. Certain fixatives act as mordants for certain stains; e.g., after the use of mercuric fixatives the staining of the tissue constituents with many dyes is enhanced. Also, fixatives usually increase the optical differentiation of cell and tissue components, at the same time rendering the cells insensitive to hypo- and hypertonic

solutions used subsequent to fixation. Since micro-organisms are composed of protein they will also be fixed preventing putrefactive changes in the tissue and minimising the risk of infection for those who handle them

The ideal fixative would prevent autolysis and bacterial decomposition, preserve tissues in their natural state and fix all components, make cellular components insoluble to liquids encountered in tissue processing, preserve tissue volume, avoid excessive hardness of diced tissue, allow enhanced staining of tissues and be non-toxic and non-allergenic. The fact that there are so many fixatives indicates that the ideal has not been found and fixatives are chosen for use that is adequate for the end result required.

2.6.2 Fixatives

The most widely used routine tissue fixative is commercial formaldehyde (formalin) in saturated form, as 10% formol-saline or 10% buffered neutral formalin. A wide variety of fixatives are used depending on the result required. These fixatives range from those containing metallic ions like mercury and chromate to pure alcoholic-, alcohol-picric acid-, alcohol-acetic acid fixatives and chloroform containing fixatives.

Bouin's fluid consists of 75 ml, 1.2% w/v (saturated) aqueous picric acid (Fluka Chemicals, Switzerland) with 23 ml formalin (Fluka Chemicals, Switzerland) and 5 ml glacial acetic acid (Fluka Chemicals, Switzerland). Bouin's fixative has its unique advantages and disadvantages. It is a good fixative for the demonstration of glycogen, it penetrates rapidly and it works well for all tissue except the kidney, which fixes badly. As a result of their picking up of the yellow colour, small fragments of tissue are easily identified. It is very suitable for Mallory's, Heidenhain's and Masson's aniline stains. Shrinkage is slight and depending on the size of the block, fixation takes about 1 to 12 hours. The complete Bouin's formula is a stable solution.

The disadvantages of Bouin's fixative is that it lyses red blood cells and reduces the amount of demonstrable ferric iron. Tissue left in Bouin's for longer than 12 to 24 hours become brittle and difficult to section. Lipids are altered and decreased. Bouin's

fixative should not be placed in water until the water-soluble picrates have been rendered insoluble by the action of two or three changes of 70% ethyl alcohol. After fixation stock tissues should be stored in 70% ethyl alcohol. The yellow colour of the sections on the slide can be removed subsequently by either placing the sections in a saturated solution of lithium carbonate in 70% ethyl alcohol, or, by treating the sections with ethyl alcohol followed by 5% sodium thiosulfate followed by washing in running tap water.

2.6.3 Tissue Processing

2.6.3.1 Dehydration and clearing

Tissues contain large amounts of water, both intra- and extracellular. This water must be removed so that it may be replaced by wax. This process of the removal of water is called dehydration. After dehydration, the tissue needs to be "cleared" (dealcoholised) so that the alcohol in the tissue is replaced by a fluid that will dissolve the wax with which the tissue must be impregnated. Among the paraffin wax solvents used are benzene, xylene, toluene, petroleum ether, chloroform, carbon bisulphide etc. The term clearing is used because, in addition to removing alcohol, many of these substances have the property of making tissues transparent. They effect this by virtue of their high index of refraction and the resultant optical changes when the clearing agents penetrate between the highly refractive tissue elements; i.e., the refractive index of clearing agents is approximately equal to that of the tissues. Good clearing agents should remove alcohol quickly and clear quickly, without over hardening and should not dissolve out aniline dyes or evaporate too quickly in the wax baths. Clearing agents should be used in amounts not less that ten times the volume of tissue. Xylene (Xylol) is an excellent and true clearing agent but tends to make tissues excessively hard and brittle and tissues should never be left in it longer than three hours, otherwise sectioning is apt to be difficult.

Toluene on the other hand is preferable to xylene because it does not harden tissues as much, it is somewhat slower than xylene or benzene in clearing and it's fumes are toxic.

Chloroform does not harden tissues excessively, causes little shrinkage, evaporates rapidly from the wax bath and is relatively inexpensive. Chloroform gives the widest latitude and is the best of the traditional clearing agents for routine use; it is also best for nervous tissue, lymph nodes, granulation tissue, and foetal and other delicate, highly cellular specimens, all of which tend to become distorted and to break up on sectioning if cleared in xylene, toluene or benzene.

A sixteen-hour processing schedule as described by Raphael is the most widely used procedure and involves placing the tissue in successive solutions of ethyl alcohol and chloroform (time in hours in brackets) 10% buffered neutral formalin (4); 70% ethanol (1); 80% ethanol (1); 95% ethanol (1); 100% ethanol (1), repeated twice more in fresh solutions; toluene or chloroform (1), repeated twice more in fresh solutions; wax, Paraplast (1) repeated once (2).

2.6.3.2 Impregnation and embedding

This process involves the impregnation of the tissue with a medium that will fill all natural cavities, spaces, and interstices of the tissues, even the spaces within the constituent cells, and that allows the cutting of suitably thin sections without undue distortion and without alteration of the spatial relationships of the tissue and cellular elements.

An additional physical advantage in the handling of small specimens, and indeed of all specimens, is provided by surrounding the specimen with a mass or block of embedding material, thus allowing the specimen to be handled and fixed to the microtome block without damage to the actual tissue. The most routinely used embedding material is paraffin wax. It is easier to handle and allows sectional cutting. Generally speaking, the volume of the impregnating medium should be at least 25 times the volume of the tissue.

Tissues, having been completely dehydrated and cleared, are impregnated with paraffin wax by immersion in a succession of molten wax baths; this is usually achieved with

agitation on an automatic tissue processor. Following impregnation, tissues are embedded in a wax block that enables them to be cut into thin sections on a microtome.

Paraplast (Sherwood Medical Industries Inc., St Louis, Mo. 63103) is a mixture of highly purified paraffin wax and several synthetic plastic polymers. It's melting point is 56 to 57 °C. Regardless of the rate of setting, the blocks it gives are more uniform than those obtained with any other medium. There is no need for rapid cooling or embedding; indeed, sections may be cut without cooling the block face, although the use of an ice cube is advantageous. With Paraplast, ribbon sectioning is easy, and it does not tend to crack like other paraffin wax substitutes. It is more resilient than paraffin wax and permits large blocks and dense bone blocks to be cut with relative ease, thus avoiding tedious double embedding.

Wax is kept molten in a wax oven kept at 2 °C higher than the melting point of the wax used. When tissue has been embedded into a mould, it is placed in a basin of cold water (10 - 18 °C) or in a refrigerator to cool. This method of cooling lessens the tendency of some waxes to crystallise when allowed to set at room temperature and gives blocks of uniform, smooth and solid consistency.

2.6.3.3 Blocking out moulds

The Tissue-Tek system is a convenient system comprising of stainless steel base moulds in which the tissue block is embedded. A plastic mould is then placed on top and filled with wax; it is left in place and fits the chuck of the microtome. The metal base mould is pre-coated with a release compound in alcoholic solution so that the wax does not adhere to it. A more advanced Tissue-Tek system makes use of a perforated plastic histology cassette (Deltalab) which not only provides a firm base for the mounted specimens and is useful for storage, but is also convenient for clamping in the microtome.

2.6.3.4 Section Cutting

Specimens of ultra-thin tissue and wax are cut in successive sections on a Reichert Jung 2030 Biocut rotary microtome (Optolabor, Randburg). Minot invented this apparatus in 1885/6 and independently by Pfeiffer at John Hopkins University in 1886. The rotary microtome is the most popular and widely used for sectioning tissue between two and eight microns thick. Keeping the knife blade in a good condition is a time consuming and laborious process. More modern techniques (as at MRC & Stellenbosch University) make use of disposable microtome blades (Feather Safety Razor Co., Ltd, Medical Division, Japan or Shandon Lipshaw, Miles Laboratories, USA) attached by a modified disposable microtome blade holder attached to the main microtome knife holder.

In general the angle formed between the cutting facets where they meet is known and the bevel angle and is normally about 27 to 32 degrees. Knives should further be inclined relative to the cutting plane so that there is five to ten degrees clearance between the cutting facet presenting to the block and the surface of the block. If there is not such clearance the cutting facet will wedge or compress the block as the latter slides under the knife; in this matter, no section may be obtained at all for two or three strokes; then the block suddenly expands and a very thick section is obtained. Missed sections or alternately thin and thick sections are most commonly the result of neglecting to allow a proper angle or clearance between the block surface and the cutting facet. Normally the plane of the knife-edge is at right angles to the plane of cutting. For most tissues, this is satisfactory; however, for large or tissues longer than twenty millimetre it is advantageous to incline the axis of the knife edge so that it is oblique, thirty to forty degrees; this gives a slicing cut with less wedging.

2.6.3.5 Cutting and the Cutting Edge

The actual mechanism by which sections are cut is not fully understood. However, it appears that the wax and wax-impregnated tissue in the block are compressed in front of the cutting edge, which then "wedges" off the section by a combination of tearing

and crushing at submicroscopic levels. Therefore, the cutting edge must be thinner than the material (section) to be cut. Actually, the cutting edge is not a true edge in the theoretical sense; it is, in fact, a tiny part of the circumference of a minute circle of radius 0.1 to 0.35 μm. A "good edge" should cut good sections from a good paraffin wax block at 2 to 3 μm thickness and such an edge will show no serration when examined under a microscope at X100. It will also comply with von Mohl's criterion; i.e., with a strong nearby source of light the cutting edge will show only a slight reflection as a very narrow, continuous straight line. Such an edge will split a hair that is drawn across it or will cut the hairs of the back of the hand against only their own resistance. The final test for sharpness is the ability to obtain good quality sections at 4 μm thick from good quality blocks.

2.6.3.6 Ribbon-Sectioning

For the proper interpretation of the developmental histology required by this study, it is of vital importance that ribbon sectioning be performed with utmost care. For this process it is important that the upper and lower surfaces of the block be cut parallel in order that straight "ribbons" of sections be obtained. The first section is raised carefully with the index finger or a pencil camel's hair brush. As the knife strikes the block to start cutting the next section, locally generated heat and pressure weld the edge of section number two to the back edge of section number one. This continues with succeeding sections so that a ribbon of sections is formed. The finger, a brush or a flat bladed forceps of spatula may support the ribbon. Once cut the ribbons are laid out in serial order on a sheet of cardboard or black x-ray wrapping paper, with each end of the ribbon secured by pressing the wax border onto the paper with the back of a scalpel blade. Sequential sections are then placed in a floating-out bath for two minutes until the sections have flattened. An appropriately numbered APES-coated slide is gently approximated to the end of the section. The slide is gently raised in an even motion and, as it touches the edge of the section; the section adheres to it as the slide is drawn upward. The slide is then stood to drain at an angle of 60 to 80 degrees for two to five minutes.

2.6.3.7 Drying

After draining, the sections are fixed to the slides by placing them in a wax oven at 56 to 60 °C for two hours; or in an incubator at 37 °C overnight; or on a hot plate at forty-five to fifty-five degrees for thirty to forty-five minutes, preferably inverted, the slide ends resting on trace like supports three to four millimetres above the plate surfice.

2.6.3.8 Adhesive Mixture for Coating Slides

Although these are unnecessary for routing staining, provided that slides are clean and grease-free, they are essential for methods entailing significant exposure of sections to acids, and especially alkalis. They are advisable for complex or prolonged sequences and for certain tissues such as nervous system and bone. The agents used act mainly by reducing surface tension and thus producing closer capillary adhesion of sections to the slide. A synthetic substance is now more widely used than the traditional proteinatious substances quoted in textbooks. 3-Aminopropyltriethoxysaline [3-Triethoxysilypropylamine] (APES) is one such substance.

2.6.4 Staining

2.6.4.1 Haematoxylin and Eosin

Once slides have dried staining of the sections occurs in order to identify specific intra- and extracellular components. The first of the sequences of fifteen sections required from each of the specimens in this study will be stained with Haematoxylin and Eosin (H & E) and the rest using immunocytochemical techniques which will be discussed in detail below.

If unstained sections of tissue are examined under the microscope with transmitted light, very little detail other than nuclear and cell boundaries can be identified. Dyeing or staining of the sections enables one to study and see the physical characteristics and

relationships of the tissues and of their constituent cells. This all the more possible since different tissues, and indeed different components of the cell, display differing affinities for most dyes or stains. These differences in staining are explicable on the basis of variations in physiochemical structure and composition of cells and tissues. However, histochemistry, the special branch of histology that attempts identification of cell and tissue components by virtue of specific chemical reactions, is of relative recent development, especially immune- and double-immune staining. The most commonly used routine staining technique is H & E.

Cell and tissue components can be demonstrated not only by the use of stains but also by impregnation and staining for specific structures and chemical substances, as is the case in this study, for the polypeptides insulin, glucagon, somatostatin, PPY and NPY. Such specific staining or immune labelling has little or no affinity for other tissue elements (Lilly 1965).

Haematoxylin is a natural dye that is extracted from the core or heartwood of the tree Haematoxylon campechianum. This tree was originally found in the state of Campeche in Mexico, where the native population was familiar with its properties as a dye. Nowadays, the tree is grown commercially, mainly in Jamaica. When the tree is ten years old it is felled, the bark and sapwood is stripped off and the heartwood is exported in logs. The natural extract obtained from the logs, haematoxylon, is not an active dyestuff, but must first be oxidised into active haematein. In the process of oxidative conversion to haematein, haematoxylon loses two hydrogen atoms and assumes a quinonoid arrangement in one of its rings. Spontaneous oxidation occurs very slowly in watery or alcoholic solution, and it takes twelve to sixteen weeks for this to be satisfactorily accomplished. The oxidation process is achieved very quickly in a commercial setting by using catalytic converters such as mercuric oxide, sodium iodate, potassium permanganate, hydrogen peroxide and calcium hypochlorite. During this process however, substances other than haematein are produced, some of which are also involved in staining. "Ripened" haematoxylin stain is a mixture of hematosylin, haematein, active oxidation products of haematein and haematoxylon, and inactive ultraoxidation products.

Without a mordant haematein is almost useless, being a weak amber dye that stains an

amber colour. Mordants are substances that, by their physiochemical structure, aid in attaching a stain or dye to the tissues. They are essential to haematoxylin staining, for which the mordants used are always divalent or trivalent salts of hydroxides of metals. The complex of stain and mordant is called a "lake". The sulphate and chloride salts of aluminium, iron, chromium, copper, molybdenum and vanadium are most commonly used. Aluminium salts give a blue lake, and increasing the amount of the aluminium salt increases the selectivity for nuclei, especially if acid is added or is used as a differentiating agent. Chemical substances that heighten the colour intensity, crispness and selectivity of a stain are called accentuators. Unlike mordants they do not link or bind to the dye or bind the dye to the tissue and seem simply to reduce surface tension.

Harris' Haematoxylin is prepared by dissolving (by heating to 60 °C) 100 g of ammonium or potassium alum in one litre of distilled water. Five grams of haematoxylin in 50 ml absolute alcohol and five grams of mercuric oxide are mixed into the still warm solution. The solution, which becomes purple, can be heated for a further one to three minutes and then cooled rapidly. The oxidation mentioned earlier is brought about mercuric oxide. When the solution has cooled, 20 to 40 ml glacial acetic acid is added and the solution filtered before use.

Eosins are acid xanthene of phthalein dyes. The most common members of the group of commercial dyes are eosin Y, eosin B and phloxine. Eosin Y is most commonly used as a background or contrast stain because it gives a useful and pleasant contrast to nuclear stains such as haematoxylin. Eosin is available in powder form and is dissolved as one percent weight/volume concentration; a large crystal of thymol or 0.25 ml of 40% formaldehyde is added to each 100 ml solution. Eosin is the most widely used cytoplasmic counterstain. With the advent of modern scientific practice, it is no longer necessary to spend many hours making up histological stains. Most chemical companies sell the stains in kit form so that the contents of bottle A is simply added to the contents of bottle B.

2.6.4.2 Routine Haematoxylin-Eosin Staining

Mounted and dried sections of the desired tissue, is so treated that all parts of it are in the same phase as, and in contact with, the solvent medium of the stain. In the case of paraffin sections, the wax must first be removed by xylene (two baths, each of 3 to 5 minutes). The xylene is, in turn removed by passing through two baths of absolute alcohol (each of 2 minutes). The sections are then brought through "down-graded" alcohol baths: one of 95% alcohol for 2 minutes, followed by 2 minutes in 75 to 80% alcohol. If the stain to be employed is in alcohol solution, the sections are transferred directly into it from the alcohol baths. However, since most routine stains are in aqueous solutions the section must be taken from 75 or 80% alcohol and placed in running tap water or in distilled water for one minute before staining is begun. The tissue is then stained for four to eight minutes in Harris' Haematoxylin and differentiated by dipping (3 to 10 seconds) into a one percent acid alcohol (1 ml HCl (conc) to 99 ml 80% ethyl alcohol) and the rinsed in water. The slide is then passed through an eosin Y dye solution, again rinsed and dehydrated by passing through three or four baths of absolute ethanol (10 to 20 seconds in each) and then two or three baths of xylene (15 to 20 seconds in each). Once dried the slide is permanently mounted with DPX (Fluka Chemika-BioChemika, Switzerland), refractive index is 1.52.

CHAPTER 3

MATERIALS AND METHODS

Introduction

Facilities

Experimental Animals

Induction and Anaesthesia

Surgery

Immunolabelling

Morphometry and Statistical Analysis

3.1 Introduction

This study has meticulously adhered to the guidelines laid down by the Helsinki Declaration concerning the treatment of experimental animals and the Guidelines on Ethics for Medical Research S.A. M.R.C (Revised Edition 1993 - Appendix B). This study further endorses the recommendations of the Declaration and the guiding principles pertaining to the care and use of animals as laid down by local control bodies i.e. SPCA and Animal Welfare Organisation (AWO) and was approved in is entirety by the Ethical Committee of the University of Stellenbosch.

3.2 Infrastructure

One of the founding aims of the project was to contribute to the research capacity development of the University of North-West (formerly Bophuthatswana).

Histology, laboratory and animal housing and facilities for the first half of this study was provided by the Department of Biology at the University of the North-West (UNW) by kind favour of Mr S.D. Phalatse (Acting Head of Department) and the School of Agriculture (UNW) by kind favour of Prof. M. Funnah (Dean of School). The bulk of the study and its conclusion was performed at the Department of Anatomy and Histology, Faculty of Medicine, University of Stellenbosch, under the able guidance of Prof. Don F. du Toit.

Technical expertise was supplied by the Chief Technical Officer at the Department of Biology Mr David Kawadza and Mr D.O. Monnye both of UNW and Christo Muller, Romeo Lyners and Johannes Mattysen of the research/histology unit at the Department of Anatomy and Histology at Stellenbosch University. Logistical support and collaborative involvement was provided by the North West Department of Agriculture and Environmental Affairs as per kind favour of Dr. Anis Karodia (Director) and Dr. Werner Giecke (Head of Veterinary Services).

Immunocytochemistry was done at the Department of Anatomy and Histology, Faculty of Medicine, University of Stellenbosch, under the supervision of the MRC per kind favour of Dr. Sonia A. Wolfe-Coote, Mrs Charna Chapman & Dr. Johan Louw. Computerised morphometry was carried out at the MRC under the watchful eye of Keith Williams.

3.3 Experimental Animals

3.3.1 General

The treatment and supervision of the treatment of experimental animals was conducted under the supervision of Dr. Werner Giecke of the North West Department of Agriculture and Environmental affairs and Dr. R.F. Bakunzi, B.VM (Makerere), M.Sc (Guelph) from the Dept of Agriculture at the University of the North-West. Forty-eight Sprague-Dawley (SD) rats, housed in a thermally controlled environment at 18 °C, in standard commercially obtainable laboratory rodent housing facilities at the department of Biology at UNW with free access to water and balanced diet (Epol S.A.), were bred from 5 breeding pairs obtained from the Medical Research Council (MRC).

Composition of animal feed: Protein 200 g/kg, Fat 25 g/kg, Fibre 50 g/kg, Moisture 100 g/kg, Phosphorus 8 g/kg, Linoleic Acid 20 g/kg, Copper 6.5 mg/kg, Manganese 5 mg/kg, Selenium 0.1 mg/kg, Iron 60 mg/kg, Zinc 75 mg/kg, Iodine 2 mg/kg, Vitamin A 6000 I.U./kg, Vitamin D 600 I.U./kg, Vitamin E 60 I.U./kg, Vitamin K 1 mg/kg, Folic Acid 0.18 mg/kg, Thiamine 1 mg/kg, Riboflavine 3 mg/kg, Pyridoxine 1 mg/kg, Pantothenic Acid 10 mg/kg, Niacin 15 mg/kg, Choline 1200 mg/kg, Biotin 0.1 mg/kg, Vitamin B_{12} 0.022 mg/kg.

3.3.2 Duct Ligation Groups

Twenty three groups of six (n = 138) randomly selected Sprague-Dawley (SD) rats underwent PDL as described below. Two rats from each of the groups served as sham operated controls (SOC's) with n = 46 as indicated in the table below.

Table 3.1: Each of the above mentioned groups were sacrificed according to the time schedule described below:

Group	Days Post Duct Ligation	Experimental Group (No of animals)	Number of SOC's	Total number per group
1	1	6	2	8
2	1.5	6	2	8
3	2	6	2	8
4	2.5	6	2	8
5	3	6	2	8
6	3.5	6	2	8
7	4	6	2	8
8	4.5	6	2	8
9	5	6	2	8
10	5.5	6	2	8
11	6	6	2	8
12	6.5	6	2	8
13	7	6	2	8
14	7.5	6	2	8
15	8	6	2	8
16	8.5	6	2	8
17	9	6	2	8
18	9.5	6	2	8
19	10	6	2	8
20	11	6	2	8
21	12	6	2	8
22	13	6	2	8
23	14	6	2	8
	Total	138	46	184

After processing two series of seven consecutive serial sections were cut from each specimen and stained as follows:

Slide 1 in each group stained with H & E.

Slide 2 in each group ICC stained for Insulin (I).

Slide 3 in each group ICC stained for Glucagon (G).

Slide 4 in each group ICC stained for Somatostatin (ST).

Slide 5 in each group ICC stained for Pancreatic Polypeptide (PP).

Slide 6 in each group ICC stained for NPY.

Slide 7 in each group ICC stained for PYY.

3.4 Induction and Anaesthesia

3.4.1 At UNW.

When the rats were three months old (240-280 g) they were anaesthetised by a single intraperitoneal dose of Intraval-sodium (Thiopentone Sodium - Rhône-Poulenc Animal Health (Pty) Ltd., Midrand) general anaesthesia (40 mg/kg). The rats were held in a gloved hand and the anaesthetic administered in the lower right aspect of the abdomen as indicated in figure 3.1 below. The level of anaesthesia was confirmed by pinching a hind leg with a forceps and the suspension of the plantar reflex. This study found that laparotomised SD rats suffered from post-operative stress and many initially succumbed within 24 hours of the surgical procedure as induction was a lengthy process and the drug kept the rats anaesthetised for anything from 4 to 6 hours. Possible factors that were considered contributory to the problem were trauma induced hypoglycaemia, dehydration, hypothermia, anaesthetic overdose and lung oedema caused by prolonged exposure to di-ethyl ether during initial induction.

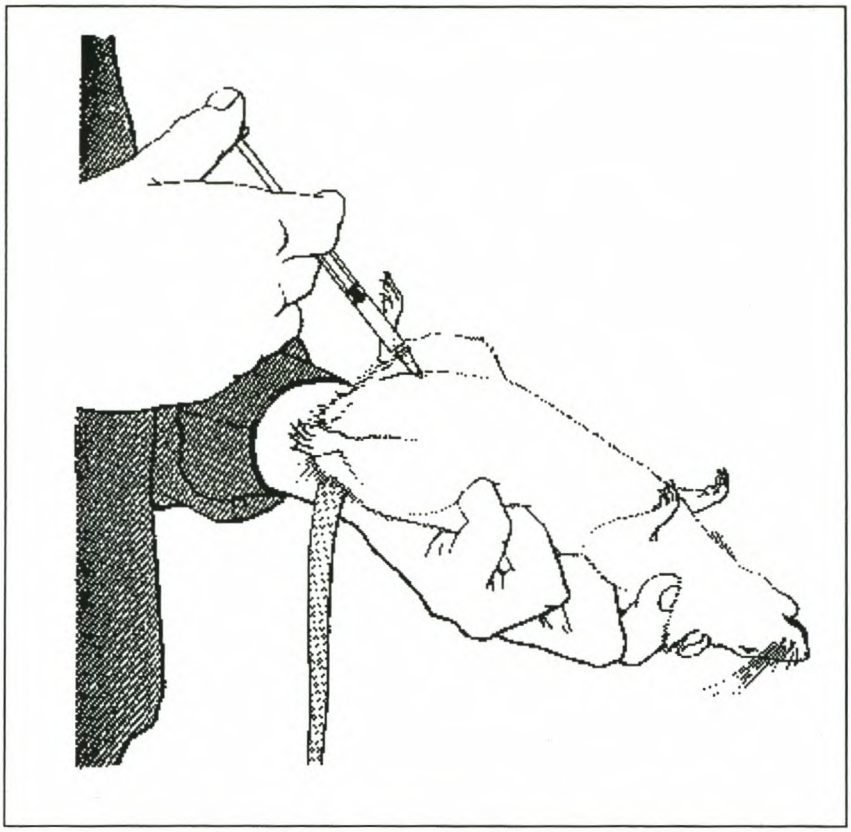


Figure 3.1: Indicates positioning for intraperitoneal administration of anaesthetic

To counter hypoglycaemia the rats were given glucose water to drink for the 12 hour pre-operative fasting period. To counter dehydration, 5ml of warm saline was left in the abdomen before suturing and recovery took place under a 60 W electric lamp to counter hypothermia.

Post operatively the rats received 2.5 mg Pethidine Hydrochloride (Intramed (Pty) Ltd, Port Elizabeth) six hourly as an analgesic, 2.5 mg Amoxil (SmithKline Beecham Pharmaceuticals, Midrand) and 0.5 mg streptomycin (Novo-Strep 5g/15 ml, Novo Nordisk (Pty) Ltd, Johannesburg) as a single dose, to guard against infection

3.4.2 At Stellenbosch University

Pre-operative induction of experimental animals was achieved using di-ethyl ether. Anaesthesia was maintained with 0.1 ml 10 mg/ml Ketalar (Warner Park-Davis Research Laboratories, Warner Lambert S.A., Main Rd, Retreat). As both ether and Ketalar are analgesics, no post-operative pain relief agents were used.

3.5 Surgery

3.5.1 Pancreatic Duct Ligation (PDL).

Pancreatic duct ligation is in itself an intricate procedure as the pancreatic duct is invisible to the naked eye and the anatomical relationship of the pancreatic duct to the common bile duct differs somewhat from humans. (see Figure 3.2 below). A dissection microscope was necessary and even then the duct is colourless and only about 0.2 mm in diameter.

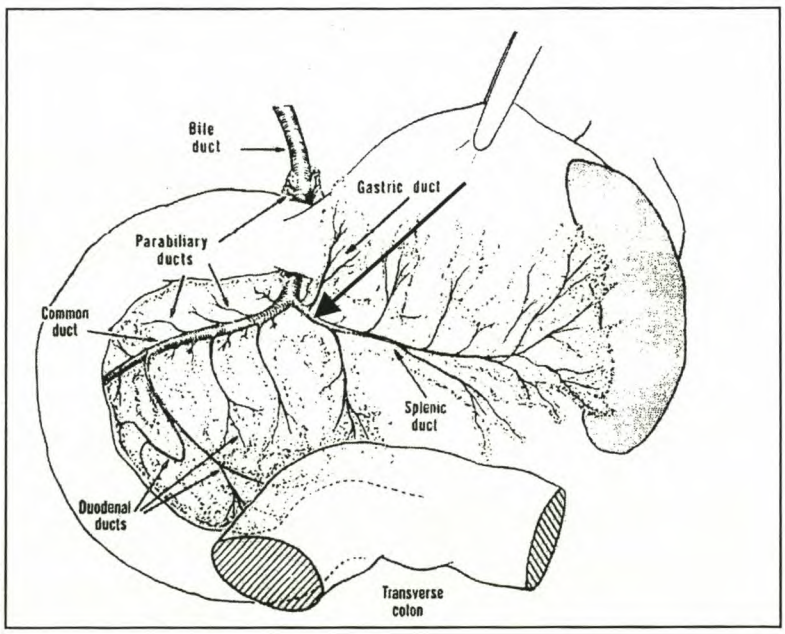


Figure 3.2: Arrangement of bile and pancreatic ducts.

Although earlier studies (Walker 1987, Walker et al. 1992) suggested that care needed to be taken as not to occlude the duct completely, subsequent more recent studies (Rosenberg 1998, Wada et al. 1995, 1997) showed that the degree of ductular occlusion was unimportant and all series were performed with total ductular occlusion. The

easiest way to gain access to the pancreas was to perform a midline laparotomy and pull out the stomach and duodenum, in this way two-thirds of the pancreas could be drawn out, and when spread out with the mesenterium on the surface of the abdomen the duct was quite easily located (van Dongen, Remie, Rensema, van Vinik 1990).

Anaesthetised rats were prepared for surgery by shaving the abdominal surface and there after cleaning it with Betadine antiseptic solution containing providone-iodine 100 mg equivalent to 10 mg/ml (Adcock Ingram Pharmaceuticals, Industria, Johannesburg) or hibitane. A full length laparotomy extending from the xiphisternum to the pelvis was utilised to gain access to the abdominal cavity. After exposing the pancreas, it was prized away from the surrounding tissue with a blunt forceps. The pancreatic duct was divided, for the purposes of this study, into four sections as suggested by Richards et al. (1964) namely, splenic, duodenal, gastric and parabilliary.

In this study the gastric and splenic segments were ligated as described by Hulquist and Jonsson (1965).

The ligation was carefully made from the point where the pancreas is attached to the first portion of the duodenum to the point just before its attachment to the transverse colon as described by Wada et al. 1995, 1997). The duct from the posterior (tail) portion of the pancreas was tied off with a single stitch of resorbable suture material (5/0 Dexon II, polycaprolate coated braided polyglycolic acid, Cyanamid (Pty) Ltd, 52 Electron Avenue, Isando, 1600). This procedure resulted in the ligation of the tail portion and accounted for 50 to 60 percent of the entire pancreas (Walker 1987; Wang et al. 1994, 1995, 1996, 1997; Watanabe et al. 1993).

After duct ligation, the abdomen was closed in layers using (3/0) Ethicon 636G chromic sterile surgical catgut (Johnson & Johnson, Halfway House) with a 19 mm reverse cutting needle, and the skin closed with (4/0) Ethicon W501 sterile black braided silk suture using a 16 mm super cutting needle. Surgical Chromic catgut was soaked in saline for 10 minutes prior to use to soften the alcohol preserved suture material. The wound swabbed with Kleenwound foaming antiseptic spray (Beige Pharmaceuticals (Pty) Ltd, Edenvale) powdered with dry penicillin and sprayed with Spray Skin flexible plastic skin (Link Laboratories, Edenvale). Post-operatively the animals were housed in standard laboratory cages and a suitable analgesic administered every six hours under the supervision of a qualified vet from the Dept of Agriculture UNW.

3.5.2 Sampling and tissue preparation

One hour prior to sacrificing, animals were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU - Fluka BioChemika, Switzerland) at a dose of 50 mg/kg body weight. BrdU is a thymidine analogue which binds to replicating DNA and indicates cell proliferation when immunocytochemically treated (Githens et al. 1976). BrdU was stored in a fridge between 4 and 10 °C. Crystals of BrdU were weighed (250 g rat received about 12.5 mg) on a chemical balance immediately prior to use and dissolved in 2 ml of a 0.9% saline solution (Sabax Sodium Chloride 0.9%, Adcock Ingram Critical Care Ltd, Johannesburg) in a 5 ml plastic syringe.

According to the predetermined schedule (Table 3.1 on page 77), duct ligated animals were anaesthetised and prepared for surgery as described above in order to harvest the pancreas for histological assessment. The whole pancreas was exposed, excised and fixed in Bouin's solution which is constituted from picric acid (75 ml, 1.2% saturated picric acid solution), formaldehyde (25 ml, 40%) and glacial acetic acid (5 ml). Bouin's fixative penetrates tissues well and is known to fixate small tissue volumes or masses rapidly. McEvoy et al (1977) strongly recommend the use of Bouin's fixative for immunocytochemistry. The tissue was processed and cut in half longitudinally through the middle line, and the exposed surfaces placed face down on the base of a labelled cassette and imbedded in histological embedding wax (Paraplast Plus, Monoject Scientific Inc), as described below.

3.5.3 Sectioning

Two series of six, 3 – 6 µm thick consecutive paraffin sections cut on a rotary microtome, were mounted on marked, frosted, APES (3-aminopropyl-trimethoxysilan, Fluka Chemicals, Switzerland) coated microscope slides. The second series was cut at least 250 µm from the first as described by Rosenberg et al. (1988) in order to avoid sectioning the same islets. The sections were incubated in an oven at 60 °C for 30 minutes, dewaxed in xylene and rehydrated through ascending series of ethanol. The first section of each sequence was stained with Hematoxylin (Certistain, Merck Diagnostica, Germany) & Eosin (Certistain, Merck Diagnostica, Germany) and consecutive sections double immunostained for BrdU and Insulin , Glucagon, Pancreatic

Polypeptide, and Somatostatin using the ABC technique described by Bouwens et al (1995). On completion, coverslips were put in place using DPX mountant for histology (Fluka BioChemika, Switzerland).

3.6 Immunolabeling Technique

3.6.1 ABC Technique

Vectastain ABC substrate was prepared by mixing together equal volumes of 0.02% hydrogen peroxide, made in distilled water from a 30% stock, and 0.1% (1 mg/ml) diaminobenzidine tetrahydrochloride (DAB) made in 0.1 M Tris buffer, pH 7.2. The hydrogen peroxide solution should be freshly prepared from concentrated stock. Many peroxidase substrates are unstable in the presence of hydrogen peroxide or when exposed to light. For best results, the peroxidase substrate is made just prior to use.

Paraffin sections were stained by firstly deparaffinising and hydrating tissue sections through xylenes or other clearing agents and graded alcohol series then rinsed for five minutes in distilled water. The sections were incubated in 0.3% H_2O_2 in methanol for 30 minutes to quench endogenous peroxidase activity. The sections were then washed in buffer for 20 minutes and then incubated for a further 20 minutes with diluted normal serum from the species in which the secondary antibody was made and the excess serum blotted from the sections.

Sections were then incubated for 30 minutes with primary antiserum diluted in buffer and then washed for 10 minutes in buffer, after which they were incubated for 30 minutes with diluted biotinylated antibody solution and then washed for 10 minutes in buffer. A 30 to 60 minute incubation with Vectastain ABC was then required before another wash of 10 minutes in buffer. Sections were incubated for two to seven minutes in peroxidase substrate solution, washed for 5 minutes in tap water, counter-stained with Toluene, cleared and mounted. (consult appendix B for post embedding labelling procedure work schedule).

Precautionary measures in order to ensure optimum success include firstly, sections should be well prepared. Fixation (generally in buffered formalin not exceeding four percent formaldehyde) was sufficient to maintain the integrity of sections throughout the staining procedure but not so harsh as to destroy the antigen under study. During the staining procedure, sections should not be allowed to dry out. Incubations should be

done in a humidified chamber.

Secondly, all reagents were used at optimal concentration. For most applications the Vectastain ABC procedure is substantially more sensitive than other immunoperoxidase techniques. Consequently, the primary antibody often can be used at higher dilution. To avoid adsorption of the antibody to the plastic or glass container in which the final dilution is made, the primary antibody may be diluted in buffers containing 0.1% crystalline-grade bovine serum albumin or dilute blocking serum which is included in the Vectastain ABC kit.

Thirdly, incubation times may be shortened. In cases where the antigen concentration in the section is high, suggested incubation times with primary antibody, biotinylated secondary antibody, and Vectastain ABC reagent may be reduced. Additional savings may be achieved by increased antibody dilutions. Incubation times as short as five minutes have been reported to be sufficient in some cases when incubation temperatures are raised to 37 °C. If the antigen concentration is low, incubation times may be lengthened to achieve maximal staining.

Fourthly, only freshly prepared buffers should be used. Bacterial contamination which can occur in buffers stored at room temperature may affect the quality of the staining. It is recommended that the Vectastain ABC reagent and substrate solution buffers be prepared with glass distilled water. Deionized water (even with low conductivities) may contain inhibitors of peroxidase and can reduce sensitivity.

Fifthly, Vectastain ABC kits should be stored under refrigeration. For best results, the kit reagents should be used before the date shown on the bottom of the box. The A and B reagents in the kits are matched. Reagent A from one kit is not to be used with Reagent B from another kit. The supplier recommends that the kits be retained in their original package boxes. If reagents are to be removed, they should be marked carefully to ensure that lots are kept and used together.

Sixthly, although the affinity-purified biotinylated secondary antibody and the normal serum provided in the Vectastain ABC kit can be purchased individually, the avidin DH and biotinylated horseradish peroxidase H are prepared especially for the Vectastain ABC kits and are matched reagents.

The seventh point is that paraffin sections should be dried in a hot air oven at 50 - 56 °C.

Some slide warmers contain "hot spots" that can overheat tissues. Paraffin tissue blocks should be stored in sealed containers in a cool location, then, complete deparaffinisation is important. Clearing agents and alcohol solutions should be changed regularly. All steps of the deparaffinisation should be sufficiently long to completely remove the paraffin from the sections, and lastly, slides should not be heated in an oven after mounting.

3.6.2 Method Controls

Method controls consisted of omission of each stage, in turn of the labelling procedure.

3.6.3 Specificity Controls

Specificity controls included adsorption of the primary anti-sera with excess homologous or heterologous antigen (10 – 100 µg per ml diluted anti-serum) for 24 hours before immunolabelling. In the case of anti-sera to peptides sharing amino acid sequences, cross reactivity of anti-sera was analysed by dot blotting and the results compare with tissue labelling, bearing in mind the different availability of epitopes between native antigen and antigen fixed in the tissue section

3.6.4 Antisera Controls

Drops of 2 µl (1 µg / µl) of antigens sharing amino acid sequences (PP, NPY, PYY) were placed on nitrocellulose strips and allowed to dry at room temperature. After the strips were fixed in Bouin's fluid for 1 hr at RT, the dot blots were stained using the Enhanced Chemiluminescence (ECL) kit (Amersham, UK) (Louw et al., 1997). The strips were incubated with unadsorbed anti-sera to assess cross reactivity and with anti-sera adsorbed with antigen combinations to try and reduce or eliminate cross reactivity.

After incubation with the detection reagents, the strips were exposed to autoradiography film to detect immunolabelling and assess antibody specificity.

Table 3.2: Details of anti-sera used for Immunocytochemistry.

Immunogen	Raised in	Supplier	Lot No.	Dilution	Specificity
Porcine Glucagon	Rabbit	Dakopatts	052	1/200	X-reacts with many mammalian species.
Synthetic C-peptide of Insulin	Rabbit	Incstar	881 6019	1/200	X-reacts with human or primate tissue.
Synthetic Cyclic (1-14) Somatostatin	Rabbit	Dakopatts	072	1/200	X-reacts with many mammalian species.
Synthetic C-terminal hexapeptide of pancreatic polypeptide	Rabbit	Bioproducts	001	1/2000	X-reacts with PP but not with PYY or NPY or any known gastrointestinal hormone.
Synthetic peptide neuropeptide tyrosine (NPY)	Rabbit	Bioproducts	3RG2	1/2000	X-reacts with 1-33 NPY and <0.01% PYY.
Porcine PYY peptide	Rabbit	Biogenesis	940 520A	1/1000	X-reacts with PYY but not with NPY or PP.
MIB-5, mouse IgG1, Kappa	Mouse	Dianova	Dia 5055	1/100	Reacts with Ki-67 equivalent in primates, monkeys, cow, goat, sheep, pig, dog, rabbit and rat.

3.7 Morphometry

3.7.1 General

Immunocytochemically stained consecutive serial sections of post-PDL pancreas were morphometrically assessed using a computerised system at the MRC per kind favour of Drs Sonia Wolfe-Coote and Johan Louw. The system consisted of a Pulnix TMC-6 CCD video camera on a standard/normal Zeiss light microscope coupled with a DT 3153 MACH Series Frame Grabber graphics card in a 486 PC. A HL Image ++ (image plus-

plus) Windows 95 compatible software package (Western Vision Software, USA) was used to capture and process microscopic images. Data was exported to MS Excel (Microsoft Inc, USA) and processed through STATISTICA (Statsoft Inc, USA).

Aspects measured included immunoreactive area in micron squared (μm^2) in each microscopic field, number of immunoreactive/brown staining areas within a particular field, individual IR-areas larger than 50 μm in diameter, and areas smaller than 50 μm in diameter associated with and/or in close proximity to a each other.

Statistical aspects, calculated on data captured, followed methods described by McEvoy et al. (1977), Lifson et al. (1985), Rosenberg et al. (1988, 1998), Ferrand et al. (1995), Kramer et al. (1985) included total area measured, total area immunoreactive for a particular hormone, percentage area of immunoreactivity in relation to total area.

Further calculations determined the number of immunoreactive areas, standard deviation, averaged deviation, median percentages of the various endocrine hormones under review in relation to the total area as well as to each other and the average reported in normal tissue. The data was subjected to the basic statistical functions contained in MS Excel. Final analysis was performed in MS Excel and STATISTICA.

Six equidistant random (using a mathematical table of random numbers Diem and Lentner 1970) fields from each slide were examined and their morphometrical data averaged and statistically analysed. Visual fields that were considered "problematic" in that they contained damaged tissue, large gaps and/or overlapped with non-ligated tissue were moved one "grid unit" around the parent area in a predetermined sequence until an appropriate tissue area was visible.. "Grid units" where determined using a millimetre square eyepiece grid in position on the microscope.

The grid contained 100 squares (10 X 10) and was positioned over the tissue before the random areas were selected and marked with a fine point transparency pen under 25X magnification. At 100X magnification each pre-selected 0,1 mm^2 area was measured (top left, top right, bottom left, bottom right) to allow for change in magnification as indicated below. Grid areas were numbered consecutively (from 1 to 100) from top left to bottom right and used as reference for area selection. Unsuitable "problem areas" (mentioned below) were treated consistently by first choosing the area directly above and working around clock-wise until a suitable area was found.

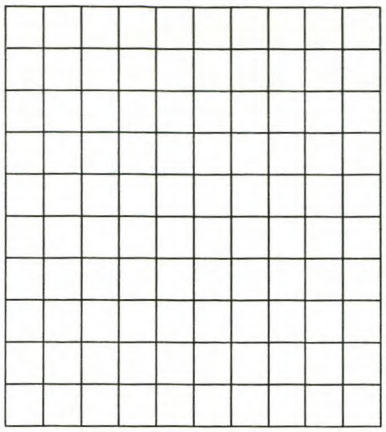


Figure 3.3: 10 X 10 (1 mm^2) eye-piece grid on morphometry unit.

3.7.2 Problem Areas

In one or two cases NPY and PYY produced "rogue" results as visually no evidence of IR-staining could be detected, while the computer analysis could not differentiate between included IR-tissue, darkened/thicker cell margins, nuclei and lymphocytes and considered these as "blobs" in spite of thresholding.

8	1	5
4	S	2
7	3	6

Figure 3.4 : S = selected area to be assessed. If unsuitable then areas bordering the area was selected starting with 1, which if also unsuitable, 2, then 3 or 4 in numerical order. If all of the above were found to be unsuitable then one of the adjacent areas numbered 5 to 8 was selected.

3.7.3 Optical Measurement

Areas of endocrine immunoreactivity of pancreas tissue (islets) were designated small, medium and large using the vernier scale on a standard light microscope and a 1 mm microscope grid captured on photographic film to measure the cross-sectional diameter through the centre of an islet in both a vertical and horizontal plane (using the natural axis of the microscopic slide fitted into the retainer on top of the specimen table of the microscope). The eventual scale used was 9 mm = 100 μm.

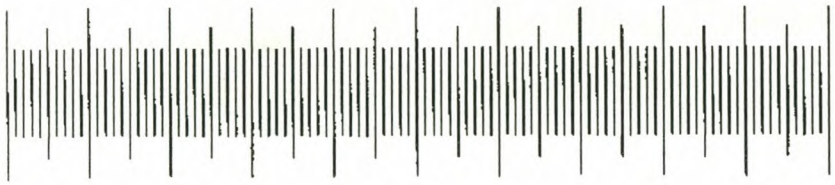


Figure 3.5: 1 mm microscopic grid captured on photographic film. Scale: 9 mm = 100 μm.

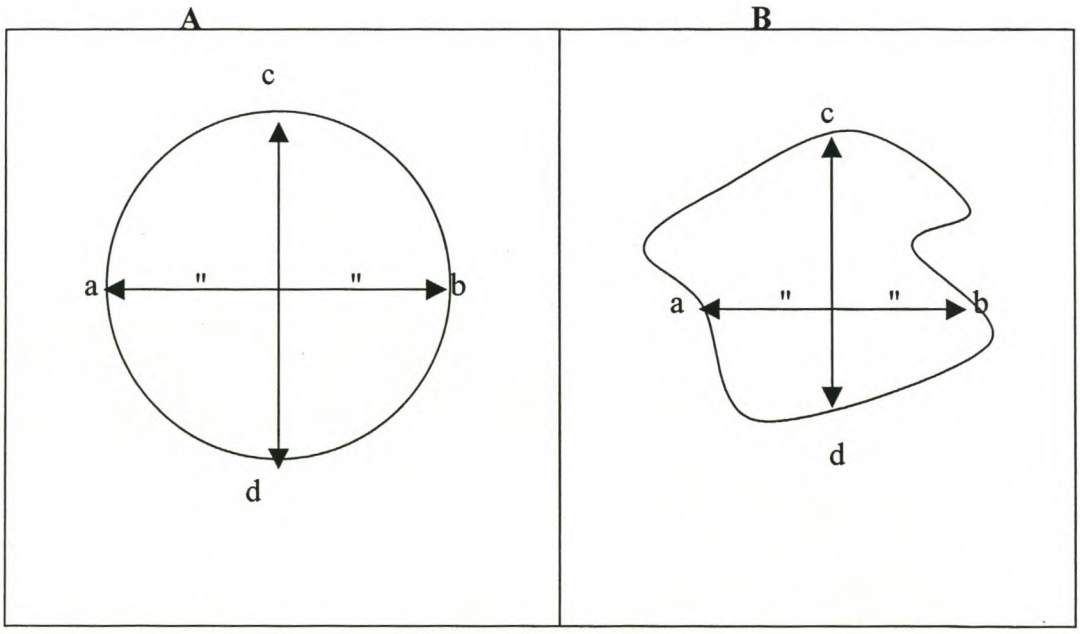


Figure 3.6: Hand drawn illustration to demonstrate horizontal and vertical axis of an islet.A = regular islet; B = irregular islet. ab = horizontal diameter; cd = vertical diameter with cd through midpoint of ab.

Islets described as small measured 10 – 100 μm; medium islets 100 - 200 μm and large islets greater than 200 μm. In 99% of cases (n = 50), islets could quite comfortably be categorised into one of the three groups and most were uniformly regular in shape. Those that were ovoid/ellipsoid/irregular were assigned the diameter that a sphere of

equal volume would have as described by Lifson, Lassa, Dixit (1985). Using the centre of a horizontal line (joining the two furthest points of an islet – line "ab" in figure 3.6 above) and a vertical axis (line "cd" in figure 3.6 above) eliminated the islets that were not spherical in shape, especially ligation induced endocrine formations (LIEF's), amalgamating structures and islets in close proximity to one another.

3.8 Statistical Analysis

The volume of the raw data obtained from the morphometry fills several hundred pages. For each series of data i.e. day one insulin for all six experimental groups were tabulated in MS Excel and the following averages calculated:- total area scanned, average area ICC positive, standard deviation, average deviation, median, maximum and minimum values to give data presented as follows:

		Total Area	Parent Area	Child Area	Roundness	XDiff	YDiff
DAY 1 INSULIN		111093.2					
	TOTAL	3400.7656	3292.22709	108.5385	7.14520828	309.7104	241.953
	AVERAGE	188.93142	182.901505	6.029917	0.39695602	17.20613	13.44184
	STDEV	392.59325	390.301902	21.14085	0.30374371	19.64635	12.73975
	AVEDEV	226.39634	216.346476	10.71985	0.24086602	12.99518	9.224158
	MEDIAN	41.864828	41.8648281	0	0.30951628	8.995824	9.084702
	MAX	1610.8914	1610.89136	88.63548	1	84.94405	48.47631
	MIN	4.8516188	4.85161877	0	0.04151437	1.941685	2.49012
% of total area = IIR		3.0611825					

Figure 3.7: An example of the format in which the morphometric data was captured and processed.

Percentage endocrine tissue, expressed as a percentage of the total area, was used to draw up the final tables, and the data was statistically evaluated using the student's t-test for independent samples.

The p-levels reported with the test represents the probability of error involved in assuming that a significant difference exists between and two particular series of data. P-values reported are designated "p" indicating the comparison between an internationally accepted norm (for which the appropriate references are quoted in the text) and experimental values and "p1" indicating a comparison between the values of

sham operated animals and experimental values. Differences between experimental results and an accepted norm or sham operated control is considered significant if $P < 0.05$. All values of $P > 0.05$ are not considered to be significantly different.

3.9 Alternative Statistical Analysis

In addition to the above computations, data were analysed as described by Bouwens et al. (1994), Wang et al. (1995), Gu et al. (1994), Louw (1995), Waguri et al. (1997). Analysis involved calculation of the total volume of endocrine cells using the stereologic morphometric method with the formula: $A/Ap = V/Vp$ where A = each endocrine cell immunoreactive area, Ap = total immunoreactive area of all endocrine cells, V = each endocrine cell volume, Vp = volume of total endocrine cells. The percentages of individual endocrine immunoreactive areas in each region were combined and values calculated for each hormone separately using the formula $A/Ap \times 100 = \%$ of each endocrine cell type per unit area. The result of this analysis is to be found in appendix C.

3.10 Cell Proliferation post-embedding Marker

Monoclonal antibody "MIB-5" reacts with the nuclear protein "Ki-67" which is present in all active phases of cell cycle and associated with cell proliferation. Specificity of MIB-5 for Ki-67 antigen has been shown by immunohistochemical, biochemical and molecular analysis. MIB-5 is especially suitable for detection of Ki-67 protein in routinely fixed paraffin material of Ki-67 protein in a variety of species different from human. Pre-treatment of tissue sections within a microwave or autoclave is necessary to perform reliable staining MIB-5 exhibits a strong nuclear staining of cells undergoing proliferation. Mouse monoclonal IgG1 reacts against the Ki-67 epitope which is present within nine out of sixteen concatemeric repeats of Exon 13 of the Ki-67 gene.

CHAPTER 4

RESULTS

Introduction and Overview

Morphometric Assessment

Comparative Overview

Histological Assessment

4 INTRODUCTION

To assist the reader the figures and tables are located at the end of this chapter (pages 131 to 149).

Histological staining (H&E) and immunocytochemical staining for insulin (I), glucagon (G), somatostatin (ST), pancreatic polypeptide (PP), neuropeptide tyrosine (NPY), peptide tyrosine tyrosine (PYY) on serial sections of duct ligated pancreas tissue were obtained from SD rats on successive days as described in materials and methods (Chapter 3). The experimental results are presented by firstly, an overview of each hormone over the duration of the study (paragraphs 4.1.1 to 4.1.5), secondly, the morphometric assessment (as section 4.2) followed by a comparative overview (as section 4.3) and finally a survey of all the daily endocrine events for each separate series (section 4.4 and all its subsections).

4.1 Overview of Staining Results

4.1.1 H & E Assessment

In normal control (unligated) tissue, islets appeared to be consistently shaped and sized. Most islets (99%) could be classified into one of three categories (small, medium or large). Insulin, glucagon, somatostatin and pancreatic polypeptide patterns were defined. One-day post ligation tissue showed very obvious signs of oedema (Figure 4.3 on page 135). Adjoining tissue segments were separated by large channels and the lumina of acini exhibited signs of distension. On a cellular organisation level, acinar tissue appeared normal and islets seemed to be unaffected. Tissue did not show any visible signs of inflammation and/or lymphocytic infiltration.

Although islets still appeared to be unaffected by the ligation process after 36 hours, acinar tissue showed signs of apoptosis (*genetically determined programmed cell death.) During apoptosis a cell starts to shrink, losing contact with the neighbouring cells. It's chromatin condenses, and its DNA breaks into nucleosomal sized fragments. Finally the cell fragments into membrane bound pieces called apoptotic bodies. These fragments are then removed by macrophages or neighbouring cells with no inflammatory reaction in the tissue, Scaglia et al. 1995*) as opposed to necrosis (*cell tissue death due to abnormal*

circumstances i.e., oedema, ischaemia, infection etc.) and distended intra-acinar duct lumina were more evident (arrows Figure 4.5 on page 136).

Ductular distension in a number (±20%) of fields at the periphery of the organ was very obvious after two days. Areas of acinar deletion/disorganisation had advanced significantly and began to resemble connective tissue. By this time more than ±70% of the acinar tissue had lost its original character. A number of islets showed signs of vacuolisation. After 2½ days the ducts exhibited a degree of distension. Areas of ductular distension (at sites of centro-acinar cells) started becoming more prominent, while islets had a similar appearance as described for day two.

At various stages in the series (days 1 to 14) existing islets showed significant signs of increased ductular activity (*defined as an increase in mitotic figures, multi-layering of cuboidal cells, and hyperchromic nuclei and cytoplasm, Zenilman et al. 1996).* In a number of areas (±20% of total tissue area), areas of increased ductular activity were found in close association to large areas of insulin immunoreactivity. Ductular activity increased dramatically by day three. Also of note was the transient infiltration of mononuclear lymphocytes by this stage and acinar destruction/degeneration. A few islets lost their clearly delineated boundaries and appeared indistinct about the edges with signs of vacuolisation of the margins as well as core increasingly evident.

By day 3½ ductular distension seemed to have reached a maximum with an average count of 4 to 5 ducts per 1000 μm^2 area (see figures 4.19 – 4.23) each averaging between 10 and 40 μm in diameter. This phenomenon tended to persist throughout the 14-day period of the study and beyond, and was still evident after 30 days. The presence and/or fluctuation in the number of ducts (as seen throughout the sequences between days 4 and 14) tended to follow a cyclic pattern. Peaks (maximum numbers) were observed on day 3½, 5½, 7½ and 14 (figures 4.19 on page 139; 4.33 on page 143; 4.35 and 4.36 on pages 143 and 144 respectively). The intervals between these periods were characterised by a decline in the dilatation and numbers of the ducts. Day 3½ coincided/corresponded with the corresponding insulin peak in figure 4.1.1(a) on page 131, which was preceded by glucagon and PP on days 2 ½ and 3 (figures 4.1.1(b) and (d) on page 131). The day 7½ peak coincided with the next PP peak in figure 4.1.1(d) on page 131, and day 14 coincided with insulin decline in figure 4.1.1(a) on page 131, the somatostatin peak in figure 4.1.1(c) on page 131, and the PP peak in figure 4.1.1(d) on page 131, both for the same period.

Recessionary intervals were observed at days 5½ coinciding with a corresponding insulin (figure 4.1.1a on page 131), somatostatin (figure 4.1.1c on page 131), PP (figure 4.1.1d on page 131) decline, and again on day 6½. Days ten to fourteen displayed progressive cellular disorganisation to give day 14 equivalent/similar to day 3½ and 7½/8. Furthermore day 4 resembled days 3, 5½, 6 and 6½ while day 3½ resembled days 4½ and 5, days 7½ and 8, and also day 14. Islets were seen to be normal (unaffected by ligation) on days one and two, and nearly so between days 5½ and 9.

4.1.2 Insulin

Two different endocrine structures were found to occur during the experimental procedure. The first was designated "pre-existing islets" some of which remained unaffected while others underwent varying amounts of change. The second consisted of non-islet endocrine tissue, consisting mainly of insulin immunoreactive cells, called ligation induced endocrine formations (LIEF's). They were usually around or in close proximity to areas of ductular distension. In all but one (day 1) of the series of islets viewed, islets consisted of a core of insulin secreting cells surrounded by uniform layer of cells, with different secretory characteristics (G, PP, ST), at varying thickness' with insulin cells illustrated in figure 4.2.

The morphometric assessment of the total insulin content, expressed as a percentage of the total tissue area measured for each series, revealed a bi-phasic curve for insulin as illustrated in figure 4.1.1(a) on page 131. Only areas of the primary endocrine pancreatic hormones (insulin, glucagon, somatostatin and pancreatic polypeptide) exhibited any form of cyclic pattern (figures 4.1.1 a, b, c and d) while NPY and PYY made a transient appearance and disappeared. Significant differences were found between control and experimental animals ($P < 0.01$) with regard to insulin positive tissue area as illustrated in table 4.1 on page 134.

4.1.3 Glucagon

Insulin accounted for 2.4 ±0.12% of the total pancreas tissue in control animals (table 4.1) and glucagon accounted for 0.78 ±0.03% (table 4.2). An insulin:glucagon ratio of 3:1 was found in the control groups. The average percentage of glucagon tissue in the pancreas in

the experimental group was calculated to be 2.52% and glucagon peaked at day 2½ (15.77 ±1.7%), 6½ (3.30 ±0.42%) and 13 (2.29 ±0.37%) representing near equidistant time intervals (4½ and 5½ days respectively).

Days 1, 2½ and 5½ conformed closely to the structural norm for islet glucagon immunoreactivity (GIR). Arrangement in normal unligated animals, with glucagon forming a virtually continuous (80 - 90%) two to three cell layer around the perimeter of an islet (figures 4.10 and 4.11 on page 137, and 4.33 on page 143; day 1 not shown). In endocrine tissue which can be considered to be LIEF's (day 8 – figures 4.37 and 4.38 on page 143, and day 8½ - figure not shown) glucagon did not form a uniform continuous band of cells around the insulin mass. At day 3½ (figure 4.21 on page 140) GIR cells were in consort with insulin IR cells but mostly as independent individual cells or cell clusters (indicated on overlay to figure 4.21).

At day 4½ "area A" (figure 4.30 on page 142) possessed more cells with GIR than insulin immunoreactivity (IIR) and areas 1, 2 and 3 conformed to the pattern of ß-cell core with glucagon cells on the perimeter, although sparse (compare figure 4.30 with 4.31 on page 142). In areas 4, 5, 6, 7 and 8 of the same figure, only cells with IIR could be found. *Comment: Tissue distension/swelling/oedema was visible/evident up until, at least, day 5. Areas of GIR (day 5½ -figures 4.32 and 4.33 on page 143) often overlapped with the insulin core (i.e., GIR cells could be found between IIR cells close to the perimeter of islets.*

4.1.4 Somatostatin

In non-ligated sham operated control (SOC) pancreas islet tissue ST was restricted chiefly to the perimeter of islets where they could be found with varying frequency as single cells as indicated in figure 4.2 on page 135, and described in paragraph 4.4.1.4. Some evidence of ST-positive cells, in apparent isolation, were present in amongst acinar tissue as described in 4.4.4.4 for day 2½ and illustrated in figure 4.12 on page 138. Somatostatin positive tissue accounted for a very small percentage of the ICC stained tissue in the sections studied with no significant difference noticed between control and experimental groups ($P > 0.05$). Values for ST between normal and control groups only differed by

0.01 (table 4.3 on page 134). *Very rarely* ST-positive cells could be found in amongst the cells making up the insulin core of islets (described in paragraph 4.4.5.4).

It is important to take note that the above description is for control and/or existing islet endocrine tissue arrangement in the first few days post ligation. In ligated tissue (paragraph 4.4.6.4) ST-IR presence in LIEF's is significantly absent but present in clusters of 2 to 5 cells, except in islets 2 and 3 in figure 4.3 above where a continuous row of ST-IR cells are to be found. Cells can be seen apparently forming the lining of a duct in the latter, and as a cluster of about ten cells within an area of insulin immunoreactivity in the former, throughout areas of cellular transition/disorganisation (See paragraphs 4.4.8.4; 4.4.10.4; 4.4.12.4; 4.4.13.4; 4.2.14.4; 4.2.18.4 for a more detailed description.

Morphometric quantification of the average area of somatostatin and pancreatic polypeptide throughout the whole series indicates significantly more of the latter (4917.902 vs. 3476.478 μm^2). This observation is confirmed by the percentages exhibited in the graphs for ST and PP in figures 4.1.1(c) and (d) on page 131.

4.1.5 Pancreatic polypeptide

Control animals possessed virtually equal numbers of PP-IR to ST-IR cells (0.12 vs 0.10%). A significant difference was found to be present in the percentage of PP immunoreactivity between control and experimental animals ($P < 0.05$) Although the accepted pattern for the placement of PP-IR cells in islet context has been established, significant quantities of PPIR cells were present in areas of acinar transformation/deletion (apoptosis?) and increased ductular activity as early as day 2½ post duct ligation (paragraphs 4.4.4.5 and 4.4.6.5), in no fixed pattern, in association with cells exhibiting insulin or glucagon immunoreactivity.

4.1.6 NPY and PYY

NPY antiserum has as yet not been characterised in the rat and has presented problems throughout the study. In spite of this uncertainty the findings of these two peptides is reported here for the sake of totality. A relationship **seemed** to exist between these two

peptides. In a number of cases they appeared to occur in close proximity to one another as illustrated in figure 4.25 on page 141 and the accompanying overlay. Neuropeptide Y was first seen on day three while both NPY and PYY were first seen as staining complete cells on day 3½ (figure 4.24 on page 141) which were in intimate relationship to (overlay day 3½ NPY – green, and PYY –purple).

In a limited number of cases, NPY/PYY appeared colocalised with dilated duct tissue and/or those areas where insulin immunoreactivity appeared as single cells or clusters of three to five cells. Each time both peptides were found in between clearly distended ducts as illustrated in figures 4.24 (on page 141), 4.25 and 4.28 (on page 142). Prior to day 7½ both peptides were associated with ducts only. Thereafter with PYY with ducts and NPY with islets. NPY was but mainly associated with structures that resembled islets and was not restricted to islet perimeters as illustrated in figure 4.39 on page 144.

4.1.7 Islet Frequency

Duct ligation induced the formation of significant amounts of immunoreactive tissue, especially for insulin, which could be seen to appear in areas where obvious acinar deletion had occurred (Figure 4.20) and where there was evidence of large-scale ductular distention.

Table 4.7: Comparison of numbers of 2 – 5 cell LIEF's in 10 random visual fields in each of the experimental groups studied (1000 μm x 1000 μm visual field.)

Visual Field	Normal n = 2	SOC n = 12	Unaffected Ligated n = 5	Ligated day 3½ n = 5
1	0	1	0	35
2	3	0	0	26
3	1	0	2	29
4	0	1	3	32
5	0	2	0	16
6	2	0	1	22
7	0	3	0	19
8	1	3	1	25
9	0	0	1	33
10	2	1	2	21
	10	11	10	258

MORPHOMETRIC ASSESSMENT

4.2.1 Introduction

Areas of LIEF, characterised by the presence of one or more large insulin areas (d > 100 μm) which were designated "parent areas" by the morphometry software package used, were often associated with large numbers of smaller adjoining subsidiary areas (d < 50 μm). These were referred to as "child areas" which appeared to occur both in consort with and separately from islets. Figures 4.1.1 (a - f) illustrates the overall average endocrine area (n = 5) for each day post-PDL respectively while figures 4.1.2 (a–f) indicate parent and child areas separately. Of note is the asynchronous relationship between the two in all but the initial stages of endocrine proliferation (correlation coefficients placed on the respective graphs for comparison).

4.2.2 Insulin (Figures 4.1.1 (a), 4.1.2 (a) and Table 4.1).

The total percentage insulin positive area during days one to three (phase 1) post-PDL show a slight rise at day 1½ after which it declined to day three. Figure 4.1.2 (a) however indicates an increase in "child areas" by/around day two even although total insulin area is declining. Between day 3½ and 7½ (phase 2) an initial peak of insulin immunoreactivity in synchrony with an area of "child" increase, not again repeated, can be seen. Maximum parent/child ratio seen at day 3½ was 5:1, and also represents the largest parent/child ratio of the series. From day 8 until the termination of the experiment (phase 3), insulin percentage area increased to 25% (day 13), while the lower than normal value at day 14 remains unaccounted for.

4.2.3 Glucagon (Figures 4.1.1 (b), 4.1.2 (b) and table 4.2).

Although the percentage glucagon immunoreactive area only has one significant peak (day 2½ = 8607.9 μm^2), its parent/child ratio approximates 8:1 and is not repeated to any degree of significance as is the case of insulin. The glucagon peak precedes the insulin peak by 24 hours.

4.2.4 Pancreatic Polypeptide (Figures 4.1.1 (d), 4.1.2 (c) and Table 4.4)

Only three significant areas of "child" activity are found at days 2½/3½, 6½/7½ and 11-14. The parent/child ratio at days 2½/3½ approximates 16:1 (which is half that of glucagon). Phase one (day 1-3) is in synchrony with glucagon and somatostatin but not with insulin. After ligation PP was found in between islet insulin core cells, as isolated pockets of PPIR cells in association with acini and around ducts and areas of endocrine proliferation.

4.2.5 Somatostatin (Figures 4.1.1 (c), 4.1.2 (d) and Table 4.3)

Phase one was characterised by a peak of STIR at day 2½, coinciding with that of glucagon and PP. Phase 2 shows no increase in ST activity while phase 3 shows an increase over the three day period (day 9½ - 13) with day 14 more that doubling that of day 2½.

4.2.6 NPY and PYY (Figures 4.1.1 (e & f), 4.1.2 (e & f) and Tables 4.5 and 4.6)

These polypeptides only manifested as isolated cells and produced slight coloration along lumina of ductular structures. No evidence of any clustering was found to be present. NPY, in association with what appeared to be an islet was a singular occurrence (figures 4.28 and 4.29 on page 142).

4.3 COMPARATIVE OVERVIEW

4.3.1 Day 4½

The specimen displayed and discussed in the text below typifies the findings of the other slides in the series.

Of the eight identified areas of insulin immunoreactivity (purple on overlay), number 4, 5 and 8 (Fig 4.30 on page 142) show no other immunoreactivity. Areas 1, 2 and 3 of the same figure, possess IR for glucagon (green), PP (blue) and somatostatin (red) as well while 6, 7, 8 and "area A" exhibit no IR for ST. In area A insulin and glucagon IR overlap substantially with only four small PP-IR cells in between. Substantially more PP-IR is to

be found in an area directly below that for insulin and glucagon, evidently part of a LIEF, but possessing only PP-IR cells running roughly in a line (could be part of a ductular wall). Islets 1, 2 and 3 all have a clearly defined insulin core with the other hormones occupying (mostly) the peripheral margins. Both islets 2 and 3 are immediately adjacent to ducts (arrows). The duct/islet interface, invariably, is made up of cells which are IIR with mostly glucagon and somatostatin (area 3) and glucagon; or insulin with PP (area 2). No glucagon or somatostatin is seen to be in contact with the duct lumina but occur close-by.

4.3.2 Day 5½

Areas of IIR (together with PP, G and ST) consist of clearly definable islets and a number (1 to 7, Fig 4.32) of areas (green) of IIR only. Islets are surrounded by significant numbers of ducts (blue) at which other islet hormone secreting cells in varying proportions make up ductular lumina in consort with insulin. In all cases only the inside luminal margin is found to have an endocrine morphology. Substantial amounts of glucagon secreting cells make up the perimeter of islets with the "gaps" made up by PP-cells. Evident in studying any of the islets is the phenomenon that insulin secreting cells also occupy a place in the perimeter with the other cell types.

4.3.3 Day 6½

Ductular activity seems to have declined considerably with the remaining evidence of ductular activity reduced to very small structures. In figure 4.34, islets 1, 2, 3 and 6 co-exhibit insulin, glucagon and PP but not ST while only in islet 5 are all the major endocrine hormones found. In and around the encircled area indicated by the bracketed number 14 {14} all the major pancreatic hormones, except insulin, are found out of islet context, in close proximity to one another.

4.3.4 Day 7½

Large insulin expressing islets in figure 4.35 are in consort with accompanying rich outer perimeter of PP- and GIR cells, with and without ST, are evident. In a largely spread-out area PP (numbers 1 to 5 and 6) and glucagon (a, b, c) can be found in association with ductular complexes. Two of the smaller islets exhibit an insulin core around which limited glucagon and no PP-IR cells can be found. Ductular activity is more evident than described for day 6½. Although the serial sections for insulin and NPY are too far

removed from one another to determine the exact location of NPY positive cells within islet context, there is some evidence of NPY-IR, mostly around the perimeter of apparent islets similar to that found in figure 4.29.

4.3.5 Day 8

Large irregular areas of IIR (Figure 4.37 on page 144) in association with duct activity were plentiful. Glucagon activity (Figure 4.38 on page 143) was minimal being restricted to one to five cells in most areas, and PP activity only evident in about seven areas (blue - Figure 4.32). There was no evidence of either ST-IR or PYY-IR activity wile NPY-IR was restricted to areas of IIR and ductular activity.

4.3.6 Day 9

Insulin IR was mostly (except in one area) independent of other hormones while GIR was restricted to loose cells. No ST-IR or PP-IR could be found in the area under consideration. PP-IR was found on its own away from other sites of immunoreactivity. No evidence of either NPY or PYY could be found.

4.3.7 Day 9½

Insulin IR was mainly restricted to large and medium well formed and clearly defined islets (Figures 4.40 to 4.42 on page 145) small areas (3 to 5 cells) of IIR were found between islets (Areas 3 and 4; 4 and 5; Figure 4.42) as well as in isolation elsewhere. Glucagon formed a visible interrupted layer of cells around an insulin positive core (Figure 4.42) while somatostatin was virtually absent and PP limited to but a few areas.

4.3.8 Day 10

A number of insulin-rich areas also possessed glucagon immunoreactivity but no PP-IR, while somatostatin immunoreactivity was confined to two areas (arrows). Refer to figures 4.43 to 4.45 on pages 145 and 146.

4.3.9 Day 14

Areas of ductular distention tended to lessen after day 8, causing series for days 9, 9½ and 10 to indicate this. Day 14 (Figure 4.46 on page 146) however showed a rejuvenation of ductular distention. A number of areas possessed IIR tissue in close association with areas

of ductular distention. Most areas of endocrine activity presented varying numbers of cells with differing morphology. Areas 1, 2 and 3 presented a preponderance of insulin IR cells with GIR cells mostly situated on the outside. Areas 4 and 5 contained many more cells with GIR than IIR while ST-IR and PP-IR cells made up the minority in all five areas mentioned. In a number of cases glucagon (G_1 and G_2), insulin (I_{1-3}) and PP (PP_{1-3}) were in isolation associated with ducts.

4.3.10 Day 30

This additional sequence was characterised by equal numbers of large and small LIEF's, making up 22% of the total area of the pancreas (Figures 4.47 to 4.49 on pages 146 and 147). The small insulin positive areas tend to make up one particular area (upper right quadrant of figure 4.49), while the larger islets another. Conspicuously absent were any signs of the ductular activity that characterised earlier slides. The size of LIEF's (numbered 2 in figure 4.47) probably due to the merging of one or more endocrine formations with one another. The majority of LIEF's were glucagon deficient and devoid of PP and ST.

4.4 HISTOLOGICAL ASSESSMENT

4.4.1 Day 1

4.4.1.1 H & E

There is a clear difference to be seen between ligated (figure 4.5 on page 135) and non-ligated (figure 4.14 on page 138) pancreatic tissue in this particular specimen. Although acinar cells and islets appeared normal there were distinctive signs of tissue distension due to oedema in the ligated tissue which was characterised by large gaps/spaces between adjoining lobules, not seen in the non-ligated tissue.

4.4.1.2 Insulin

Pre-existing (designated as such due to a well defined insulin core surrounded by a uniform glucagon mantle) large ($d > 200$ μm) and medium sized (diameter between 100 and 200 μm) islets of Langerhans (cells), as well as islet cell clusters (2 - 5 cells – $d < 50$ μm) in

ligated tissue stained heavily for insulin. Islet tissue was however sparsely scattered throughout the exocrine substance with 9 - 14 elements of varying size per visual field at 40X magnification at the most dense area and 5 - 8 in areas of lower endocrine density.

4.4.1.3 Glucagon

Cells staining positively for glucagon were restricted to the periphery of islets and in most cases formed a near continuous band/layer of cells surrounding the ß-cell core. All endocrine tissue exhibited a glucagon presence, i.e., cell clusters, small, medium and large islets. In many cases, especially medium and large islets, glucagon positive cells formed a double layer of cells around the perimeter. Areas of endocrine activity could be differentiated as follows:

2-5 cell clusters - approximately equal numbers of insulin positive glucagon positive cells,

small islets – virtually equal numbers of insulin and glucagon positive cells (d = 10 - 100 µm),

medium sized islets - approximately 25% of cells glucagon positive (d = 100 – 200 µm),

large sized islets - approximately 15% of cells glucagon positive (d = >200 µm) See materials and methods – Chapter 3, paragraph 3.7.2.

Isolated (single) glucagon positive cells were sparsely scattered throughout the pancreas without any apparent connection/association with established small to large islets and/or clusters, and could be a result of a tangential section through only the mantle of an islet lying deeper in the tissue.

4.4.1.4 Somatostatin (100X)

Somatostatin positive cells were restricted to one or two cells per islet cell cluster, four to five cells per small islet, four to six cells per medium islet and four to eight cells per large islet as seen in cross-section. Somatostatin positive cells were also present in two to three cell clusters between acinar cells, without any apparent association with existing/clearly

defined islets. As was the case for I-, and G-cells, isolated (single) cells staining positive for ST were present between acinar cells/tissue (no photo/figure shown).

4.4.1.5 Pancreatic Polypeptide (PP)

PP-positive cells (in the comparable section of the pancreas to I, G and ST) were isolated single cells not associated with any apparent islets (no photo/figure shown).

4.4.1.6 Neuropeptide tyrosine (NPY)

No evidence (on the same or similar pattern of I, G, ST or PP) was found of cells showing any immunoreactivity for NPY in any part of the pancreas. Apparently healthy exocrine cells in a number of areas however, exhibited the presence of brown deposits, with individual cells staining quite heavily (Figure 4.6 on page 136).

4.4.1.7 Peptide tyrosine tyrosine (PYY)

Cells staining positively for PYY (in the same way as I, G, ST, and PP) were not found. Exocrine cells in the ligated portion of the pancreas however, exhibited the same brown cytoplasmic vesicles as indicated above for NPY which was absent from endocrine tissue as well as non-ligated exocrine pancreas tissue and a section of attached spleen (no photo/figure shown).

4.4.2 Day 1½ (36 hours)

4.4.2.1 H&E (Figure 4.5 on page 136)

Cross- and longitudinally sectioned ductular distension were clearly visible. The centro-acinar spaces appeared enlarged, especially along the periphery of the organ. Islets appeared normal and unaffected.

4.4.2.2 Insulin

Heavy insulin staining was evident in all islets visible as described in 4.4.1.2.

4.4.2.3 Glucagon

Cells in periphery of islets stained only lightly for glucagon. A number of isolated glucagon positive staining cells (apparently not associated with clearly defined islets) were found scattered amongst exocrine tissue. In this slide ductular/acinar distension and/or what look like areas of increased ductular activity were visible.

4.4.2.4 Somatostatin

Very little evidence of somatostatin immunoreactivity (ST-IR) was evident. Only single cells (2 - 3) within an islet and single cells (3 - 4) in whole slide except for one other area where a cluster of eight to twelve ST-positive cells could be viewed.

4.4.2.5 Pancreatic Polypeptide

Only as zero to ten cells around outer periphery of clearly defined islets and as single cells in apparent isolation from other endocrine tissue could be seen. Tissue distension was clearly evident along the periphery of the tissue. Islets appeared normal and unaffected by PDL.

4.4.2.6 NPY (Figure 4.6 on page 136)

Once again (as in 4.4.1.6 above) no cells within the entire series classically exhibited any immunoreactivity both within islet or acinar context. There was however (as above) evidence of some vesicular /brown droplet activity in ligated portions of the specimens which were absent in non-ligated areas. Vesicular/brown droplet activity was however not associated with any islet cells of adjoining unligated tissue.

4.4.2.7 PYY (Figure 4.7 on page 136)

Massive (very obvious) evidence of vesicles and/or brown droplets in the cytoplasm of acinar cells in ligated area of pancreas. Along margins of the organ, where ductular/acinar distension is evident the amount of staining is significantly less. Globules/vesicles/droplets are not present in "process control" slides and considerably less in sham operated controls (SOC's). See figures 4.7 and 4.8 for comparison.

4.4.3 Day 2

4.4.3.1 H&E (Figure 4.9 on page 137)

Cellular (acinar) disorganisation had become evident. In areas where one would expect to find the centro-acinar lumina, distension/vacuolar/ductular activity can be seen to be taking place.

4.4.3.2 Insulin

Islets clearly stained for insulin (only up to medium (d = 100 – 200 μm in size).

4.4.3.3 Glucagon

As scattered cells only (4-5 cells/islets) around periphery. None in acinar tissue as described on days 1 and 1½.

4.4.3.4 Somatostatin

Only a small number of cell clusters positive for ST usually associated with small islet cells.

4.4.3.5 Pancreatic Polypeptide

Large numbers of PP-positive cells around perimeter of clearly defined islets.

4.4.3.6 NPY

Droplets/vesicles/globules present in ligated cells but not present in non-ligated portion. No evidence of vesicles in or around islets.

4.4.3.7 PYY

As for 4.4.1.7 and 4.4.2.7 above.

4.4.4 Day 2½

4.4.4.1 H&E

Exocrine tissue in ligated portion of the pancreas exhibit signs of increased cellular disorganisation (compared to tissue in non-ligated area) characterised by oedema, transient (and mild in some specimens in this group) lymphocyte infiltration, acinar swelling and the increased ductular distension and activity. A number of large islets (d > 200 μm) present in three of the five groups (group 6 unsuitable) showed signs of vacuolisation which could be ascribed to oedema, although not all islets were equally affected. The clear dividing line between islets and adjoining exocrine tissue was now seen to be becoming blurred (under H & E staining), as the glucagon mantle lost definition.

4.4.4.2 Insulin (Figure 4.10 on page 137)

In spite of apparent disturbances to the H & E profile of islets, they appeared as well defined structures and were intact, and stained strongly positive for insulin. Large, medium, small islets and islet cell clusters were present. There were however many more islet cell clusters (of 2-5 cells) as had been evident in any of the previous slides with the exception of day one. Transient mild lymphocyte infiltration was also evident in a number of specimens. There were also, what could be interpreted as isolated areas of exclusively insulin activity, in close proximity to ducts and/or areas of ductular distention.

4.4.4.3 Glucagon (Figure 4.11 on page 137)

Glucagon staining was heavy and extensive and in at least a half of the islets as illustrated in figures 4.1.1 on page 131 and 4.11 on page 138. Staining was more extensive than described under day one (4.2.1.3). Glucagon positive cells were mostly restricted to the periphery of islets, but were evident in islets/endocrine tissue of all sizes as well as in apparent isolation. There were also present in areas where transient mild lymphocyte infiltration and increased ductular activity occurred. The amount/number of cells/tissue staining positive for glucagon exceeded that which had been evident in normal pancreas tissue.

COMMENT. There were areas of insulin and glucagon activity that I have termed ligation induced endocrine formations (LIEF's) in areas of cellular disorganisation and/or not in

association with existing or obvious islets; not always although sometimes, exhibiting dual immunoreactivity (I & G).

4.4.4.4 Somatostatin (Figure 4.12 on page 138)

Somatostatin positive cells were not restricted to the perimeter of clearly well defined islets but also occurred within the insulin core. In large islets (d = >200 μm) the number was approximately 8 - 20 cells in medium islets (d = 100 – 200 μm) 6 - 12 ST- positive cells and in small islets (d = 10 – 100 μm) 4 - 8 ST-positive cells. Then there were also large numbers of single/isolated ST-positive cells and 3 - 5 cell clusters scattered within, what appeared to be endocrine tissue. There were approximately 20 - 30 ST-positive cells/field of vision at 100X magnification anywhere on the slide. Cells (enterocytes) in an adjoining piece of small intestine contained large numbers of ST-positive cells. Mild lymphocyte infiltration was evident in a minority of specimens, while heavy infiltration was evident in only one (Figure 4.12 on page 138).

4.4.4.5 Pancreatic Polypeptide

Large numbers of PP-positive cells, on a similar scale as for days one through two, were visible. Most were associated with islets and were situated around the perimeter of each islet. Isolated areas of one to five cells outside islet context were numerous and associated with what could be appeared to be areas of increased ductular activity (in areas of evident cellular disorganisation). Refer to figure 4.12 on page 138. As for somatostatin, PP was present with ST-positive cells within what appeared to be endocrine tissue.

4.4.4.6 NPY

No outstanding obvious NPY activity associated with either existing islets or developing ductular complexes at 100X magnification was evident; neither was there any obvious vacuolar/vesicular/droplet staining present as had been the case in previous slides (days 1, 1½ and 2). The only evidence in the entire series were single cells which stained NPY positively (lightly) and which seemed to be associated with ductules. This was the first evidence of a cell staining NPY-positive until now, similar to that for day 3½ (figure 4.24 on page 141).

4.4.4.7 PYY

Little vacuolar/vesicular/droplet activity in ligated tissue was to be seen. There seemed to be a higher incidence of the presence of cytoplasmic droplets in one particular area of non-ligated pancreas, in one sequence in the series.

4.4.5 Day 3

4.4.5.1 H&E (Figure 4.15 on page 138)

Ligated tissue differed substantially from adjacent non-ligated pancreas tissue in the same animal. Cellular disorganisation, cell deletion, mild lymphocyte infiltration, ductular distension and/or distension of centro-acinar lumina are all obviously visible.

4.4.5.2 Insulin (Figure 4.16 on page 139)

Clearly defined immunoreactive areas staining positive for Insulin are evident as large (d > 200 μm – no. 3 on figure), medium (d = 100 – 200 μm) and small (d < 100 μm; no's 2 – 7 on figure) structures and single insulin positive cells (not shown) and islet clusters scattered throughout the tissue. Ductular distension is more evident in a generally disorganised exocrine pancreas, but also within islets. Only insulin and glucagon were found together in one large islet (no. 3; around their periphery) but absent from the remaining areas of IIR (1, 4, 5, 6 and 7). Little evidence of GIR was to be seen, in figure 4.18 on page 139.

4.4.5.3 Glucagon (Figure 4.17 on page 139)

Perimeter cells of islets staining positive for glucagon were visible but substantially less pronounced than for normal islets. Mild lymphocyte infiltration was evident. Large sections of the slide have a brown tinge, consistent with the same slide in days 1, 1½ , 2, 2½ and SOC, but absent in process (method) control.

4.4.5.4 Somatostatin

Cells around periphery of islets, as well as single cells, more towards the centre of islets stained positively for ST, but was restricted to two to five cells per existing islet but no-

where else. Closer scrutiny (400X) revealed ST-positive cells near a blood vessel (in one specimen) and in the vicinity of a number of distended ducts, however very scarce.

4.4.5.5 Pancreatic Polypeptide (Figure 4.18 on page 139)

A number of PP-positive cells were associated with clearly defined islets. Elsewhere islets were found with a number of PP-positive cells in their periphery and groups of three to five cells in surrounding connective tissue. The figure however indicates the presence of PP-IR amongst acinar tissue, a finding in three out of five specimens in the series.

4.4.5.6 NPY

Four to five NPY-positive cells (surrounding one islet only) could be found, similar to figure 4.29 for day 4.

4.4.5.7 PYY

No evidence of any PYY immunoreactivity what so ever to be found in this series.

4.4.6 Day 3½

4.4.6.1 H&E (Figure 4.19 on page 139)

Large numbers of clearly distended ducts and/or areas of increased ductular activity. Each was surrounded by 6 - 15 epithelial cells (thus: varying in size) had become evident. Poorly defined islets were also evident, both with and without ductular activity. No lymphocyte infiltration was evident. Immunoreactivity for insulin, glucagon, somatostatin and pancreatic polypeptide was mostly not in association with one another. Ligated tissue situated next to normal tissue in the slide emphasised the changes that had taken place.

4.4.6.2 Insulin (Figure 4.20 on page 140)

Tissue (in 4 out of 5 specimens in the series) was characterised by a profusion LIEF's, ICC positive for insulin. These endocrine formations were of all sizes, and in close proximity to each other as already mentioned in paragraph 4.1.1. All LIEF's, small clusters and single cells were all associated with distended ducts and areas of increased ductular

activity, ductular segments and peripheral smudging. Significant numbers of individual insulin positive cells, in close association with ductules, were to be seen (Figure 4.19).

4.4.6.3 Glucagon (Figure 4.21 on page 140)

Significantly less glucagon immunoreactivity in two areas where insulin positive LIEF's can be distinguished, compared to the glucagon content at days one to 2½ are to be seen. Glucagon positive cells are also associated with (longitudinal and circular) ducts/ductules and in those areas where insulin positive cells are plentiful. A number of ducts are in close association with, or in the centre of LIEF's. The pattern of glucagon immunoreactivity described in paragraph 4.4.1.3 above can be found in sections of pancreas tissue not having undergone PDL. In adjoining normal tissue GIR formed a thick (almost continuous) band two to three cells thick, around a core of ß-cells.

4.4.6.4 Somatostatin (Figure 4.22 on page 140)

Somatostatin immunoreactivity in non-ligated portions of the pancreas, in these specimens, were more profuse than described for any of the proceeding days (ligated and/or non-ligated tissue, as described in 4.4.1.4, 4.4.2.4, 4.4.3.4, 4.4.4.4 and 4.4.5.4) with 10 - 20 cells per islet, mostly around the periphery (mostly inside glucagon positive cell layer) but not necessarily so, while existing islets in the ligated portion of the tissue were devoid of ST-positive cells. Somatostatin activity is found in groups of 2 - 5 cells scattered randomly throughout the stroma, in association with ductular activity. Cells also vary in their intensity/magnitude of ST immunoreactivity with some cells staining more heavily than others (at 250X). At 400X magnification ST-positive cells exhibit a distinctly granular appearance. At two areas where LIEF's are reasonably well defined (A & B in Fig. 4.20) there is no somatostatin immunoreactivity and both islets are in close association with a large duct.

4.4.6.5 Pancreatic Polypeptide (Figure 4.23 on page 140)

PP-IR appeared as one or two cells around the periphery of clearly definable areas of IIR. Single cells in association with ductules however also exhibited PP immunoreactivity, so also did cells (staining lightly) in the connective tissue, in areas which appeared to be LIEF's. Some of these areas stained positively for I, G and ST as well. At a number of places PP-positive cells were clearly in association with ductules. PP-IR in unligated

adjoining tissue was restricted to the periphery of clearly defined islets with seven to twelve cells/islet (no photo), as was to be expected in normal islets.

4.4.6.6 NPY (Figure 4.24 on page 141)

A number of NPY positive cells were to be found in clusters of 3-5 cells, not however associated with existing or well defined islets but only with areas of increased ductular activity and connective tissue. Normal, unaffected acinar tissue however, did not possess such cells, but resembled the phenomenon described in 4.4.1.6 and 4.4.2.6 above.

4.4.6.7 PYY (Figure 4.25 on page 141)

A number of slides exhibit limited PYY activity, restricted to the ligated areas only, with 2 - 5 cells per field of vision at 250X magnification, not associated with any areas of endocrine activity but appear randomly in connective tissue.

4.4.7 Day 4

4.4.7.1 H&E (Figure 4.26 on page 141)

Cellular disorganisation and the increase in ductular activity appeared to have reached a stationary phase. Areas of endocrine activity were not easily discernible in this group. The tissue appeared diffuse and non-specific.

4.4.7.2 Insulin (Figure 4.27 on page 141)

A number of IIR areas all with a diameter of less than 100 μm were evident in these specimen. Insulin immunoreactivity is also evident in clusters of cells and single cells in devoid of any other endocrine presence, in association with surrounding connective tissue and between areas of ductular activity.

4.4.7.3 Glucagon

As in 4.4.5.3 above, glucagon immunoreactivity, in the specimen on exhibit, is much reduced compared to proceeding days post PDL. This was a typical finding of the remaining samples in the series. Single cells around the periphery of an area of insulin immunoreactivity (designated area "A") are glucagon positive. Single cells and/or groups

of cells around regions of increased ductular activity and in their surrounding connective tissue, exhibiting varying degrees of immunoreactivity, are indicated on the accompanying overlay

4.4.7.4 Somatostatin

Somatostatin immunoreactivity (ST-IR) is evident only as faint smudges in two areas. Firstly associated with duct lumina, both in and out of islet context and secondly in amongst IIR cells.

4.4.7.5 Pancreatic Polypeptide (Figure 4.28 on page 142)

Large numbers (8-15 cells) of PP-positive cells were often found not only around large (and what appear to be) islet-like structures, but randomly throughout the insulin positive tissue of LIEF's. PP-positive cells were also to be found as single cells or small clusters in connective tissue and/or in association with poorly constituted islet-like structures and ductular tissue. In two areas clusters of 10 - 30 closely clumped heavily staining PP-positive cells could be seen in this specimen.

4.4.7.6 NPY (Figure 4.29 on page 142)

Three to five very lightly staining NPY-positive cells could be found around the outer perimeter of approximately two out of every five islets visible under 100X magnification.

4.4.7.7 PYY

As for paragraph 4.4.6.7.

4.4.8 Day 4½

4.4.8.1 H&E

Significantly more ductular activity was evident in this group compared to the previous day (day 4). Islets appeared largely unaffected although ductules were in contact with the perimeter of areas of endocrine activity as well as inside the line of the cell boundaries.

4.4.8.2 Insulin (Figure 4.30 on page 142)

Large numbers of very large areas of insulin immunoreactivity (larger than normal islets found in adjacent non-ligated tissue) staining positively for insulin are prominent (Area A, 1, 2 and 3). Smaller IIR areas are also present (4 - 8), but of importance is the presence of a duct, albeit small, with all the cells, making up it's periphery, positive for insulin. Clumps/clusters of insulin positive cells are also present between adjoining ductules (not shown). Until this stage, islets have tended to be round or oval. For the first time also we find islets clearly encroaching on each other and clusters of islets fusing to form larger irregular structures as is the case in figure 4.47 on page 146, area "2". (See also Figure 4.31 on page 142.

4.4.8.3 Glucagon (Figure 4.31 on page 142)

The duct mentioned in 4.4.8.2 above and indicated on figure 4.31, showed no immunoreactivity (IR) to glucagon. Glucagon IR (GIR) until now (4.4.1.3) has been restricted mainly to a uniform but approximated single cell layer in large islets, and double cell layer in medium to small islets, surrounding a core of ß-cells. In the majority of cases (90% +) the G-positive cells have surrounded a ß-cell core of clearly and well defined islets. In this slide GIR is not restricted to the periphery of islets, neither is it only a single or double uniformly distributed cell layer but is it randomly scattered with a lack of uniformity, and in a number of instances, stretches across the cellular gap of adjoining areas of endocrine activity. The greater amount of GIR is in association with areas of increased ductular activity and connective tissue.

4.4.8.4 Somatostatin

Slight to moderate staining of cells, around and what appear to be existing islets. There are also areas of increased ductular activity where there are smudges of ST activity. Many cells in connective tissue are also ST-positive.

4.4.8.5 Pancreatic Polypeptide

Significantly smaller numbers of cells in and around clearly defined islets are PP-IR. Less (reflected in figure 4.1.1d), compared to adjacent unligated tissue and/or any of the preceding days post- PDL especially days 2, 2½ and 3½. Most cells were restricted to the perimeters of areas of insulin immunoreactivity, ductular activity and connective tissue.

In normal adjacent non-ligated pancreatic tissue islets possessed 8 - 12 PPIR cells, around the perimeter of islets.

4.4.8.6 NPY

Clear cells, IR for NPY, can be found around the perimeter of a limited number of what appear to be, islet-like (endocrine) structures, and in association with areas of ductular activity. This is confirmed by the corresponding morphometric quantification illustrated in figure 4.1.1(e).

4.4.8.7 PYY

No PYY immunoreactivity was evident.

4.4.9 Day 5

4.4.9.1 H&E

Specimens exhibiting intense ductular activity is evident in three out of five samples in this series. Larger well defined and prominent islets seemed unaffected by the morphological changes occurring around them.

4.4.9.2 Insulin

A number of medium to small islets ($d < 100$ μm), mostly associated with ductules, stained IR for insulin. Significant numbers of small IIR areas and cell clusters were visible, so also single cells.

4.4.9.3 Glucagon

Glucagon positive cells, not conforming (one or two cells per area of IIR and/or clusters of only GIR cells) to the normal pattern discussed in 4.4.1.3 were numerous in all specimens in this series. Significant numbers of G-positive cells associated with areas of ductular activity were to be seen. In one serial section overlapping of GIR with ST-IR could be seen. GIR was also evident as part of duct lumina (two places).

4.4.9.4 Somatostatin

As for paragraph 4.4.8.4.

4.4.9.5 Pancreatic Polypeptide

The pattern for the distribution seen thus far (described under day 2) of PP-positive cells was evident in all specimens in this series. Cells with PP-IR are more numerous around vaguely/ill-defined areas of insulin immunoreactivity (most probably LIEF's) than described in 4.4.1.5. Most PP-positive tissue is however not associated with islets but with areas of ductular activity and/or areas of general cellular disorganisation. Cells with PP-IR were often to be found in amongst IIR cells.

4.4.9.6 NPY

Only 5-10 cells in two areas on one slide of the series (per field of vision at 100X) are IR for NPY. A half of the cells are lightly stained and the remainder more heavily without any apparent connection to any areas of other endocrine activity, but LIEF's and areas of increased ductular activity.

4.4.9.7 PYY

Single cells with IR for PYY in association with both islets (existing and emerging) and

ducts can be detected in this slide, which was the only specimen in the series to demonstrate PYY activity.

4.4.10 Day 5½

4.4.10.1 H&E

Significantly more islets are visible per field of vision at 100X than in previous groups. Areas of IIR are also large (d > 200 μm) to medium (d = 100 – 200 μm) in size and well defined. Many duct-like structures, in what were areas of acinar cell occupation, are visible.

4.4.10.2 Insulin (Figure 4.32 on page 143)

Insulin IR-cells make up the most of the entire lumina of a number of individual ducts (indicated by the arrow on the figure). Many clearly defined islets with strong immunoreactivity for insulin are visible in all the slides for this series. Numerous small islets and islet cell clusters in association with emerging islets and areas of ductular activity were also present.

4.4.10.3 Glucagon (Figure 4.33 on page 143)

More cells per islet are present in this section than in the proceeding two groups (days 4½ and 5). The placement and number appear to be moving towards the pattern of cell distribution mentioned in 4.4.1.3. Glucagon positive cells could also be found inside islets and 2-3 cells in association with ductules (not evident on this specimen but on others in the series), areas of increased ductular activity, insulin immunoreactivity and LIEF cells.

4.4.10.4 Somatostatin

Only small numbers of ST-positive cells (lightly stained) are visible in what appears to be an aggregations of loose cells. No ST-positive cells are associated directly with insulin positive endocrine tissue or areas of increased ductular activity.

4.4.10.5 Pancreatic Polypeptide

The most clearly defined areas of insulin activity possess 5 - 8 PP-positive cells around the outer perimeter. Most (90% +) of PP-positive cells are to be found in association with these areas. There are however also PP-IR cells associated with areas of increased ductular activity and connective tissue.

4.4.10.6 NPY

Two to five NPY-positive cells are situated in the same peripheral band around a ß-cell core in insulin immunoreactive areas as G-, ST-, and PP- cells in two specimens. NPY positive cells are also associated with areas of increased ductular activity.

4.4.10.7 PYY

Cells positive for PYY are only just visible as single cells and two to three cell clusters in association with or usually in close proximity to islets and duct lumina in only one specimen.

4.4.11 Day 6½

4.4.11.1 H & E

Ligated tissue consists of existing, clearly defined healthy islets in a sea of disorganised tissue. The latter consists of ductules (lumen surrounded by cells) of varying sizes, from small (3 - 5 cells making up the circumference of the duct) to medium (5 - 8 cells) and large (8 - 12 cells). Also present are acinar-like clusters of cells with darker cytoplasm and more prominent nuclei than surrounding cells, resembling a circle of cells which present no lumen.

4.4.11.2 Insulin (Figure 4.34 on page 143)

Ligated tissue is clearly distinguishable from surrounding unligated tissue. Areas of IIR are large and well defined. Smaller areas of IIR and clusters of 3-5 cells around ducts and in connective tissue are also evident.

4.4.11.3 Glucagon

Glucagon positive cells are situated in the periphery of clearly defined areas of LIEF's, ICC positive for insulin, although not in a uniform layer around the perimeter of these structures. Large IIR areas average 10 - 20 G-positive cells, while medium IIR positive areas average five to ten and small islets, three to five. Very little G-activity can be found outside of insulin positive areas.

4.4.11.4 Somatostatin

Substantial amounts of ST-positive cells are present in an adjoining piece of intestinal tissue in one of the specimens. In normal pancreatic tissue ST is restricted to the

perimeter of areas of IIR (2 - 5 cells/islet) and in ligated tissue there is much less ST activity, restricted to isolated cells in small clusters (2 - 3 cells).

4.4.11.5 Pancreatic Polypeptide

Pancreatic polypeptide positive cells are mostly situated around the outer perimeter of islet-like structures in duct ligated pancreas tissue but also with less prominent areas of IIR and/or endocrine tissue. There is more PP activity in ligated than non-ligated tissue (6 - 12 vs. 3 - 5 cells/area respectively).

4.4.11.6 NPY

No NPY activity is obvious in non-ligated tissue in any of the slides in this series. Only three to four cells (visible as single cells) in one area of ligated tissue could be seen in one of the specimens.

4.4.11.7 PYY

No PYY activity is evident in normal or ligated section of any of the slides in the series.

4.4.12 Day 7½

4.4.12.1 H & E

Large numbers of duct-like structures, of all sizes, are scattered profusely throughout ligated tissue. Many small areas of IIR are scattered in amongst the duct-like structures. All evidence of acinar cells has disappeared from ligated tissue. Many large well established islets apparently unaffected by the morphological changes in the surrounding tissue were found in 3 of the 5 specimens in the series, are also present. Compared to adjoining normal tissue there is visibly much more endocrine tissue to see in this section.

4.4.12.2 Insulin (Figure 4.35 on page 143)

Many large, medium and small endocrine areas staining insulin positive. LIEF's are closely associated with areas of increased ductular activity.

4.4.12.3 Glucagon

As for 4.4.11.3.

4.4.12.4 Somatostatin (Figure 4.36 on page 144)

In non-ligated (healthy/normal) adjoining pancreas tissue ST-positive cells can be found in the outer perimeter of islets (2 - 6 cells/islet). This was however not the case in all islets. No ST activity outside of the islets was detected in normal tissue. In ligated tissue however, islets were virtually devoid of ST-positive cells (<1 cell/islet), while significant numbers of ST-positive cells were found clustered together in 10 - 20 cells in a number of areas, apparently detached from any other endocrine or ductular activity.

4.4.12.5 Pancreatic Polypeptide

As for 4.4.11.5.

4.4.12.6 NPY

Quite frequent staining of cells located around the perimeter of areas of IIR but less prominent in rest of the ligated pancreas except in one section of ill defined connective tissue could be seen.

4.4.12.7 PYY

No PYY activity in association with areas of endocrine or ductular activity.

4.4.13 Day 8

4.4.13.1 H & E

Tissue in ligated portion of the slide is characterised by the same increase in ductular activity as described in 4.4.11.1 and 4.4.12.1.

4.4.13.2 Insulin (Figure 4.37 on page 144)

Clearly defined IIR areas possess an inner core of insulin positive cells. Also present are large numbers of small islets and emerging islets in close association with ductules. In

this group, areas of IIR had substantially less surrounding glucagon rich mantle cells than in non-ligated tissue.

4.4.13.3 Glucagon (Figure 4.38 on page 144)

Cells with glucagon immunoreactivity are in close association with areas of obvious islet and insulin activity. Glucagon positive cells are however not restricted to a clearly defined outer perimeter as previously described (4.4.1.3) but more loosely scattered amongst insulin positive tissue.

4.4.13.4 Somatostatin

Areas that have exhibited insulin and glucagon immunoreactivity show no IR for somatostatin. The only ST-positive cells visible in the specimens are in the connective tissue and around areas of increased ductular activity.

4.4.13.5 Pancreatic Polypeptide

Immunoreactivity for PP in a number of insulin positive endocrine areas is restricted to two or three cells per area and not necessarily associated with the perimeter of the area. PP-cells also vary in the magnitude of their immunoreactivity, i.e., some cells stained more heavily than others.

4.4.13.6 NPY (Figure 4.39 on page 144)

Wide spread NPY-IR, both in association with areas of IIR, increased ductular activity and in connective tissue, are visible in only the preparation exhibited. Islet NPY cells are located mainly around the perimeter of areas of insulin immunoreactivity and is the most of any of the sequences up until now.

4.4.13.7 PYY

Negligible PYY activity (very slight) is in connective tissue in one of the specimens.

4.4.14 Day 9

4.4.14.1 H & E

Areas of pre-ligation acinar lobules are clearly definable. The space occupied by acinar tissue is now filled with connective tissue, fat cells, lymphocytes, enlarged and irregular islet-like structures and reduced numbers of distended duct-like structures on a larger scale as seen here to now. Many more areas of IIR (large ($d > 200$ μm) and clearly defined) are visible in ligated tissue (2 - 3 at 100X) compared to non-ligated (normal) adjoining tissue (0,5 at 100X). Connective tissue aggregates surrounding these areas appeared to be reforming into acinar tissue (at 250X) in one of the specimens. Ductules/ductular activity no longer had large open spaces but the cells making up their lumen seemed to be gaining cytoplasmic substance.

4.4.14.2 Insulin

ICC for insulin reveals many more areas of IIR in ligated portion of the pancreas as was evident under H & E as mentioned in 4.4.16.1 above. Insulin rich areas (IRA) varied significantly in shape and size compared to the relative uniformity of islets visible in adjoining non-ligated tissue.

4.4.14.3 Glucagon

Islets in non-ligated portion of the pancreas conform to the pattern described in 4.4.1.3 with glucagon positive cells occupying a band of cells on the outer perimeter of clearly defined IRA's. Areas of insulin immunoreactivity in the ligated portion of the pancreas however differ substantially from this pattern and also from one another. In many islets GIR is random throughout islets and only really came near to the pattern for non-ligated islets in those islets that were very large. Substantial amounts of GIR were also to be found, out of any apparent connection with other endocrine cell types i.e., as three to five cell clusters within surrounding connective tissue.

4.4.14.4 Somatostatin

No ST immunoreactivity was evident in either ligated or non-ligated portions of the (tissue) pancreas. There were however large clusters of ST-positive cells in the intestinal section (to be expected) and also in two adjoining lymph nodes and in the spleen?

4.4.14.5 Pancreatic Polypeptide

PPIR in the non-ligated portion of the pancreas was restricted to cells in the outer perimeter of islets. Most islets in the ligated portion of the pancreas exhibited IR for PP, with PP-positive cells restricted mainly to the perimeter of islets, but also found in clusters of three to five cells in connective tissue.

4.4.14.6 NPY

No NPY immunoreactivity is evident in any preparations.

4.4.14.7 PYY

No PYY immunoreactivity evident except as faint brown smudges around the ß-cell core of islets in normal non-ligated portion of the pancreas tissue in one specimen.

4.4.15 Day 9½

4.4.15.1 H & E (Figure 4.40 on page 145)

This representative slide is similar to the account given in 4.4.14.1 above. There is however evidence of compacting i.e., interlobular and inter IRA connective tissue seems to be less, resulting in IRA's being closer together (±8 to 12 IRA's per field of vision at 100X magnification).

4.4.15.2 Insulin (Figure 4.41 on page 145)

The above comment (4.4.15.1) is evident from the large amount of insulin positive tissue visible at 100X magnification. Areas of IIR are not restricted to islet-like structures but can also be found as nests/clusters of cells within the connective tissue surrounding and/or in between IRA's (15 to 18 nests/clusters per field of vision at 100X).

4.4.15.3 Glucagon (Figure 4.42 on page 145)

Glucagon was clearly in association with clearly defined IRA's. In a number of areas, small IIR positive cell clusters had no association with glucagon. Conversely, there were a number of extra islet GIR positive cell clusters with no apparent association to insulin.

Glucagon immunoreactivity associated with islet-like structure was present as an irregular mantle around a very prominent insulin core.

4.4.15.4 Somatostatin

Refer to comments in paragraph 4.4.14.4.

4.4.15.5 Pancreatic Polypeptide

Significant (more than normal) numbers of PP-positive cells are to be found around the perimeter of IRA's as well as within the ß-cell core. Many PP-positive cells are also associated with areas of apparent ductular activity and/or and connective tissue.

4.4.15.6 NPY

Individual cells around the perimeter of clearly visible IRA's stain positively for NPY in one specimen. NPY-IR causes a "brownish" tinge to many islets. No NPY activity could be detected in areas of apparent ductular activity or LIEF's.

4.4.15.7 PYY

No evidence of PYY activity could be found around IRA's or in areas of ductular activity in any of the specimens in the series.

4.4.16 Day 10

4.4.16.1 H & E (Figure 4.43 on page 145)

The area selected on this slide consists of a mass of connective tissue with a number of ducts with clearly defined cells making up their lumen, two medium sized IRA's and one small IRA is in the field of vision at 100X magnification (See overlay transparency).

4.4.16.2 Insulin (Figure 4.44 on page 146)

The same area stained for insulin immunoreactivity reveals a further eleven areas of IIR not identifiable as islets under H & E (see 4.4.16.1). An investigation into other regions of the slide revealed a massive increase of IRA's (of all sizes) in relative close proximity to each other.

4.4.16.3 Glucagon (Figure 4.45 on page 146)

Most of the areas of insulin IR also possess glucagon IR. Although the glucagon positive cells did not conform to the "norm" described in 4.4.1.3, it would appear that cellular reorganisation is taking place to achieve this.

4.4.16.4 Somatostatin

Only two-to-three ST-positive cells are associated with each IRA (as per 4.4.16.1). Present however, are also individual and two to three cell clusters of ST-IR in areas where endocrine tissue exists (as per 4.2.16.2) but not in clearly defined islet context.

4.4.16.5 Pancreatic Polypeptide

In the area of the slide under review, PP-IR is to be found around the perimeter of these (three in number) IRA's that are clearly definable. Smudges of PP-IR can also be seen in those areas of IR for other endocrine hormones (mentioned above).

4.4.16.6 NPY

Relatively large amounts of NPY positive cells were found in the perimeter area of a number of IRA's of the section under consideration. Elsewhere in the slide NPY-IR could also be found in amongst connective tissue (not associated with islets) as clearly defined NPY-positive cells, the rest of the series was devoid of any NPY activity.

4.4.16.7 PYY

No evidence of any PYY-IR could be found in any of the preparations.

Day 11

4.4.16.1 H & E

A number of specimens showed signs of acinar reformation (two out of six). Endocrine tissue was not readily visible. No signs of inflammation of oedema evident.

4.4.16.2 Insulin

Large number of small ($d < 100$ μm) and medium ($d = 100 – 200$ μm) IRA's

4.4.16.3 Glucagon

Glucagon restricted to a thin outer mantle around islet-like structures.

4.4.16.4 Somatostatin

Slide devoid of somatostatin immunoreactivity.

4.4.16.5 Pancreatic Polypeptide

Slides devoid of PP-IR activity.

4.4.16.6 NPY

No evidence of any NPY-IR could be found in any of the preparations.

4.4.16.7 PYY

No evidence of any PYY-IR could be found in any of the preparations.

4.4.16 Day 12

4.4.17.1 H & E

4.4.17.2

As for paragraph 4.4.16.1 above.

4.4.17.3 Insulin

As for paragraph 4.4.16.2 above.

4.4.17.4 Glucagon

As for paragraph 4.4.16.3 above.

4.4.17.5 Somatostatin

As for paragraph 4.4.16.4 above.

4.4.17.6 Pancreatic Polypeptide

As for paragraph 4.4.16.5 above.

4.4.17.7 NPY

No evidence of any NPY-IR could be found in any of the preparations.

4.4.17.8 PYY

No evidence of any PYY-IR could be found in any of the preparations.

4.4.17 Day 13

4.4.18.1 H & E

A number of specimens showed signs of acinar formation (three out of six). Endocrine tissue was not readily visible. No signs of inflammation or oedema evident.

4.4.18.2 Insulin

Large numbers of IRA's in close proximity to one another.

4.4.18.3 Glucagon

Glucagon restricted to a thin outer mantle around islet-like structures.

4.4.18.4 Somatostatin

Tissue in all specimens devoid of ST-IR.

4.4.18.5 Pancreatic Polypeptide

Slides devoid of PP-IR.

4.4.18.6 NPY

No evidence of any NPY-IR could be found in any of the preparations.

4.4.18.7 PYY

No evidence of any PYY-IR could be found in any of the preparations.

4.4.19 Day 14

4.4.19.1 H & E

Although islets in the ligated tissue are discernible, they are accompanied by a renewed proliferation of ductular activity. Established islets seen thus far in series for day prior to day 14 have not retained their identity. In their place are islets with irregular appearance in close association with ducts/areas of ductular proliferation.

4.4.19.2 Insulin

Areas, not discernible as islets, of IIR, large, medium and especially small with a fragmentary appearance were to be found in close (sometimes intimate) relationship to ductules.

4.4.19.3 Glucagon (Figure 4.42 on page 145)

Areas of GIR did not conform to the pattern heretofore viewed, instead GIR was associated in and around areas of IIR (not restricted to the perimeters of IIR tissue). Glucagon-positive cells were often in close association with ductules.

4.4.19.4 Somatostatin

Somatostatin-positive cells were found in and around areas of endocrine cell activity, mostly associated with ductules.

4.4.19.5 Pancreatic Polypeptide

Cells immunoreactive to PP (clearly visible) were around the perimeter of emerging/

developing concentrations of endocrine tissue and occasionally out of islet context around ductular activity.

4.4.19.6 NPY

No NPY activity found.

4.4.19.7 PYY

No PYY activity found.

4.4.20 Thirty Days

4.4.20.1 H & E

The tissue in this group was characterised by the continued absence of acinar tissue and the diffuse appearance of the surviving stroma, which resembled loose connective tissue. Distinctly separate round, oval or ellipsoid (islet-like) structures of varying size were observed.

4.4.20.2 Insulin (Figures 4.47 on page 146 and 4.49 on page 147)

Insulin positive areas were abundantly evident. In most specimens though, the insulin positive core exhibited distinct signs of vascular oedema. Many large and small areas of IIR were scattered throughout an undifferentiated stroma. A number of islet-like structures could be seen to be merging.

4.4.20.3 Glucagon

Glucagon immunoreactivity was restricted to a thin mantle around many of the larger areas of insulin immunoreactivity but blatantly absent from smaller areas of IIR.

4.4.20.4 Somatostatin

All specimens were totally devoid of ST-IR.

4.4.20.5 NPY

All specimens were totally devoid of NPY-IR.

4.4.20.6 PYY

All specimens were totally devoid of PYY-IR.

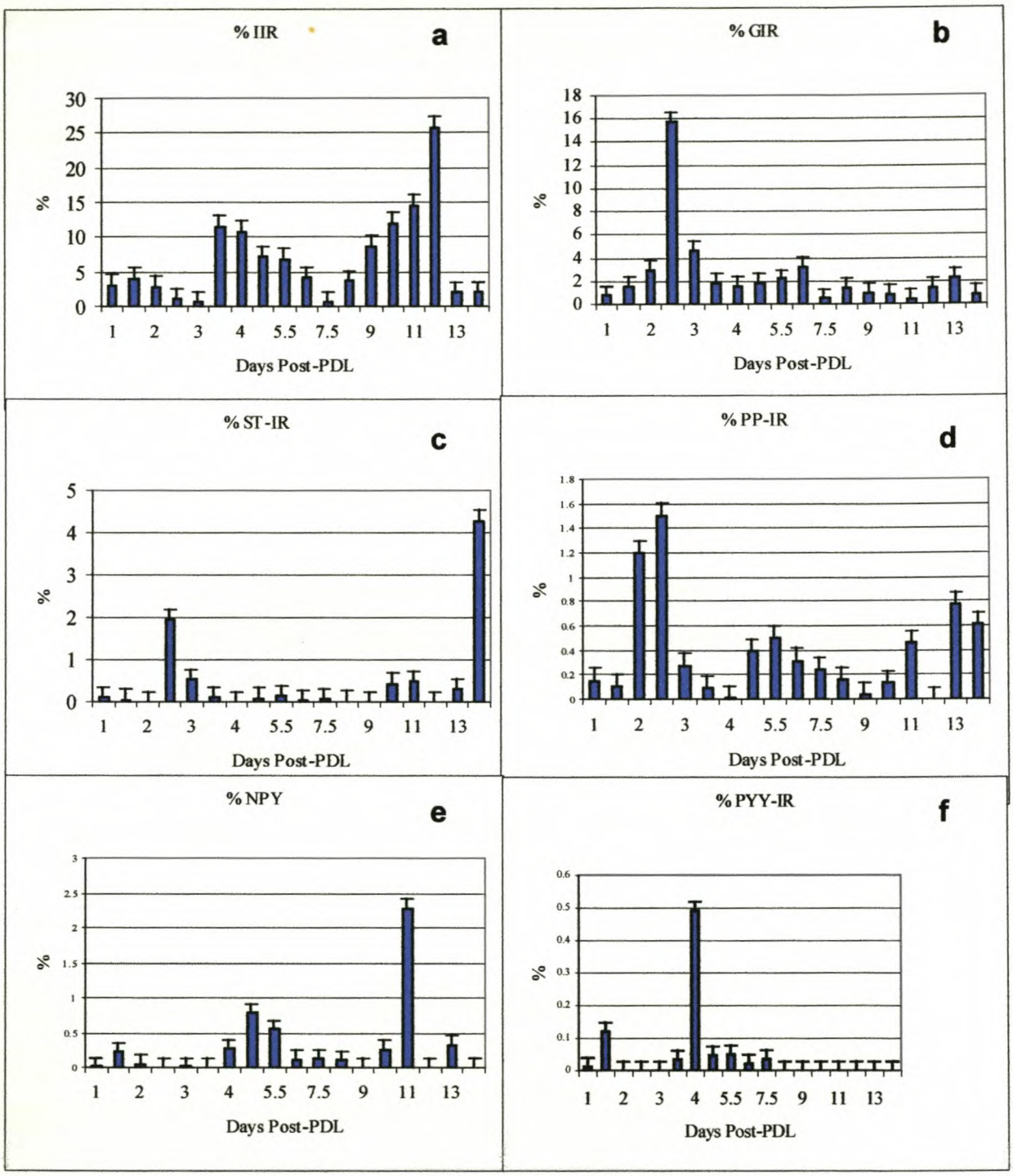


Figure 4.1.1: Series graphs indicating endocrine positive areas in ligated tissue expressed as a percentage of the total area studied for Insulin(I), Glucagon(G), Somatostatin(ST), Pancreatic Polypeptide(PP), Neuropeptide tyrosine (NPY), Peptide tyrosine tyrosine (PYY).

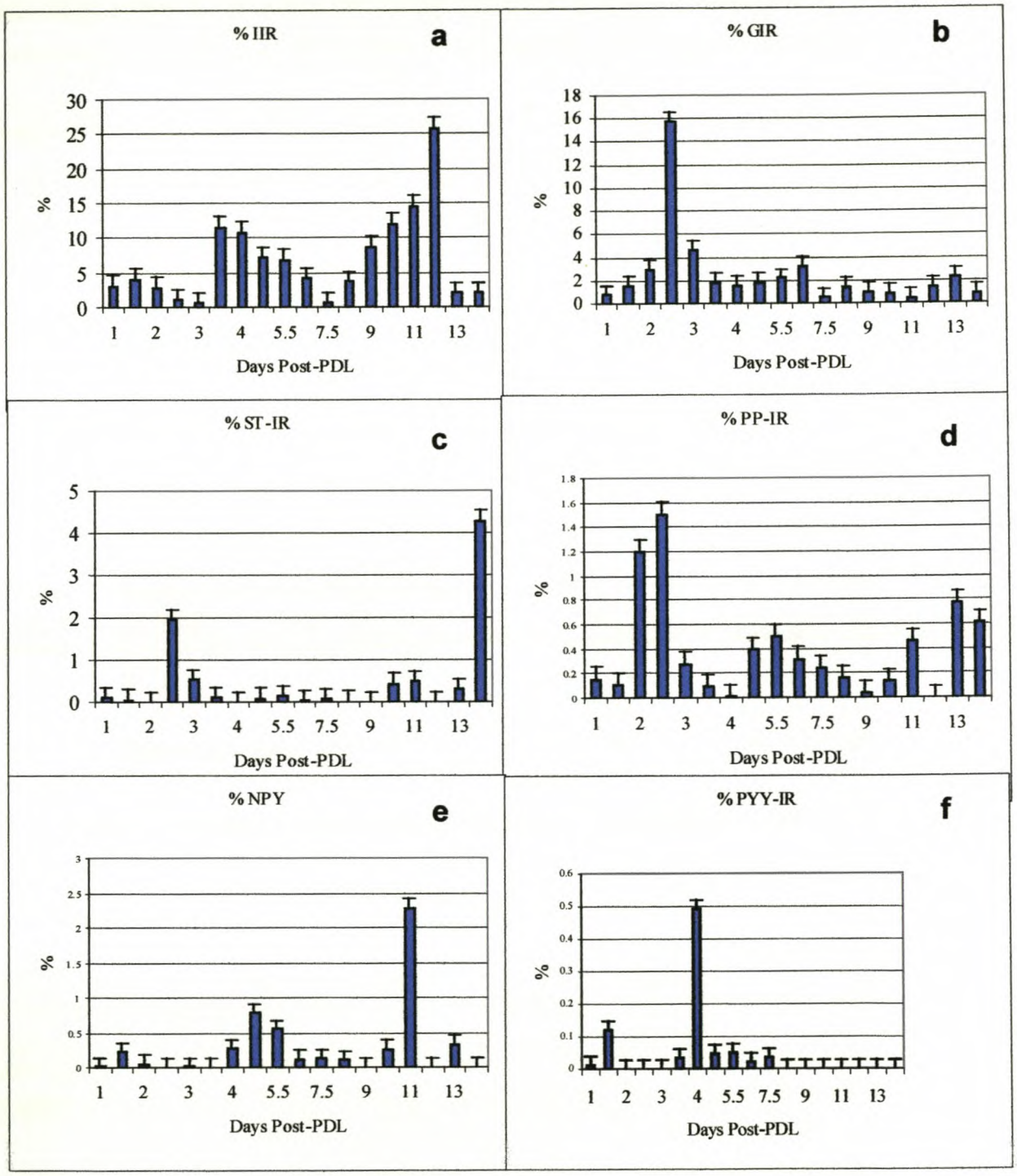


Figure 4.1.1: Series graphs indicating endocrine positive areas in ligated tissue expressed as a percentage of the total area studied for Insulin(I), Glucagon(G), Somatostatin(ST), Pancreatic Polypeptide(PP), Neuropeptide tyrosine (NPY), Peptide tyrosine tyrosine (PYY).

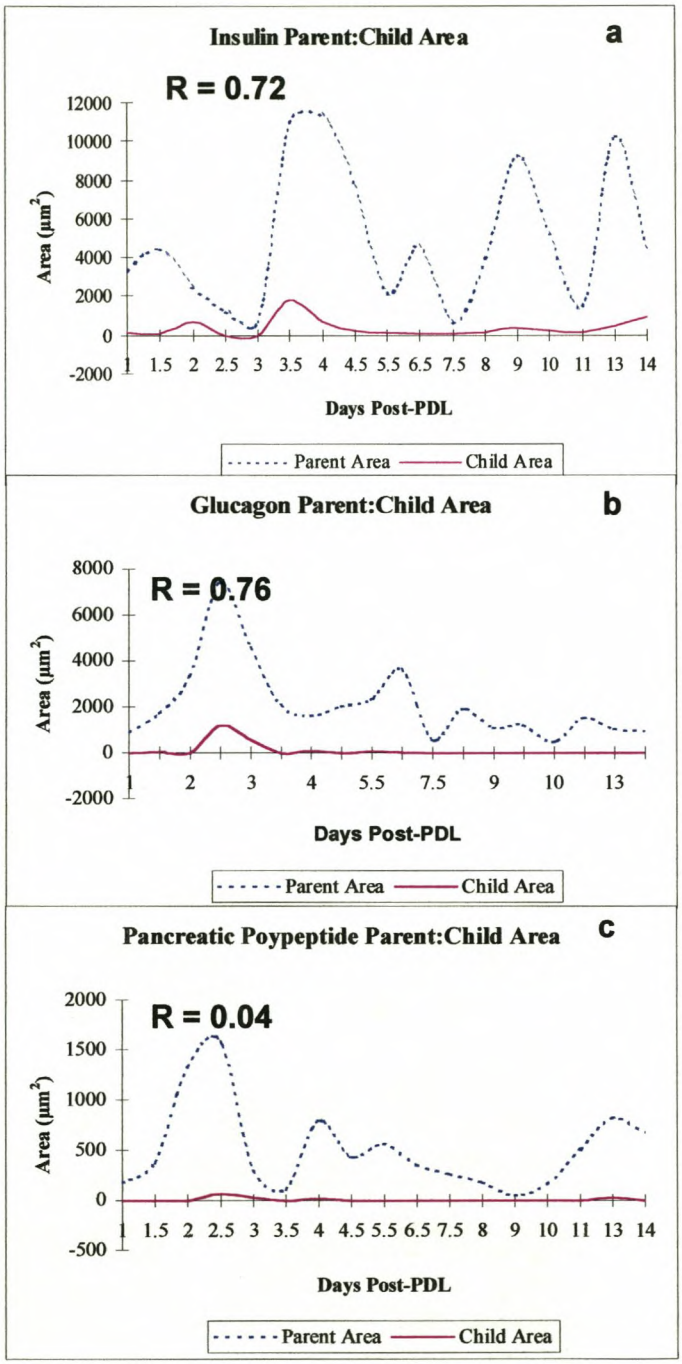


Figure 4.1.2: Series of graphs indicating parent/child area.

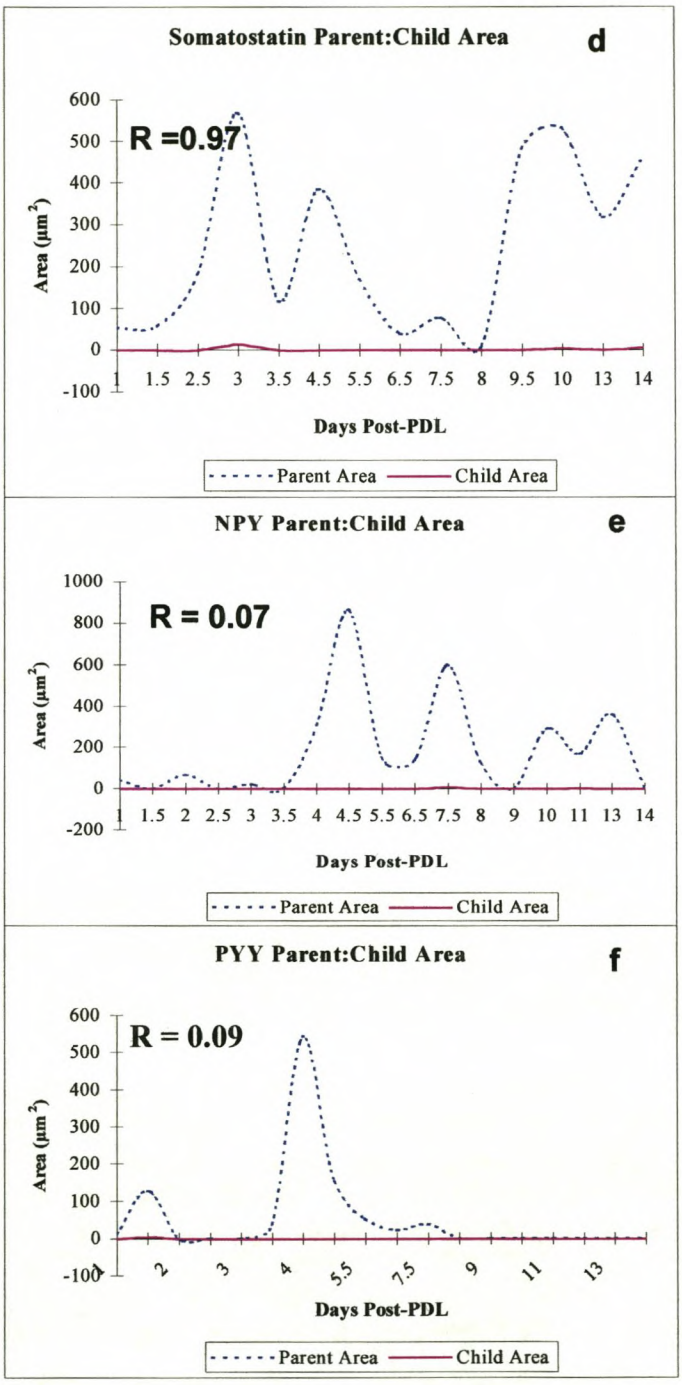


Figure 4.1.2: Series of graphs indicating parent/child area.

	McEvoy	SOC_Ins	PDL
DAY	%N_INS	% Insulin	% IIR
1	1.04	1.35	3.0611825
1.5	1.04	0.92	4.0895537
2	1.04	1.02	2.8846678
2.5	1.04	1.32	1.0965934
3	1.04	0.86	0.6530252
3.5	1.04	0.74	11.535875
4	1.04	1.29	10.878879
4.5	1.04	1	7.1588916
5.5	1.04	1.12	6.8462324
6.5	1.04	0.98	4.1992044
7.5	1.04	0.92	0.5897498
8	1.04	0.96	3.6806476
9	1.04	1.02	8.7185705
10	1.04	1	11.992362
11	1.04	1.4	14.549144
12	1.04	1.04	25.863703
13	1.04	0.82	2.106693
14	1.04	0.64	2.106693

Table 4.1: Comparative values for % insulin. McEvoy et al. (1977) is taken as normal. SOC = sham operated controls; PDL = experimental group. P = 0.0016; p1 = 0.0098 p = Walker vs. PDL; p1 = SOC vs. PDL

	McEvoy	SOC	PDL
DAY	% N_GLU	% GLU	% GIR
1	0.18	0.11	0.7965279
1.5	0.18	0.15	1.6104185
2	0.18	0.2	3.0215369
2.5	0.18	0.21	15.768879
3	0.18	0.17	4.7006467
3.5	0.18	0.14	1.8577375
4	0.18	0.11	1.5312186
4.5	0.18	0.1	1.851795
5.5	0.18	0.16	2.1975147
6.5	0.18	0.2	3.304385
7.5	0.18	0.11	0.5055957
8	0.18	0.14	1.4530195
9	0.18	0.12	0.9896316
10	0.18	0.23	0.9122137
11	0.18	0.14	0.41359
12	0.18	0.188	1.377024
13	0.18	0.159	2.2932617
14	0.18	0.22	0.844677

Table 42: Comparative values for % glucagon. McEvoy et al. (1977) is taken as normal. SOC = sham operated controls; PDL = experimental group. p = 0.01; p1 = 0.048.

	McEvoy	SOC	PDL
DAY	% N_ST	% ST	% ST-IR
1	0.09	0.1	0.1064855
1.5	0.09	0.1	0.0516288
2	0.09	0.1	0
2.5	0.09	0.1	1.9588841
3	0.09	0.1	0.5280539
3.5	0.09	0.1	0.1071131
4	0.09	0.1	0
4.5	0.09	0.1	0.0872216
5.5	0.09	0.1	0.1552374
6.5	0.09	0.1	0.0369541
7.5	0.09	0.1	0.0712145
8	0.09	0.1	0.0090295
9	0.09	0.1	0
10	0.09	0.1	0.4345457
11	0.09	0.1	0.4893864
12	0.09	0.1	0
13	0.09	0.1	0.2909617
14	0.09	0.1	4.2727702

Table 4.3: Comparative values for % somatostatin. McEvoy (1977) is taken as normal. SOC = sham operated controls; PDL = experimental group. p = 0.136; p1 = 0.145.

	Louw	SOC	PDL
DAY	% N_PP	% PP	% PPIR
1	0.216	0.12	0.1557799
1.5	0.216	0.12	0.1117674
2	0.216	0.12	1.2008031
2.5	0.216	0.12	1.505593
3	0.216	0.12	0.2779256
3.5	0.216	0.12	0.0969395
4	0.216	0.12	0.0130191
4.5	0.216	0.12	0.3889556
5.5	0.216	0.12	0.5052097
6.5	0.216	0.12	0.3204433
7.5	0.216	0.12	0.2394371
8	0.216	0.12	0.1587737
9	0.216	0.12	0.0370849
10	0.216	0.12	0.1403202
11	0.216	0.12	0.461539
12	0.216	0.12	0
13	0.216	0.12	0.7775208
14	0.216	0.12	0.616858

Table 4.4: Comparative values for % PP. Walker (1993) is taken as normal. SOC = sham operated controls; PDL = experimental group. p = 0.09; p1 = 0.014.

	Louw	SOC	PDL
DAY	%NPY	% NPY	% NPY
1	0.24	0	0.0208204
1.5	0.24	0	0.2232237
2	0.24	0	0.0500031
2.5	0.24	0	0
3	0.24	0	0.0186875
3.5	0.24	0	0
4	0.24	0	0.2770323
4.5	0.24	0	0.7898132
5.5	0.24	0	0.5484091
6.5	0.24	0	0.1242801
7.5	0.24	0	0.1347567
8	0.24	0	0.1116618
9	0.24	0	0
10	0.24	0	0.2666029
11	0.24	0	2.2885255
12	0.24	0	0
13	0.24	0	0.3342404
14	0.24	0	0

Table 4.5: Comparative values for % NPY. Louw (1995) is taken as normal. SOC = sham operated controls; PDL = experimental group. p = 0.7; p1 = 0.038.

	Louw	SOC	PDL
DAY	% N_PYY	% PYY	% PYY
1	0	0	0.0108521
1.5	0	0	0.1203159
2	0	0	0
2.5	0	0	0
3	0	0	0
3.5	0	0	0.0335183
4	0	0	0.4919841
4.5	0	0	0.0462773
5.5	0	0	0.0492122
6.5	0	0	0.020124
7.5	0	0	0.0349802
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	0	0	0
14	0	0	0

Table 4.6: Comparative values for % PYY. Louw (1995) is taken as normal. SOC = sham operated controls; PDL = experimental group = 0.11; p1 = 0.11

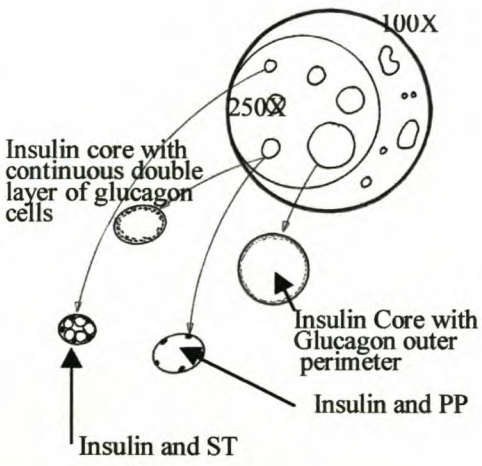


Figure 4.2: Freehand sketch of islet cell relationships as seen on day one post duct ligation pancreas tissue. 100X.

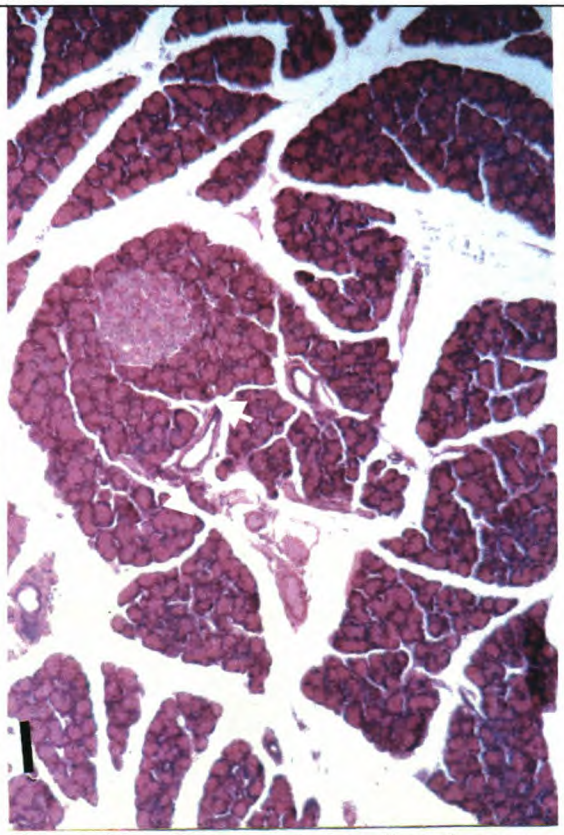


Figure 4.3: Day 1 post duct ligated pancreas tissue. Stained with H & E. 100X. Bar = 100 μm.

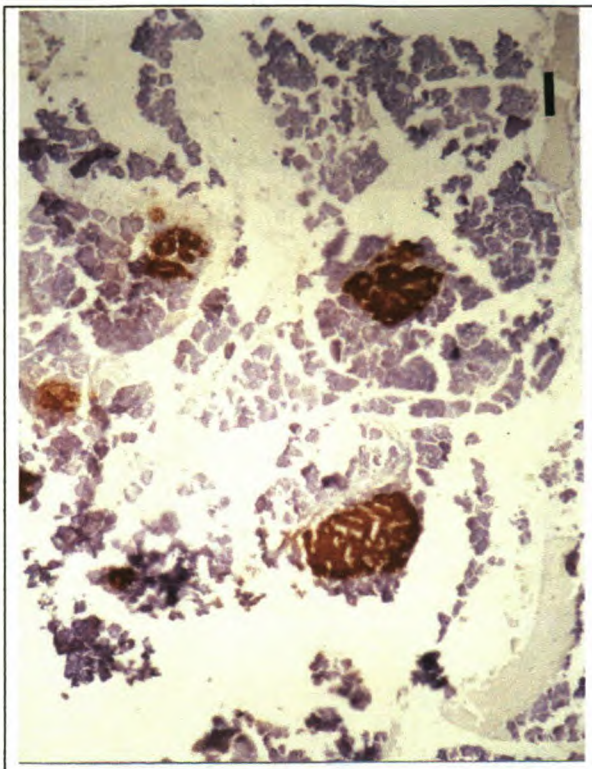


Figure 4.4: Day 1 post duct ligated pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm.

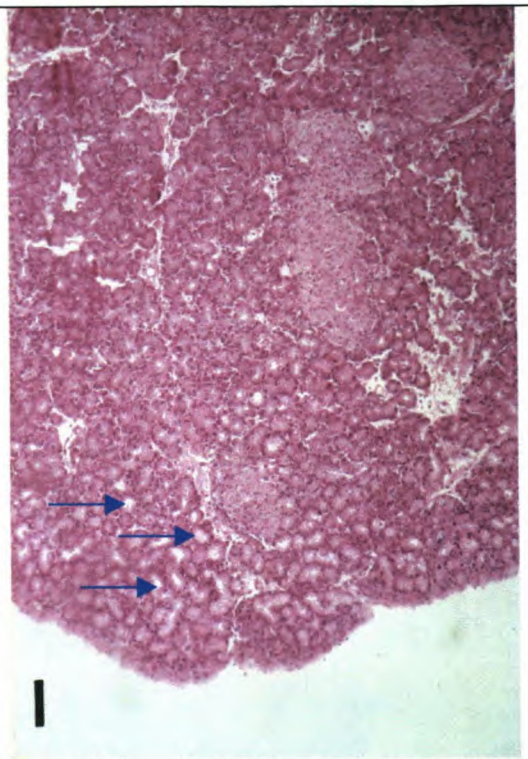


Figure 4.5: Day 1½ post duct ligated pancreas tissue. H & E. 100X. Bar = 100 µm. Arrows indicate duct/acinar lumina.

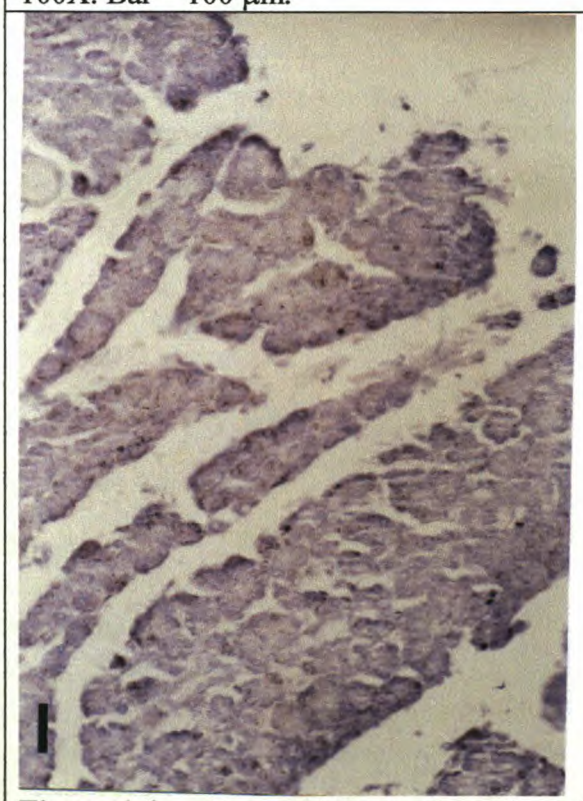


Figure 4.6: Day 1½ post duct ligated pancreas tissue. ICC stained for NPY. 250X. Bar = 40 µm.

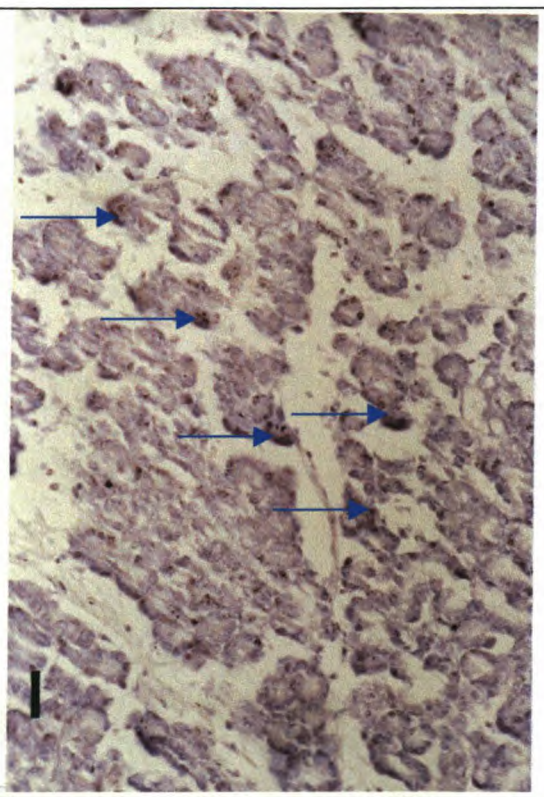


Figure 4.7: 1½ days post-PDL pancreas tissue. ICC stained for PYY. 250X. Bar = 40 µm. Arrows indicate darkened margins.

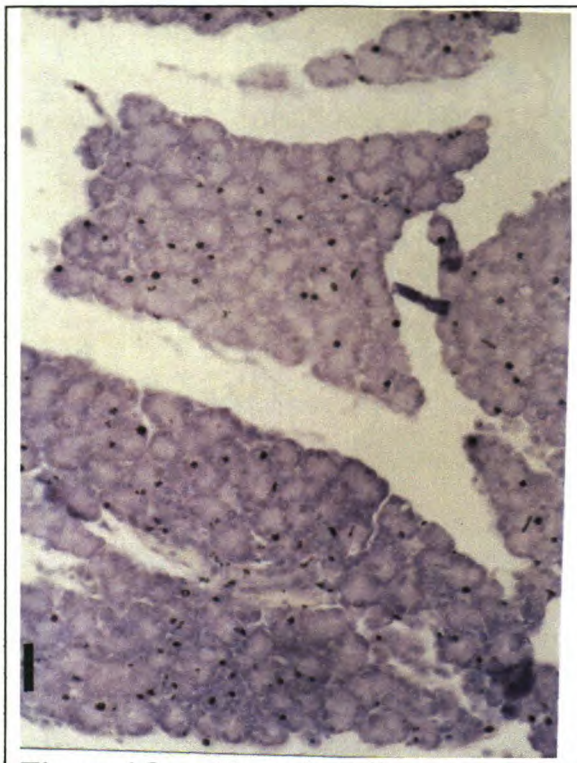


Figure 4.8: Normal unligated pancreas tissue. ICC stained for PYY. 250X. Bar = 40 μm.

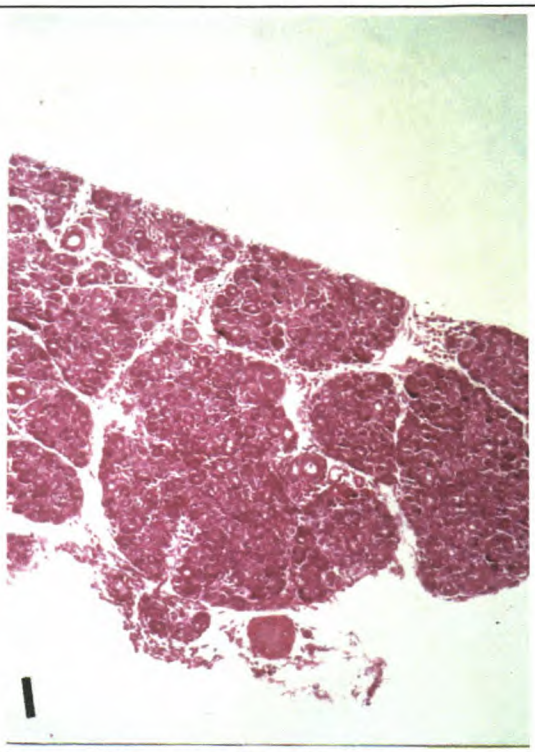


Figure 4.9: Day 2 post-PDL pancreas tissue. H & E. 100X. Bar = 100 μm.

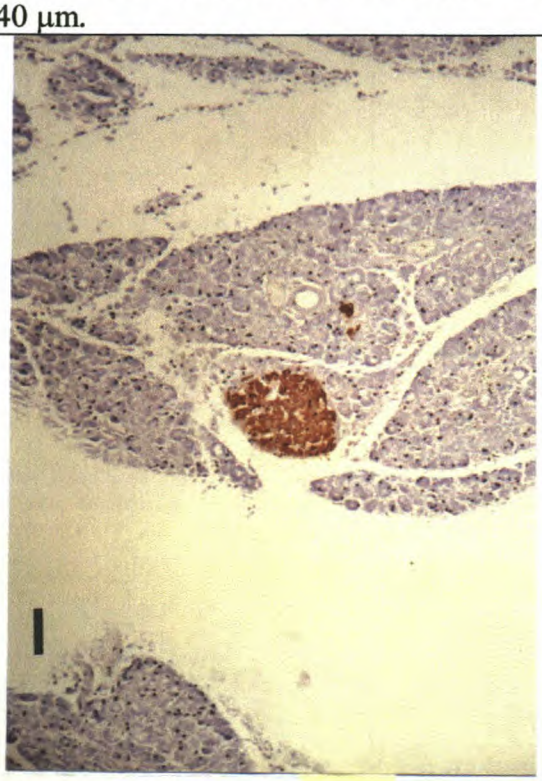


Figure 4.10: Day 2½ post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 μm.

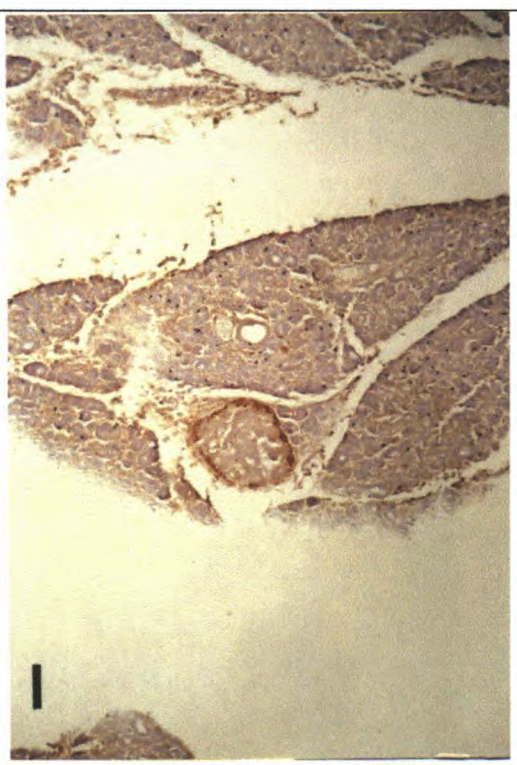


Figure 4.11: Day 2½ post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 μm.

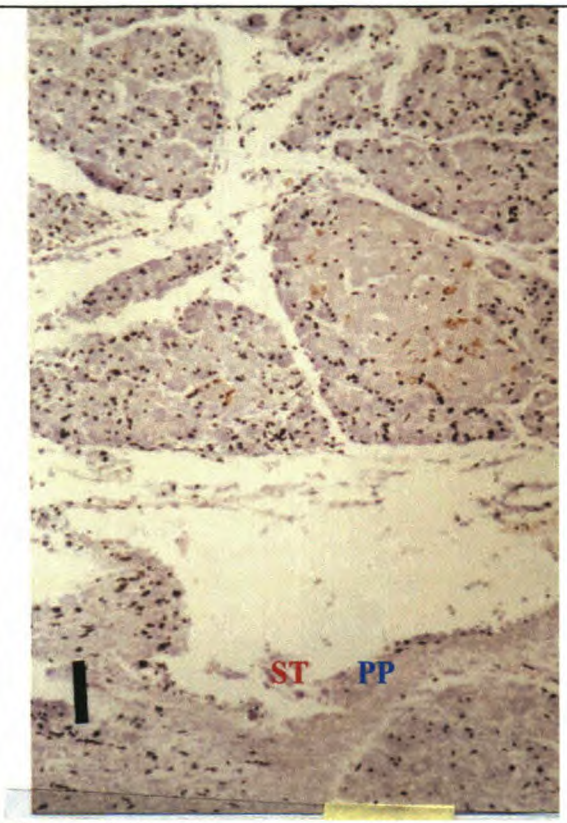


Figure 4.12: 2½ day post-PDL pancreas tissue. ICC stained for somatostatin. 100X. Bar = 100 µm.

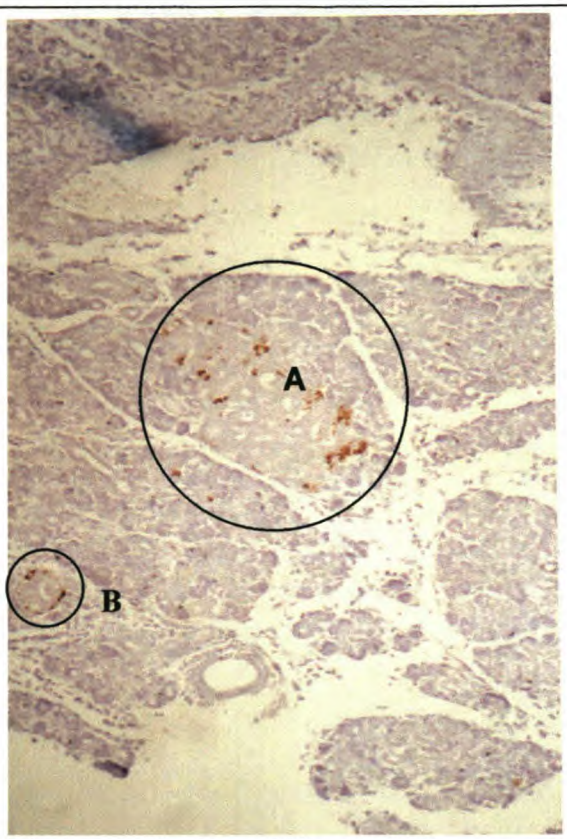


Figure 4.13: Day 2½ post-PDL pancreas tissue ICC stained for PP. 100X. Overlay indicates intra-islet PP. Bar = 100 µm.

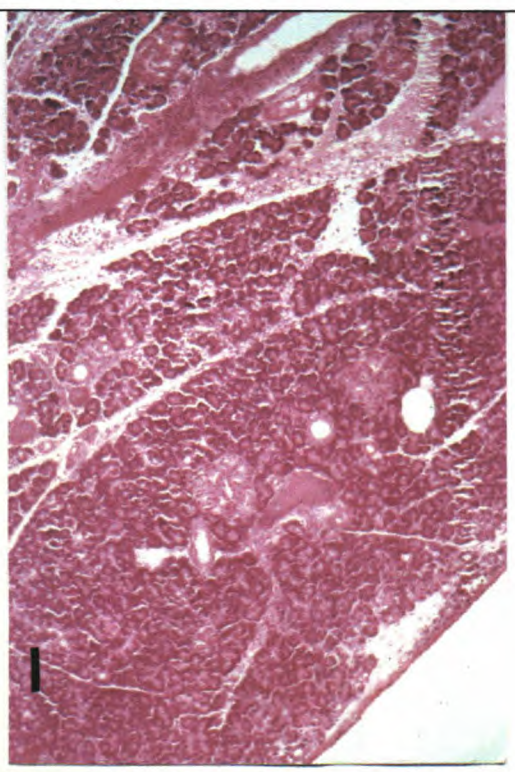


Figure 4.14: Normal pancreas tissue. H & E stained. 100X. Bar = 100 µm.

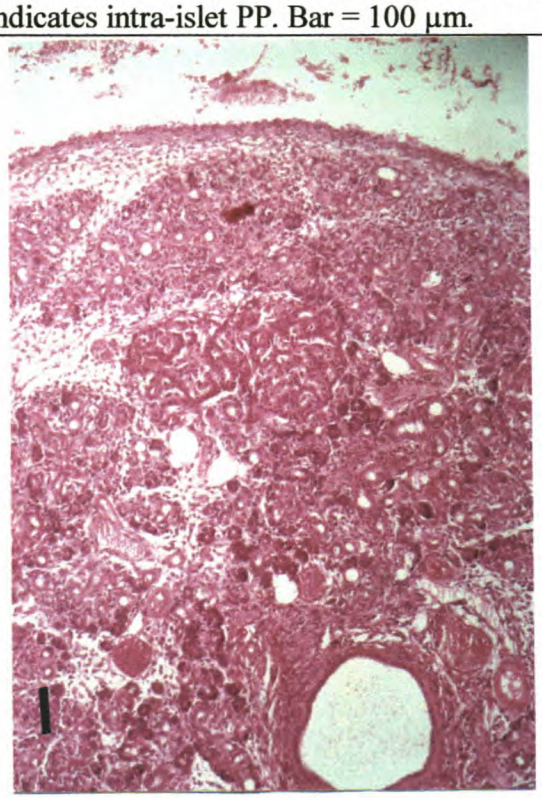


Figure 4.15: Day 3 post-PDL pancreas tissue. H & E stained. X100. Bar = 100 µm.

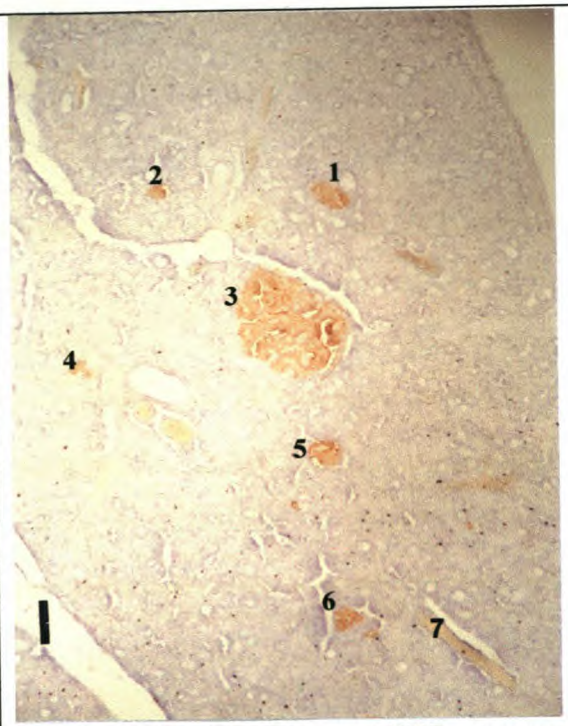


Figure 4.16: Day 3 post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm.

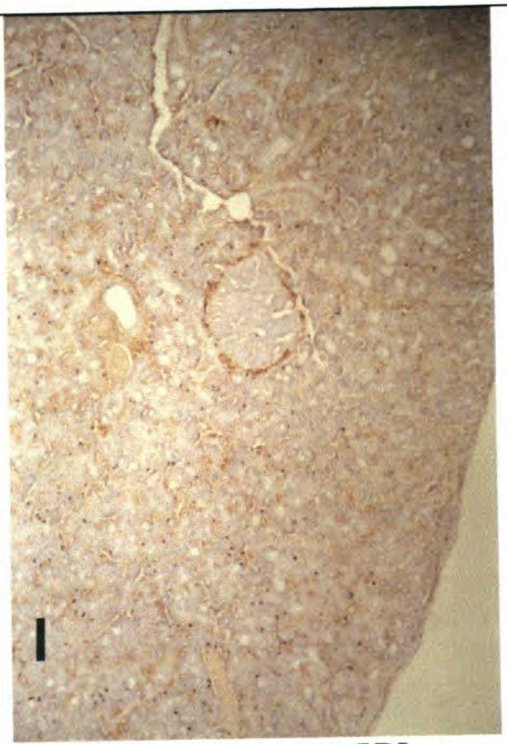


Figure 4.17: Day 3 post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm. Overlay indicates relative position of other endocrine hormones.

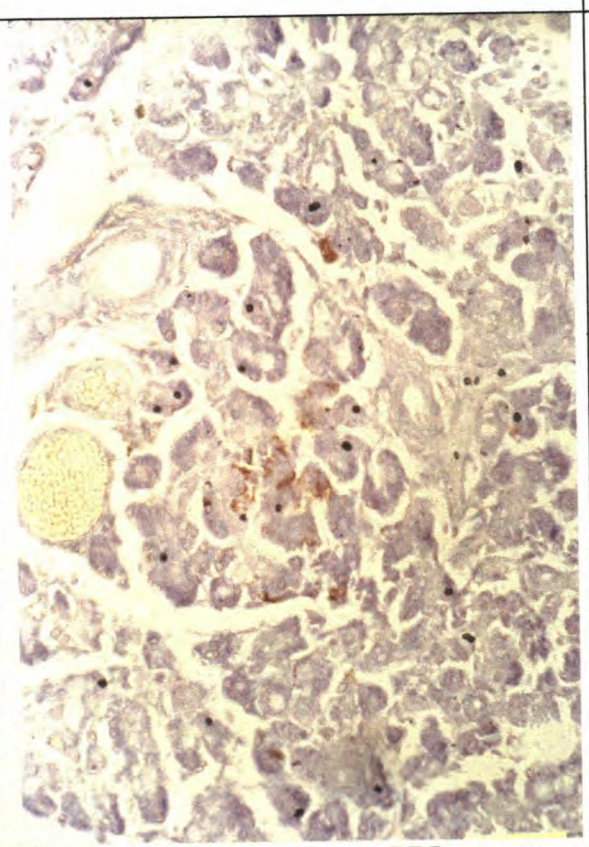


Figure 4.18: Day 3 post-PDL pancreas tissue. ICC stained for PP. X250. Bar = 40 µm.

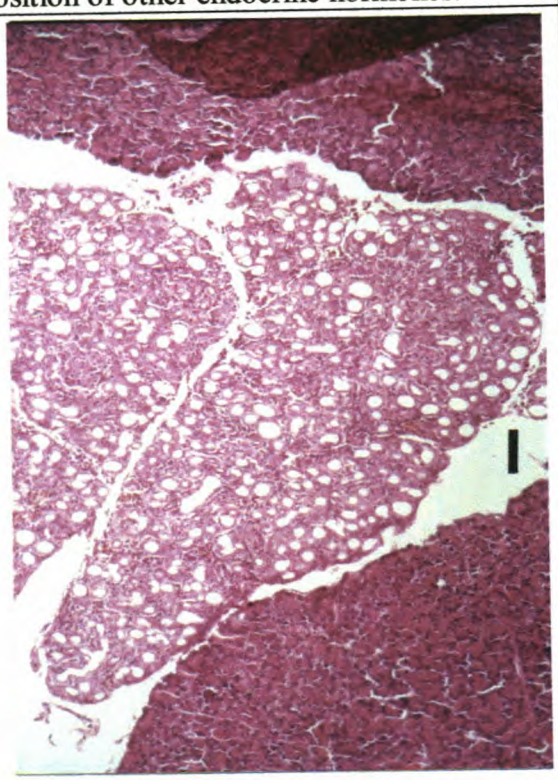


Figure 4.19: Day 3½ post-PDL pancreas tissue. H & E stained. 100X. Bar = 100 µm. Note the contrast between normal (darker) and ligated (lighter) tissue.

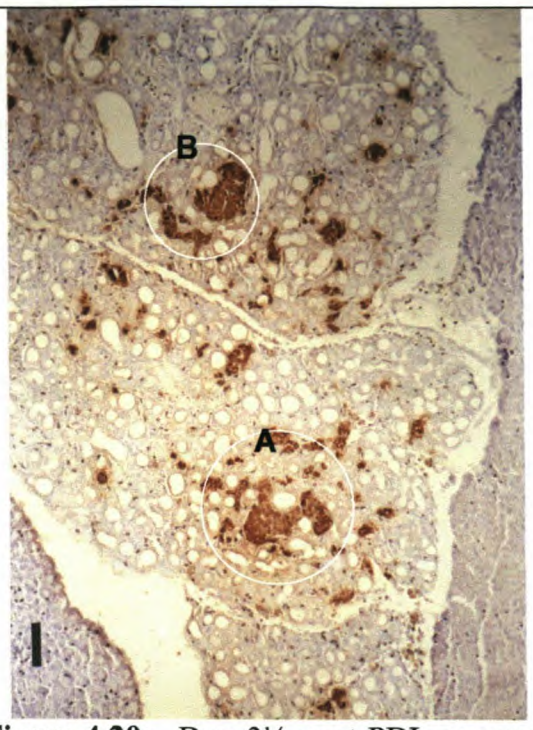


Figure 4.20: Day 3½ post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm.

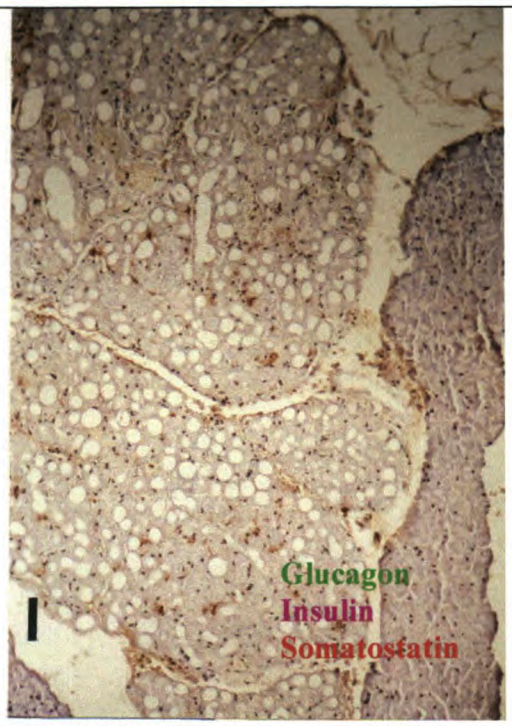


Figure 4.21: Day 3½ post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm. Overlay indicates the relative positioning of insulin, glucagon, somatostatin and PP.

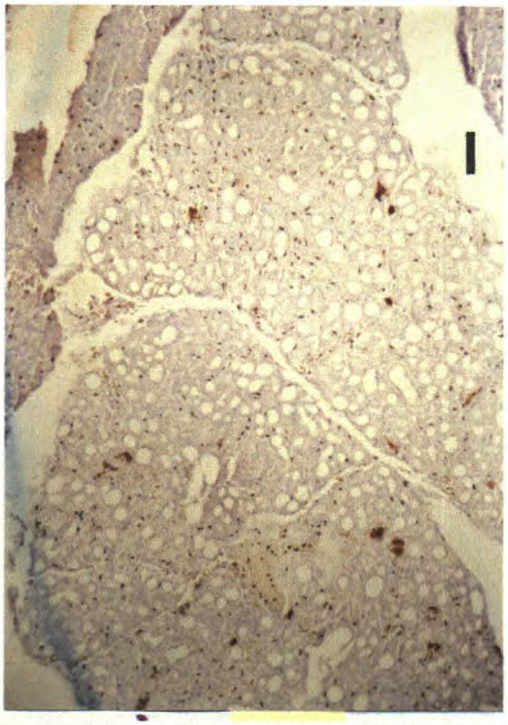


Figure 4.22: Day 3½ post-PDL pancreas tissue. ICC stained for ST. 100X. Bar = 100 µm.

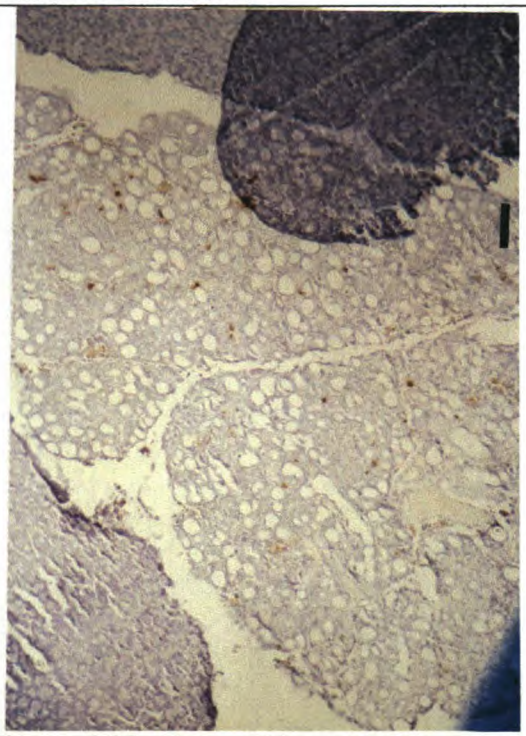


Figure 4.23: Day 3½ post-PDL pancreas tissue. ICC stained for PP. 100X. Bar = 100 µm.

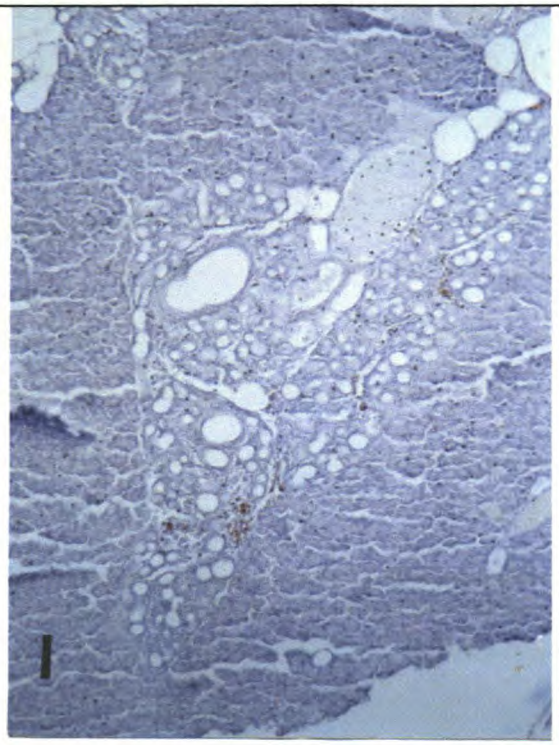


Figure 4.24: Day 3½ post-PDL pancreas Tissue. ICC stained for NPY. 100X. Bar = 100 µm.

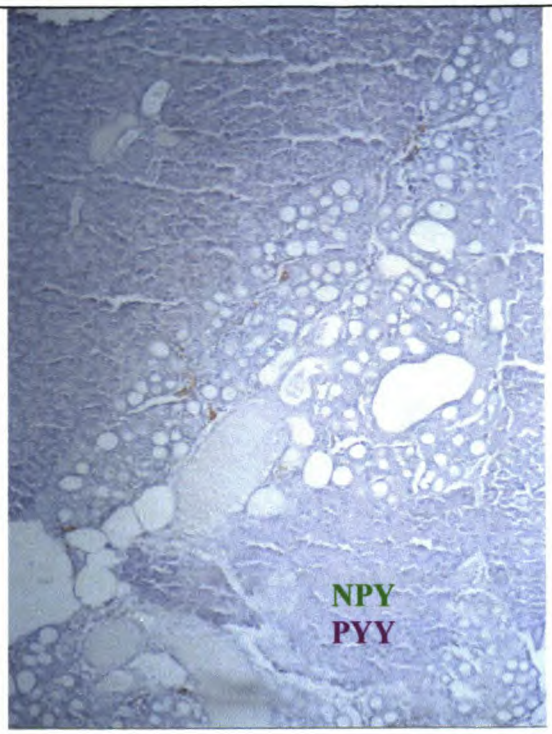


Figure 4.25: Day 3½ post duct ligated pancreas tissue. ICC stained for PYY. 100X. Bar = 100 µm Overlay indicates NPY/PYY relationship.

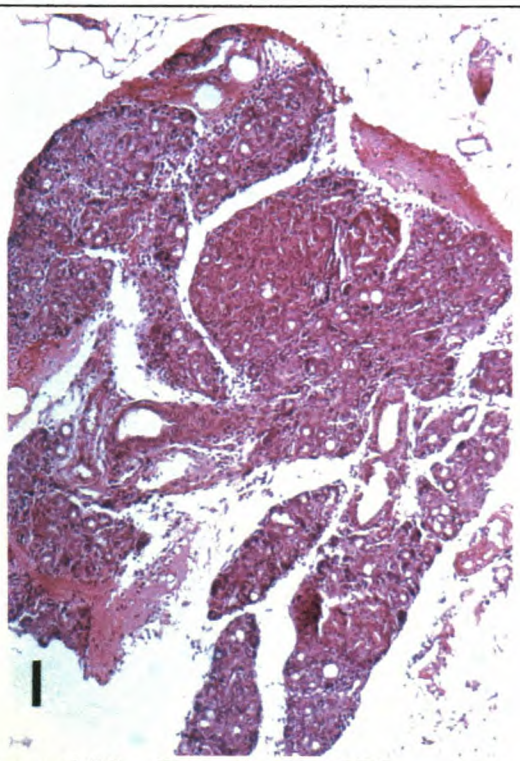


Figure 4.26: Day 4 post-PDL pancreas tissue. H & E stained. 100X. Bar = 100 µm.

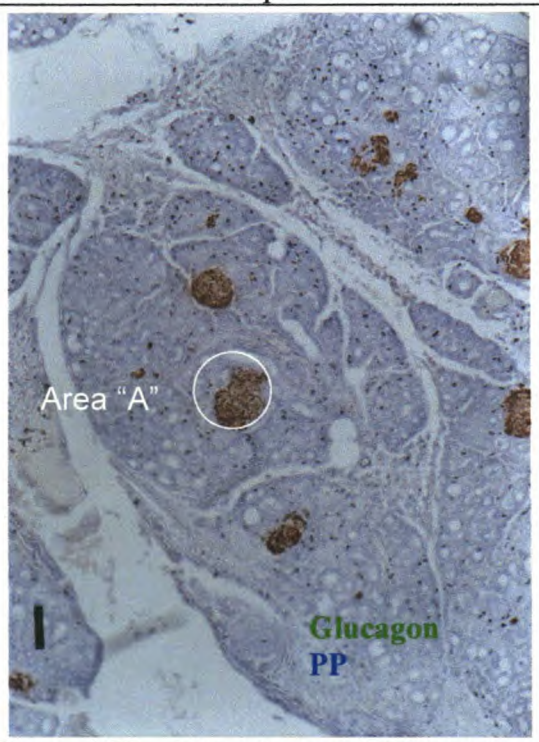


Figure 4.27: Day 4 post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm. Overlay indicates relative placement of other endocrine cells.

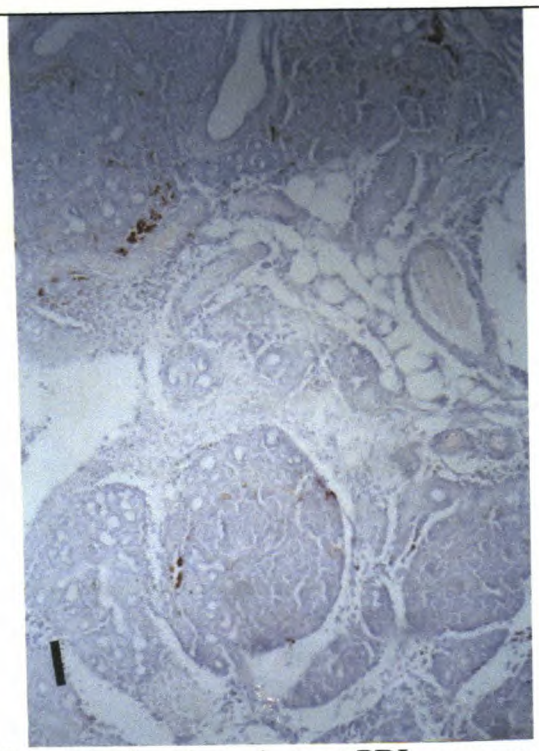


Figure 4.28: Day 4 post-PDL pancreas tissue. ICC stained for PP. 100X. Bar = 100 µm.

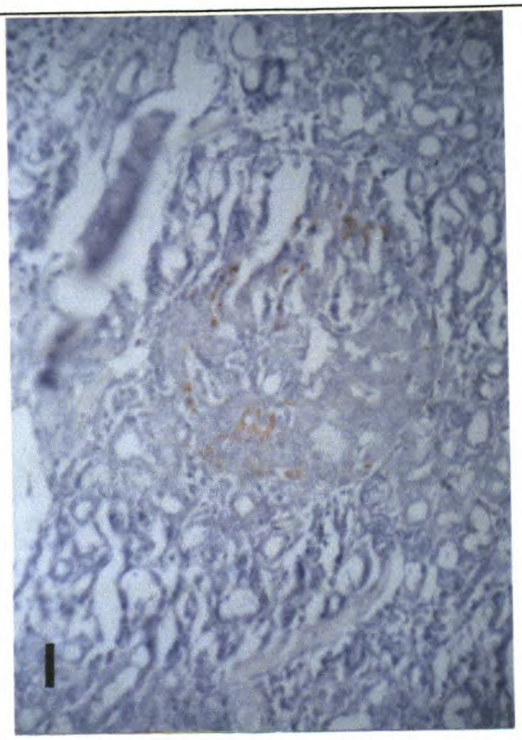


Figure 4.29: Day 4 post-PDL pancreas tissue. ICC stained for NPY. 400X. Bar – 25 µm.

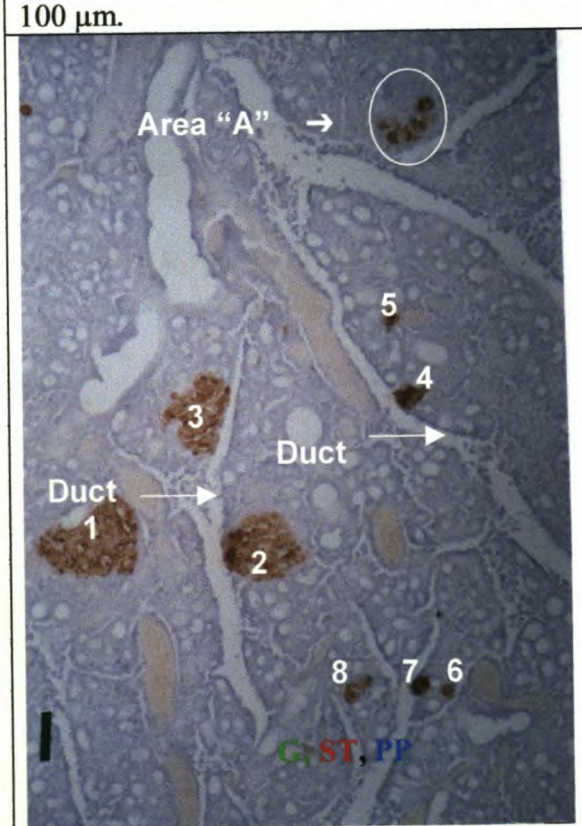


Figure 4.30: Day 4½ post duct ligated pancreas tissue. ICC stained for insulin. 100X. Bar = 100µm. Overlay indicates the relative position of other pancreatic hormones.

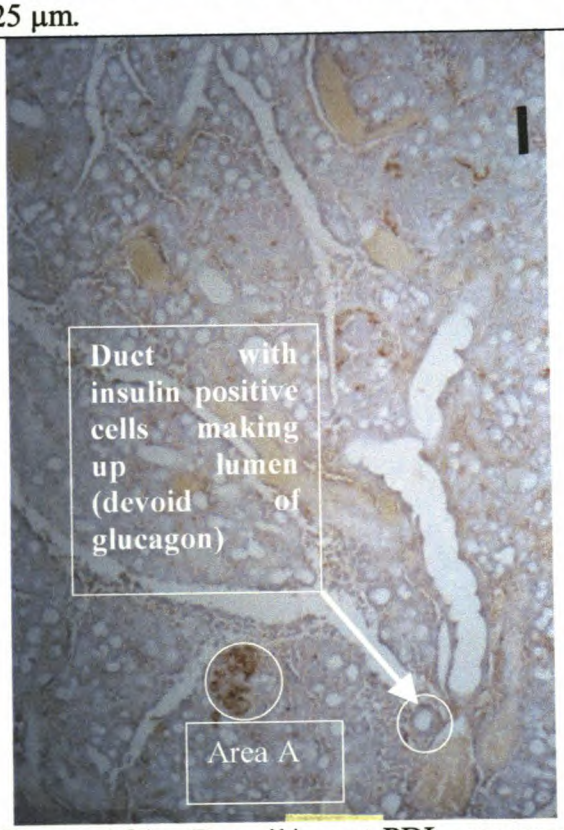


Figure 4.31: Day 4½ post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm.

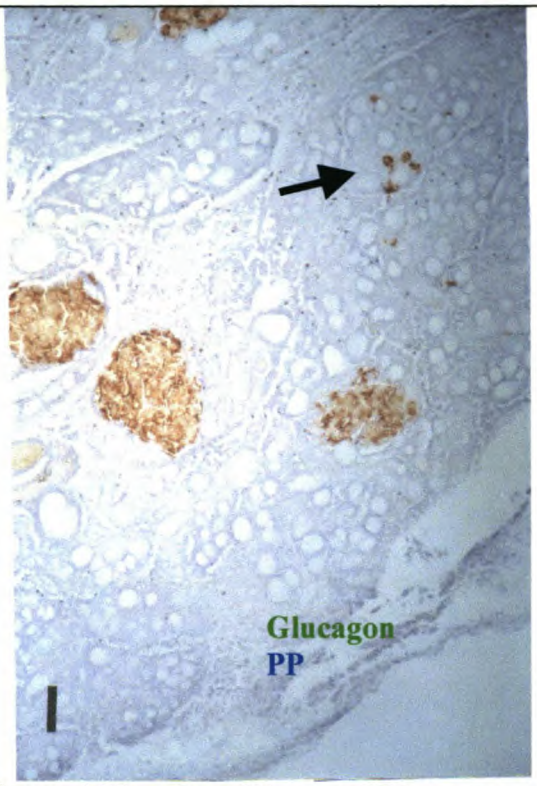


Figure 4.32: Day 5½ post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm. Overlay indicates relative position of various endocrine cells to one another.

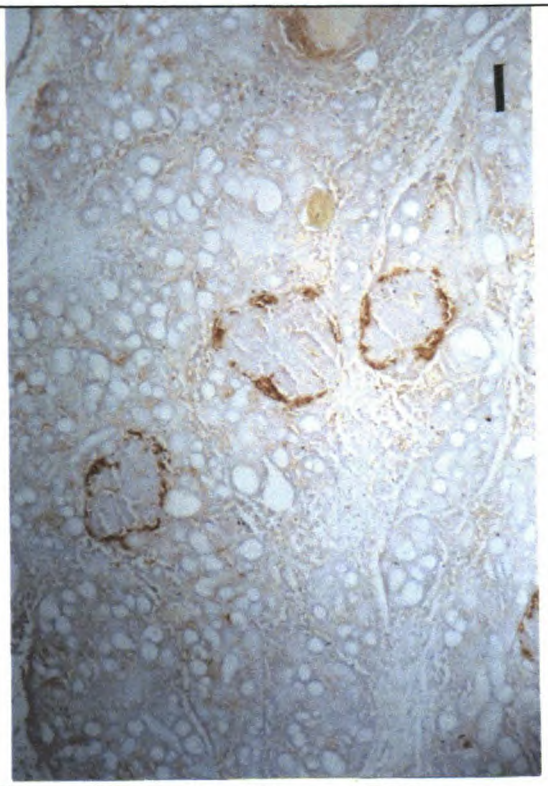


Figure 4.33: Day 5½ post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm.

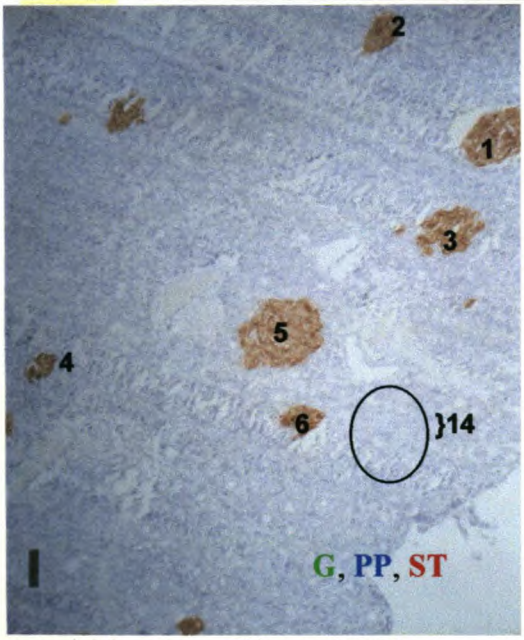


Figure 4.34: Day 6½ post-PDL pancreas tissue. ICC stained for insulin. 100X Bar = 100 µm. Overlay indicates relationship of insulin to other pancreatic hormones.

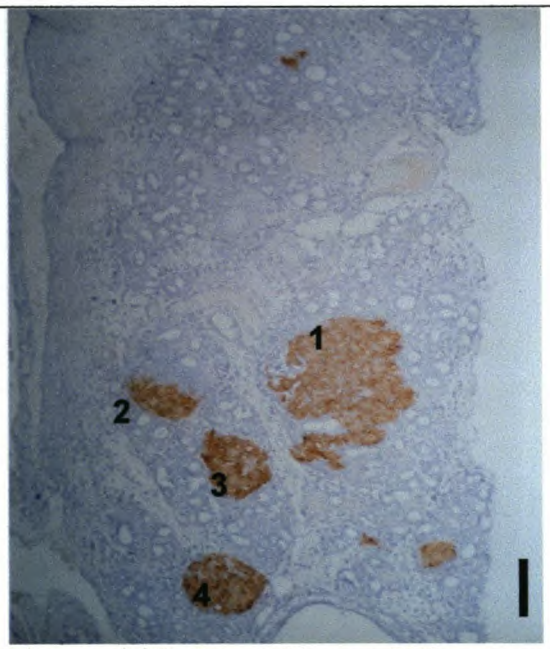


Figure 4.35: Day 7½ post-PDL pancreas tissue. ICC stained for insulin. X100. Bar = 100 µm.

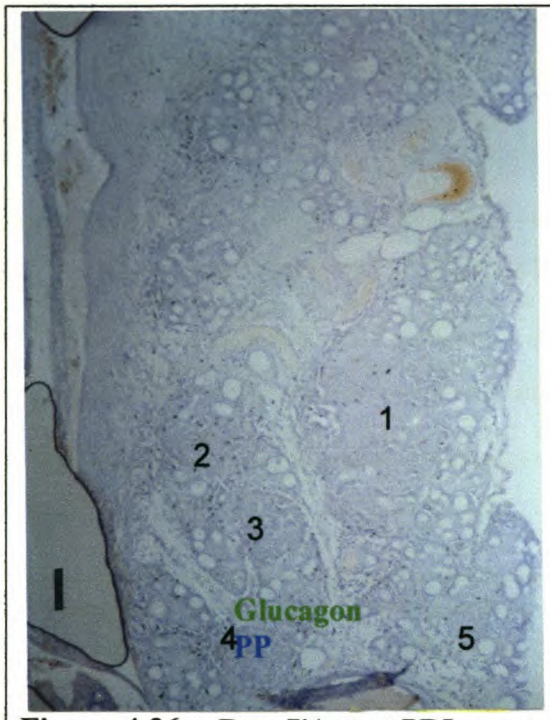


Figure 4.36: Day 7½ post-PDL pancreas tissue. ICC stained for somatostatin. 100X. Bar – 100 µm. Overlay indicates relative position and quantity of other hormones.

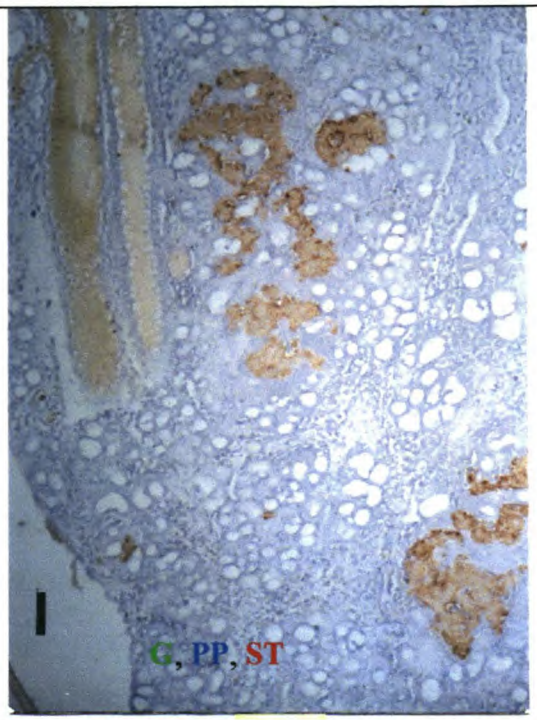


Figure 4.37: Day 8 post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm. Overlay indicates relationship of insulin to other endocrine cells.

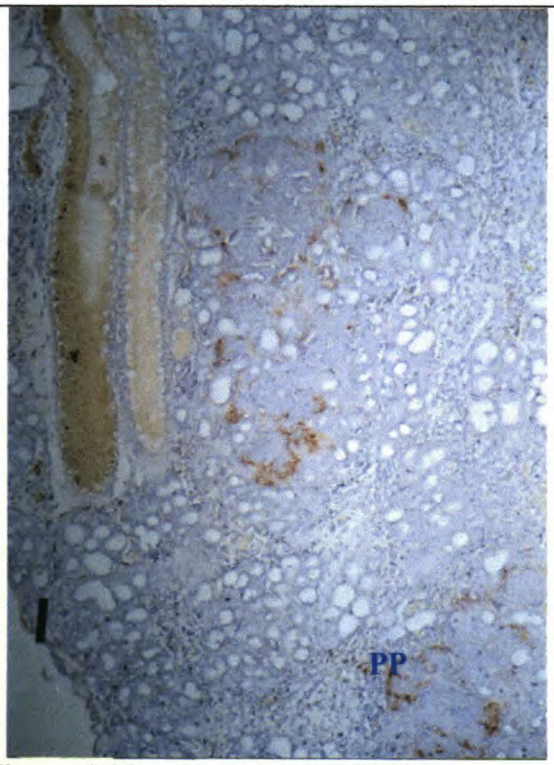


Figure 4.38: Day 8 post-PDL pancreas tissue. ICC stained for Glucagon. 100X. Bar = 100 µm.

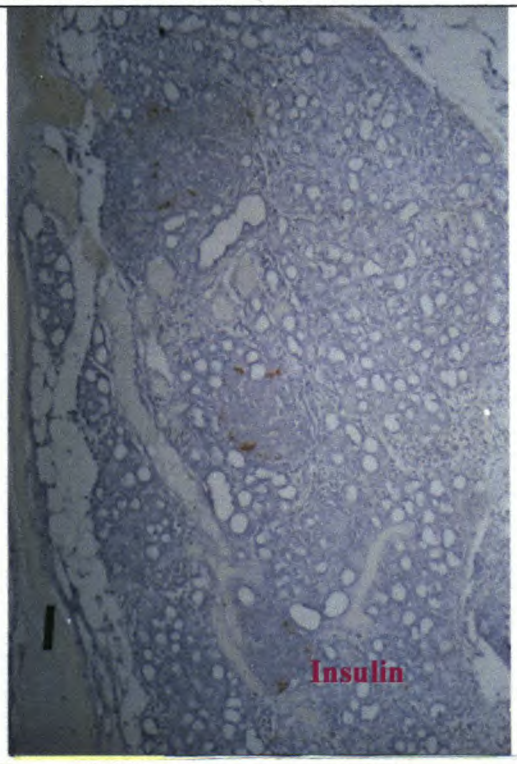


Figure 4.39: Day 8 post-PDL pancreas tissue. ICC for NPY. 100X. Bar = 100 µm.

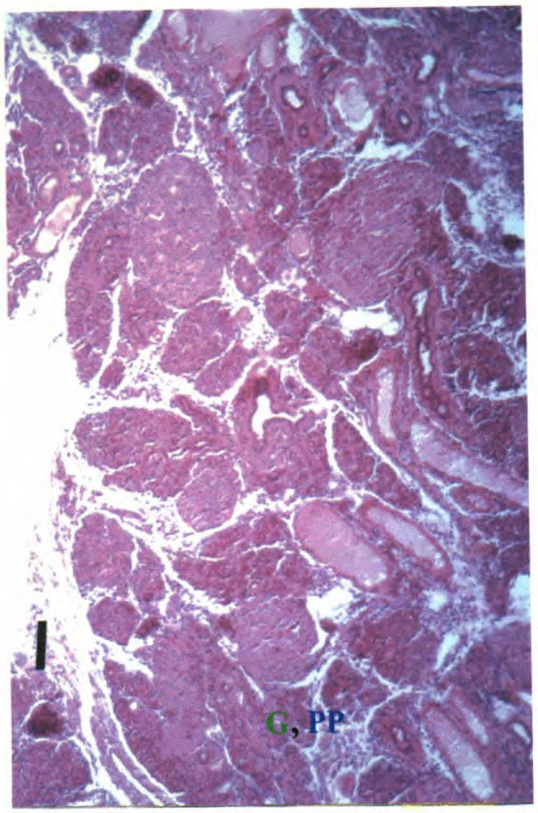


Figure 4.40: Day 9½ post-PDL pancreas tissue. H & E. 100X. Bar = 100 µm.

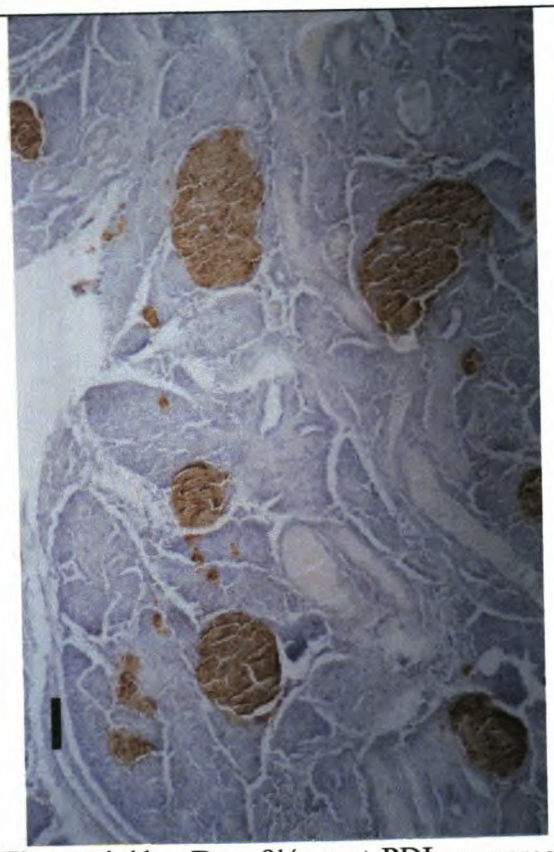


Figure 4.41: Day 9½ post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm.

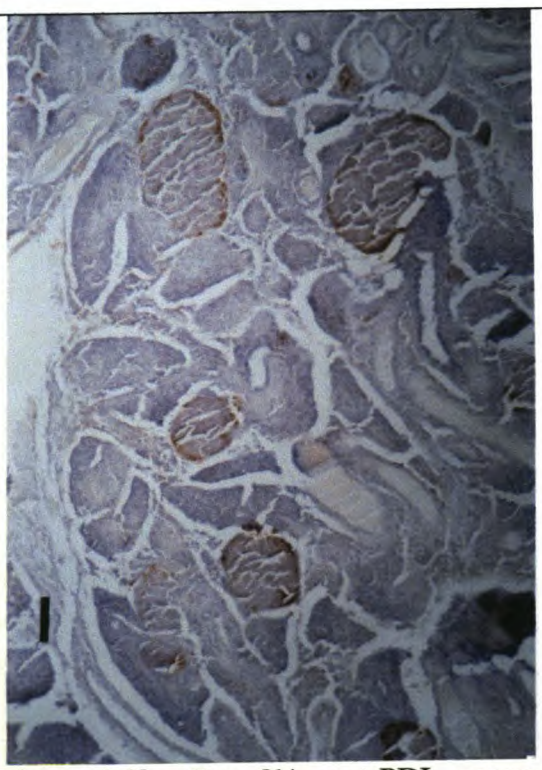


Figure 4.42: Day 9½ post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm.

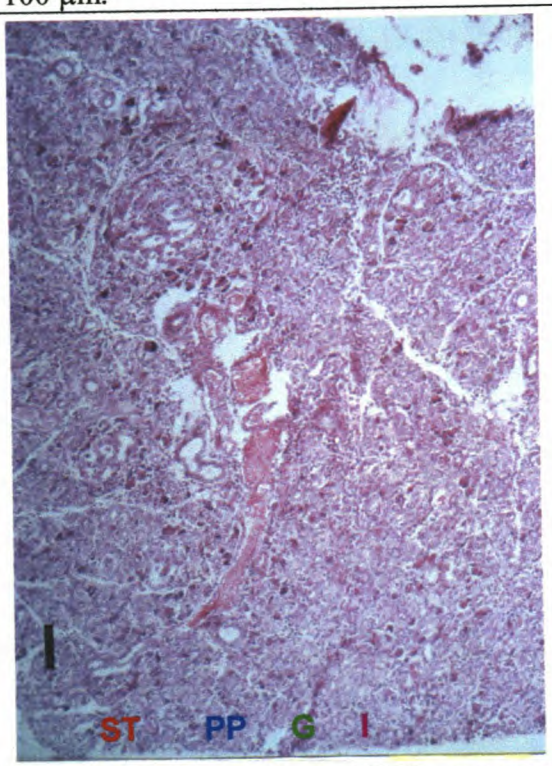


Figure 4.43: Day 10 post-PDL pancreas tissue. H & E. 100X. Bar = 100 µm.

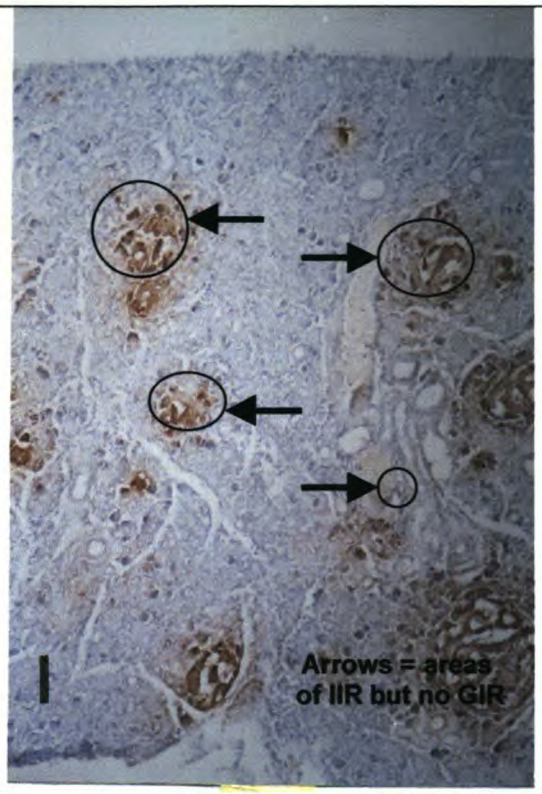


Figure 4.44: Day 10 post-PDL pancreas tissue. ICC stained for insulin. 100X. Bar = 100 µm.

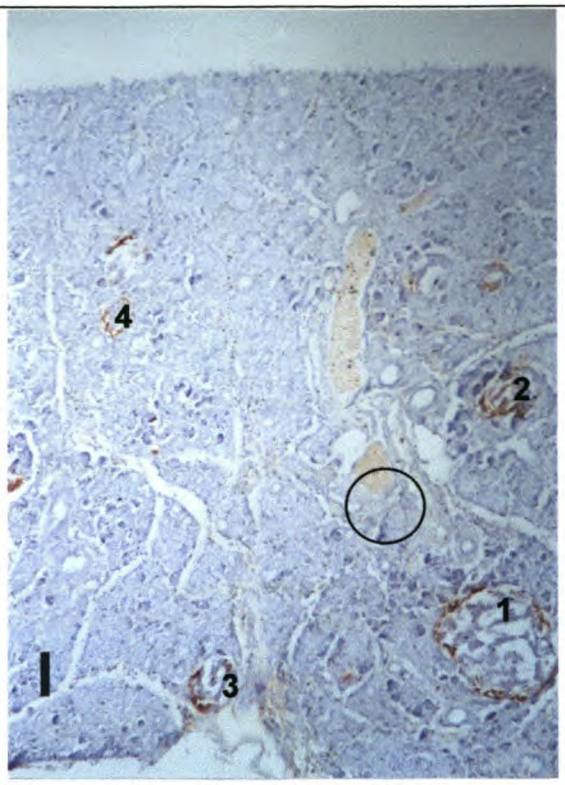


Figure 4.45: Day 10 post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm.

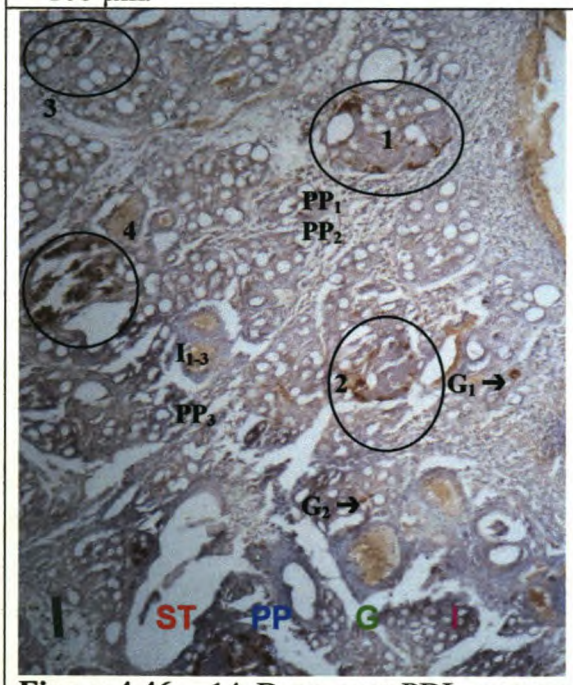


Figure 4.46: 14 Days post-PDL pancreas tissue. ICC stained for glucagon. 100X. Bar = 100 µm. Overlay indicates relationship of glucagon to insulin, PP and ducts.

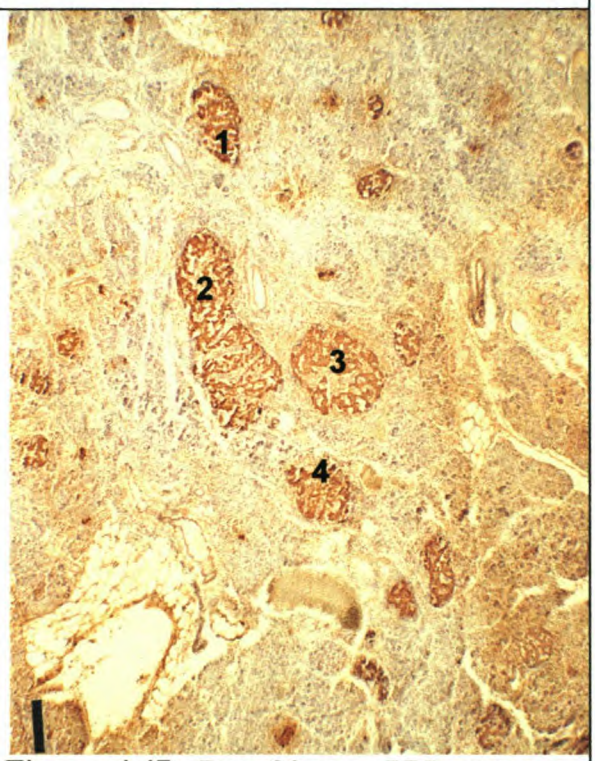


Figure 4.47: Day 30 post-PDL pancreas tissue. ICC stained for insulin. X40. Bar = 150 µm.

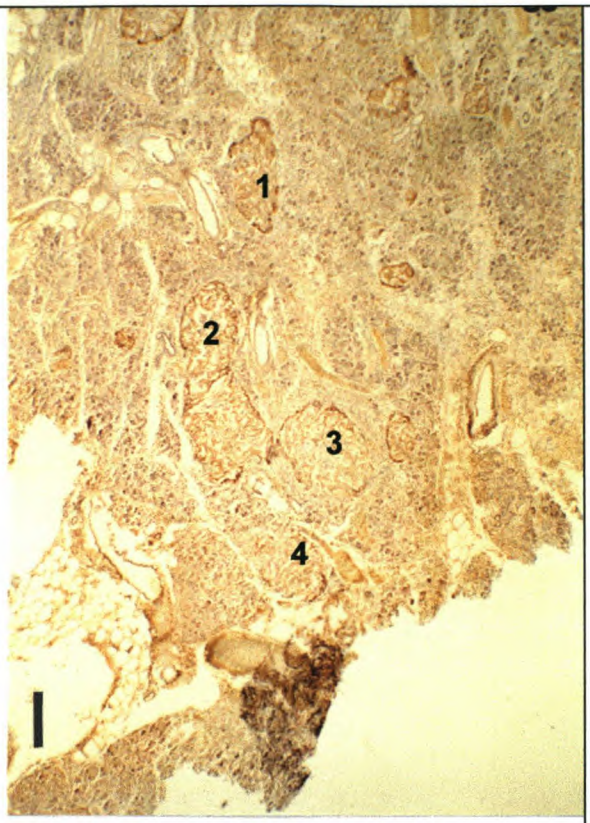


Figure 4.48: Day 30 post-PDL pancreas tissue. ICC stained for glucagon. X40. Bar = 150 µm.

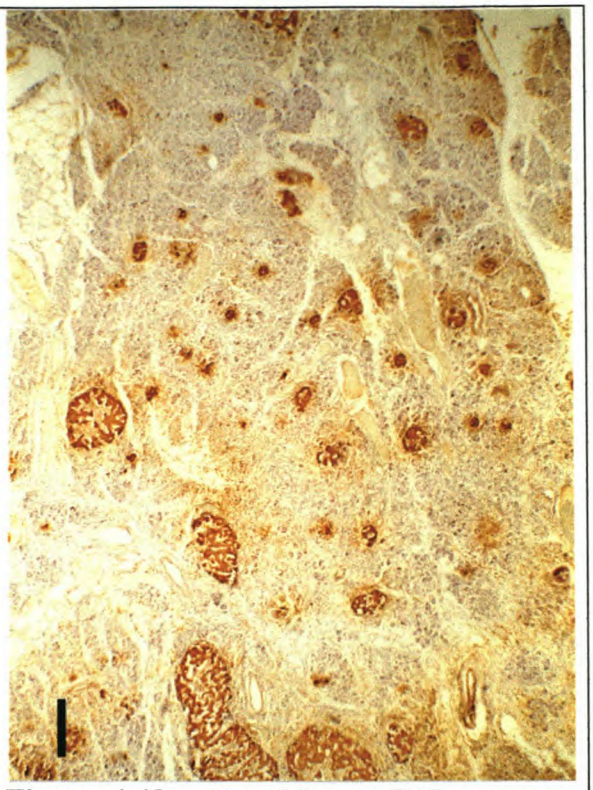


Figure 4.49: Day 30 post-PDL pancreas tissue. ICC stained for insulin. X40. Bar = 40 µm. Area adjacent to figure 4.47 indicating profusion of small islets not immunoreactive for G.

Table 4.7: Composite tabulated summary of all events occurring in each of the compartments of duct ligated pancreatic tissue from day one through 14 and day 30. This and the following page should be viewed side-by-side.

Day	Acinar Compartment	Existing Islets	Emerging Islets	Ducts	Insulin
1	Normal	Normal	None	None	Existing islet core
1½	Oedema evident	Normal	None	None	Existing islet core
2	Inter-acinar PP+	I, G & ST+ core G+ mantle	None	None	Islet Core[1]
2½	Inter-acinar PP+ & ST+	I, ST & PP+ core G+ mantle	None	None	Islet Core[1]
3	Inter-acinar PP+ & ST+	I, ST & PP+ core G+ mantle	None	Ligated tissue NPY+	Islet Core[1]
3½	Acinar deletion completed	I, ST & PP+ core G+ mantle	I+	Proliferation NPY/PYY+ I, G, ST, PP+	Scattered
4		I+ core G/PP+ mantle	I+	NPY/PYY+ I, G, ST, PP+	Reconverg -ing into islets
4½		I+ core G/PP+ mantle	I+	NPY/PYY+ I, G,	Reconverg -ing into islets
5½		I+ core G/PP+ mantle	I+ core G/PP/ST+ mantle	NPY/PYY+ I	Reconverg -ing into islets
6½		I+ core G/PP+ mantle	I+ core G/PP/ST+ Mantle	NPY+	Islet core Islet Clusters
7½		I+ core G/PP+ mantle (No ST!)	I+ core G/PP/ST+ mantle	NPY+	Islet Core
8		I+ core G+ mantle	I+ core G/PP/ST+ mantle	NPY+	Islet Core
9	Limited acinar reappearance	I+ core G+ mantle	I+ core G/PP/ST+ mantle	NPY+	Islet Core
14		I+ core G+ mantle	I+ core G/PP/ST+ mantle	NYP+	Islet Core
30	None	Indistinguishable from new I+ core thin G+ mantle	Indistinguishable from new I+ core thin G+ mantle	None	Indistingui shable from new I+ core thin G+ mantle

1 = in existing islets.

Continuation of Table 4.7.

Day	Glucagon	Pancreatic polypeptide	Somatostatin	PYY	NPY
1	Islet mantle	Islet mantle	Islet mantle	None	None
1½	Islet mantle	Islet mantle	Islet mantle	None	None
2	Islet Mantle	Intra-acinar	Islet Mantle	None	None
2½	Broken Mantle	Intra-islet PP & ST	Intra-islet PP & ST	None	None
3	Broken Mantle	Intra-islet PP & ST	Intra-islet PP & ST	Ducts	Islets
3½	Scattered	Scattered	Scattered	Ducts	Ducts +++
4	Scattered & re-converging into islets	Scattered & re-converging into islets	Scattered & re-converging into islets	None	Islets
4½		Islet Mantle	Mantle & inter-islet	Islets & Ducts	Islets
5½	Mantle	Mantle	Mantle & inter-islet	Islets & Ducts	Islets & Ducts
6½	Mantle	Mantle	Mantle & inter-islet	None	Ducts
7½	Mantle	Mantle	Mantle & inter-islet	None	Islet mantle & intra-islet
8	Mantle	Mantle	Mantle & inter-islet	None	Islet mantle & intra-islet
9	Mantle	Mantle	Mantle & inter-islet	None	Islet mantle & intra-islet
14	Mantle	Mantle	Mantle & inter-islet	None	Islet mantle & intra-islet
30	Mantle	None	None	None	None

CHAPTER 5

DISCUSSION

Duct Ligation

Islet Structure

Islet Size

Islet Frequency

Experimental Procedures

Cell Transition Theory

Recapitulation Theory

Duct Ligation Induced Tissue Remodelling

Cell Proliferation (BrdU/MIB5)

Special Appendix

5.1 PANCREATIC DUCT LIGATION (PDL)

5.1.1 Variations in Surgical Ligation

Two variations on pancreatic duct ligation technique have been described in literature. The first (Zenilman, Perfetti, Swinson et al. 1996; Walker 1987) involved the placement of a "loose" silk (3/0) suture around an avascular section of the pancreas close to, or at the junction of the common bile duct with the pancreatic duct. The ligature however included pancreatic tissue and could therefore be considered to be similar to the cellophane wrapping described elsewhere. It caused only partial occlusion of the pancreatic duct and acted as a tissue irritant. This concurs with the sentiments of Rosenberg (1998) on the issue.

The second, applied in this study and described by Edström and Falkmer (1968), Wang et al. (1995) placed a resorbable (5/0 Dexon/Chromic) suture around only the pancreatic duct to cause total occlusion as described in materials and methods.

5.1.2 Acinar Compartment Change in Response to Ligation

5.1.2.1 Introduction

It would be a simple matter to explain the early extra-islet (day 3) presence of pancreatic polypeptide amongst acinar tissue and (day 2½) inter-islet somatostatin in the light of findings from this study (figure 4.12 – day 2½, ICC for ST; and figure 4.18 - day 3, ICC for PP) if acinar cells in the ligated portion of the pancreas were undergoing regression toward duct cells as proposed by Bouwens and Rooman (1997), Arias and Bendayan (1993). If this were the case, they might go though the reverse order of lineage proposed by Gittes and Rutter (1992), Bertelli and Bendayan (1997) of G, I, ST, PP. Alternatively, existing duct cells and/or oval cells or pluripotent stem cells within the islet core or amongst acinar tissue may be reacting to a ligation induced stimulus and be taking the first step in islet neoformation. No alternative explanation is forthcoming to explain the presence of both PP and ST within the islet or acinar tissue.

Evidence will be presented at a later stage, which might lend credence to the view that both transdifferentiated acinar tissue as well as existing islets, are involved in islet neogenesis through as yet unresolved mechanisms. It could however be possible that elements of both exocrine (acinar) and endocrine (islet) pancreas undergo regression to form stem cells which will initiate and sustain neogenesis. This could explain the presence of acinar-endocrine, endocrine-acinar and endocrine cells with dual expression described by Melmed et al. (1972), Melmed (1978), Wolfe-Coote et al. (1988), Bertelli & Bendayan (1997), Gu, Lee, Krahl, Sarvetnick (1994), Gu and Sarvetnick (1993), Rosenberg et al. (1996b), Sarvetnick and Gu (1992), Wang et al. (1995), Corbett et al. (1997), Jackerott et al. (1996).

BrdU pulse labelling of day 2½ and 3 post ligation exocrine/acinar tissue produced negative results, suggesting that post-ligation acinar tissue was not in a state of proliferation and could therefore only be in a state of regression and/or transdifferentiation. The apparent dedifferentiation of acinar cells to acinar-endocrine cells expressing PP (or ST), as well as intra-islet cells immunoreactive for PP and ST observed between days 2 and 3 and thus far described, did not occur in isolation.

5.1.2.2 Oedema

Oedema is a consistent although transient observation in this study. The phenomenon is also consistent with the findings of similar studies (Edström and Falkmer 1968; Walker 1987). It is also the most likely hypothesis for the percentage decline in especially insulin during the phase one (days one to three) portion of the graph in figure 4.1.1a . If the surrounding tissue were experiencing oedema, the resulting tissue expansion would be reflected in the relative (not absolute) decline in the percentage of endocrine tissue per area measured. So also during phase two (days four to seven, figure 4.1.1a) when the effects of acinar deletion would contribute to substantial tissue shrinkage. Both these observations have, as far as could be ascertained, not as yet been linked to one another or discussed in literature.

5.1.2.3 Pancreatitis

In humans, pancreatic duct obstruction results in pancreatitis. The clinical symptoms of pancreatitis (in humans) are described as "severe, relentless abdominal pain, accompanied by fever, tachycardia, shock, intestinal ileus and elevated serum and urinary amylase levels" (Rubenstein and Wayne 1981). The pancreas is described as becoming swollen, necrotic and often haemorrhagic. Histologically there is necrosis of both exocrine and endocrine tissue sometimes accompanied by thrombosis of pancreatic vessels leading to ischaemic necrosis superimposed on the autolytic effects of pancreatic enzymes.

One could therefore expect the results of the ligation to present itself in a similar way in rats, as functional or mechanical obstruction of the pancreatic duct resulting in acute pancreatitis in a clinical setting. Yet this would appear not to be the case. Experimental animals were restful and showed no obvious signs of discomfort, had healthy appetites and cavorted about their cages at night, suggesting that they either did not suffer from pancreatitis in a similar way as humans or did not develop clinical pancreatitis or peritonitis. This presumption is confirmed by the lack of inflammation in ligated parenchyma tissue as discussed below.

The acinar deletion observed, could also be construed as proteolytic assimilation of the pancreas by the action of its own enzymes as mentioned by Rubenstein and Wayne (1981). However, the acinar deletion occurring in the exocrine compartment of the rat pancreas was not associated with the lymphocyte and macrophagal infiltration that accompanies chronic inflammatory response to pancreatitis.

5.1.2.4 Acinar Deletion

Ligation induced acinar deletion is characterised by the rapid destruction of single cells by a process of budding of membrane bound apoptotic bodies that are phagocytosed by neighbouring parenchyma cells or macrophages (Gukovskaya et al. 1996). This widely supported concept is used to explain/describe the very rapid cellular change occurring in the exocrine compartment (Abe, Watanabe 1995; Doi et al. 1997; Gukovskaya et al. 1996; Wada

et al. 1995; Watanabe et al. 1995). The mechanism of cellular deletion/replacement is however not a consistent phenomenon as species differences have been shown to occur.

A number of prominent scholars maintain that the pancreatic acinar tissue is "thoroughly" deleted by apoptosis and/or necrosis or a combination of the two (Abe et al. 1995; Gukovskaya et al. 1996; Idezuki et al. 1969; Walker 1987; Yamaguchi et al. 1993; Wang et al. 1995) and/or autolysis caused by intrapancreatic protease activation (Merriam et al. 1996).

The pancreas in early post ligation was characterised by rapid acinar deletion. The lack of an inflammatory response during the early stages in this study tends one to conclude that acinar deletion was a result of apoptosis. This then concurs with the findings of similar studies quoted in the text above. A further aspect that needs to be considered is the balance between acinar apoptosis (and deletion) and acinar transdifferentiation as suggested by (Bouwens 1998) an could possibly be confirmed by ICC labelling for apoptosis.

Although it is true that no acinar cells are detectable from day 3½ post-duct ligation, it is perhaps simplistic to ascribe the absence to only apoptosis and/or necrosis. Argument will be presented to support the presence of PP-IR (pancreatic polypeptide immunoreactivity) in the pancreatic interstitium. Whether this presence is due to a transitional change in acinar tissue, reflective of transdifferentiation in the direction of insulin secreting cells or toward deletion, it is impossible to tell. It would therefore appear from the early results of this study as if PP was the first pancreatic endocrine hormone to appear within the acinar parenchyma after ligation and is paralleled by synchronous changes in the endocrine compartment. This hypothesis is schematically illustrated in figure 5.1 on page 181 in the special appendix at the back of this chapter.

5.1.2.5 Increased Ductular Dilatation

Experimentally induced increased ductular activity, per se, has been described by Yamaguchi et al. 1993; Rosenberg 1998; Bertelli et al. 1997; Zenilman et al. 1996; Walker 1987; Wang et al. 1995; Rosenberg et al. 1988. It would however seem as if the ductular changes induced by duct ligation in the study presented here occurred on a scale not previously described. In

figures 4.19 to 4.22 ducts varying in size from between 5-10 μm to 40 or 50 μm in diameter had totally invaded areas previously occupied by acinar tissue. This observation is corroborated by Bouwens (1998). In close association with these distended ducts were areas of substantial amounts of insulin positive tissue. It has long been popular opinion that experimentally induced endocrine neoformation results from the budding of cells making up the duct lumina (Yamaoka and Itakura 1999; Larsson 1998). This then leads to the postulate that the insulin rich areas (IRA's) observed to develop in close association with areas of increased ductular activity are intimately connected.

In most cases the space (vacuity) left in the intercellular compartment resulting from acinar depletion was rapidly filled by increasingly dilation of ducts. It has been reported by Bouwens et al. (1994, 1996, 1997) to be epithelial cell proliferation which is responsible for the formation of ductular complexes, from which extensive endocrine development occurred (Wang et al. 1997; Wang et al. 1995, Rosenberg et al. 1996a). This phenomenon is said to be typical of ductular proliferation , and as suggested by Yamaoka and Itakura (1999), is the essence of LIEF. No duct endocrine cell marker was used to identify these so-called ducts in the study presented here. It is therefore merely a hypothesis that these structures are most probably representative of the structures referred to above by the authors mentioned.

By day 3½ there was a noticeable and prominent eruption of structures described by Bouwens (1998) as ductular complexes (Figures 4.19 to 4.24). Whereas on the previous day (Day 3, Figures 4.16 to 4.18) acini and islets with relatively little extra-islet endocrine activity were still distinguishable, this was no longer the case. A probable postulation for this phenomenon is that in an extremely short time period (12 hours) the so-called "ductular proliferation" was accompanied by a dramatic and simultaneous appearance of cells immunoreactive for all four major pancreatic endocrine hormones in an apparently random and disorderly manner with insulin predominating.

5.1.3 Endocrine Compartment Change

5.1.3.1 Post Ligation Endocrine Events

A number of different experimental interventions will be discussed in the text ahead (paragraph 5.5), they include ß-cell destruction by STZ, cellophane wrapping and subtotal pancreatectomy. The various interventions described were performed to induce changes in pancreatic morphology that all eventually lead to the formation of additional endocrine tissue. The pathway followed after each of the interventions involved different initiators, series of events, different animal species and endocrine cell lineage development, perhaps to now considered to be uniform, yet different. These reported differences are most probably due to the substantial variation in experimental technique employed, age and species of animals, hence the difference of opinion on cell lineage development as suggested by Rosenberg (1998).

5.1.3.2 Insulin and Glucagon

Reviewed here are the changes in insulin and glucagon distribution in normal islets as a result of duct ligation. In this study, under duct ligation conditions, the conventional islet pattern (illustrated in figure 4.2) appears to disappear very rapidly. The first signs of change are evident in figures 4.10 and 4.11 (day 2½) and reveal breaks in the continuity of the α-cell mantle surrounding the ß-cell core, and disappearance of both PP and ST from the mantle region. These changes include the appearance of PP and ST within the ß-cell core (figures 4.12 and 4.13 on page 138) as illustrated in the schematic representation of these events in figure 5.2 in the special appendix at the end of this chapter.

If acinar and endocrine transdifferentiation can be assumed, it would be highly unlikely if it was to be restricted to only PP- and ST-cells. It is therefore also possible that cells occupying any position in the pancreatic cell lineage, could under experimental conditions, be induced to transdifferentiate toward any of the other pancreatic cells as proposed in figure 5.3 in the special appendix at the end of the chapter. This reciprocal relationship between the various pancreatic cells is supported by Melmed et al. (1972); Melmed (1978) – Figure 5.10; Gu et al.

(1994) and Bouwens (1998) – Figure 5.4. All clearly indicated that cells existed with characteristics of two and sometimes three different pancreatic cells concurrently, as already discussed above.

These could all be explained if all pancreatic cells have the ability, under the influence of the correct signal (Yamaoka and Itakura 1999), to transdifferentiate into any of the other pancreatic cells mentioned as advocated by Bertelli and Bendayan (1997). Observations made in this study include the unusual presence of PP- and ST-cells in the ß-cell core described above, the appearance of gaps in the glucagon mantle (as portrayed in figures 4.11, 4.17 and 4.33) and the development of a dilapidated outer margin of islets mentioned in paragraph 4.1.1.

A progressive and permanent deletion and/or transdifferentiation (retrodifferentiation – Scarpelli and Rao 1980) of large numbers of cells in the islet periphery (α-cells) becomes more evident as time progresses as illustrated in figures 4.11 (day 2 ½ on page 137) with 4.17 (day 3 on page 139). In the latter figure, many more breakages in the glucagon continuity are evident. By day 3½ (figures 4.20 and 4.21 on page 140) very little evidence of a glucagon mantle around an islet ß-cell core could be detected. Although there is an apparent decline in the glucagon component of islets, both Scaglia et al. (1997) and Wang et al. (1995) report an increase in the relative endocrine mass.

The architecture of existing islets (indicated as "A" and "B" on figure 4.20 on page 140) can be viewed from two perspectives. The first, is that the figure in question represents a "pre-existing" islet whose structure is severely compromised by the total absence of the glucagon/PP/ST mantle and the partial islet invasion by dilated duct-like structures (complexes). In which case cell deletion or transdifferentiation could be responsible for the change. The second perspective involves considering the insulin rich area in question as an "emerging" endocrine structure. A still to be determined process would then be involved in the process of endocrine (insulin) neoformation.

In the above mentioned figures, fragmented areas of insulin immunoreactivity dominate the landscape with G/PP/ST occurring mainly as single cells with IIR and GIR often in close association with one another. This distinct change in the existing endocrine profile reported

in other similar studies varies from the findings reported here. Both Scaglia et al. (1997) and Wang et al. (1995) report a "near doubling" of the ß-cell volume which is a low estimate for the five-fold increase reported here, and a 20 – 30% increase in α-cell populations, which is clearly not seen here.

The apparent doubling of endocrine tissue area, especially IIR, post duct ligation in this study requires some comment. The endocrine tissue area increase in this study was in some cases ten times their normal value and five times higher (10% vs 2%) than those reported by Bouwens (1998). Walker (1987), Walker et al. (1992), Wang et al. (1995) report no decrease in the overall pancreas volume after ligation as adipocyte infiltration was shown to take place in areas of acinar apoptosis.

The point, post ligation, at which acinar deletion, increased ductular dilatation and an increase in endocrine formation had occurred was 3½ days in this study compared with four days described by Bertelli, Bendayan (1997); five days by Walker (1987); seven days by Yamaguchi et al. (1993). Whether or not this variance is of any significance would only perhaps require resolution if the use of PDL-tissue were under consideration for replacing foetal tissue as currently is the case in our laboratory.

5.1.3.3 Pancreatic Polypeptide

In this study in which there was total occlusion of the pancreatic duct the first out of character immunoreactive pancreatic endocrine hormones detected were PP and ST at days 2, 2½ and 3 post-PDL, where "out of character" can described as any endocrine presence outside normal islet configuration. Pancreatic polypeptide appeared both as inter-acinar and intra-islet areas of immunoreactivity while ST was restricted to the intra-islet compartment. The normal PP/ST distribution and cell quantity in relation to the other endocrine was not observed in these specimens. The appearance of a substantial increase in extra-islet PP-IR cells, even as acinar disorganisation was occurring, has been the topic of controversy for quite some time as a number of studies have reported it to be rather NPY by Teitelman et al. (1993) in cell lineage context or PYY by Upchurch et al. (1994), Jackerott et al. (1997) during embryonic development.

A number of studies report opposing findings for initial endocrine expression both during embryonic development and post surgical intervention [refer to the work of Dr Wolfe-Coote and her team on cellophane wrapped vervet monkey pancreas, Herrera et al. (1991) also Jackerott et al. (1996), (1997), Yamaoka and Itakura (1999) and Teitelman et al. (1993)] The out-of-character PP and ST (associated with islets and acini) within 48 hours seems to exclude any ductal origin and would rather suggest transdifferentiation as suggested by Bertelli and Bendayan (1997). Although it should also be said that this does not preclude any alternative origin for either PP or ST.

In this study, pancreatic polypeptide did not occur within the islet ß-cell core in control animals, nor in any quantity outside the peripheral mantle zone of islets, or as isolated PP-immunoreactive cells within the deleted acinar parenchymal stroma (outside the peripheral mantle) as was observed in experimental animals. No quantities of more than 4 or 5 cells per islet (3 - 6 μm thick) section (even in the largest islets) as illustrated in figure 4.2 on page 135, were observed.

In figure 4.13 (on page 138) many more islet associated PP-positive cells were visible scattered around the perimeter of both a clearly defined conventional islet (A) and an ill-defined islet (B). The traditional islet associated PP to other endocrine cell distribution was given as zero to two percent mentioned in paragraph 2.4. The figure indicates a clear increase in expected PP population. The distribution of PP-positive cells in the rest of the figure (similar to that depicted in figure 4.18 for day 3 on page 139) is out of character with the accepted pattern of distribution especially the PP-positive cells found within islet "B" by day 3½ (figure 4.20 on page 140). Very soon hereafter the "out-of-character" PP-IR had disappeared and/or dispersed and the PP-IR present was co-localised with ST.

Similar comments on extra-islet appearance and disappearance of pancreatic polypeptide can be made for day 3 post-PDL. Figure 4.18 on page 139 indicates PP-IR cells out of islet context, most of which appear to be in association with ductular structures, in which case they would be duct/endocrine cells as described by Bouwens and Klöppel 1996; Wang, Klöppel, Bouwens (1995); Yuan, Rosenberg, Paraskevas et al. (1996).. It is however also possible to argue that the PP presence is derived from acinar cells undergoing either apoptosis and/or transdifferentiation. If this is the case, then it is possible that it represents the presence of

intermediate pancreatic cells described as either acinar/endocrine or endocrine/acinar cells by Melmed et al. (1972); Melmed (1978); Bertelli and Bendayan (1997)

A hypothesis that would seem to explain these findings, is that acinar cells (which may possess dormant pluripotent/stem cell ability, (Bouwens 1998b) react to a ligation-induced stimulus. The first step (PP secretion) in a possible endocrine cell lineage development process, which begins with PP expression as described by Wolfe-Coote et al. (1998) and Herrera et al. (1994) in organogenesis. As to whether the hormone is PP/NPY/PYY has been the centre/focus of a long standing difference of opinion.

5.1.3.4 NPY/PYY

Embryonic cell lineage studies suggest that stem cells first express NPY and/or PYY on their way to becoming either insulin or glucagon cells. Most reports however focus on the existence of ductular and/or acinar cells sharing intermediary characteristics with insulin IR and not PP-IR as in this case or as maintained by Hererra et al. (1991) and Wolfe-Coote et al (1990b, 1992, 1998). Upchurch et al. (1994) views NPY and PYY as stem cell markers (in much the same way as Bouwens et al. (1995) views cytokeratins as duct epithelial cell markers) of islet precursors expressed in rodent embryonic pancreas during endocrine differentiation.

Dual, and even triple (Rosenberg 1998; Jackerott 1996) expression in cells has however been shown to be a common phenomenon in pancreatic morphology (Lopez et al. 1995). The PP/NPY/PYY issue would appear to revolve around two aspects: firstly, antibody cross-reactivity between PP/NPY/PYY bedevilling ICC results, and secondly, whether it is embryogenesis or experimentally induced tissue remodelling that is being discussed. The very definite presence of hormonal and neuronal factors influencing the post procedural pancreatic transformation has been established (McEvoy 1981; Myrsen et al. 1996; Rafaeloff et al. 1996; Ling et al. 1996b; Min et al. 1998; Barro et al. 1998 etc., (for further references refer to paragraph 3.2.2 of Chapter 2 – literature survey).

An important issue regarding cross immunoreactivity of bovine raised PP antibodies with NPY has been ongoing for quite some time and requires some mention at this stage. Herrera, et al. (1991) reported the very early presence of PP in developing murine foetal pancreas, which was disputed by Teitelman (1993). Dr Teitelman ascribed the report of PP-IR in developing pancreatic tissue to antibody cross reactivity. The antiserum used in this study is raised to the synthetic C-terminal hexapeptide of PP.

The antiserum has been characterised (Louw 1995) to be specific for PP, not cross reactive with PYY or NPY or any other known gastrointestinal hormone. Similarly anti-porcine PYY, raised in rabbits does not cross-react with NPY or PP. Any misinterpretation therefore in the current finding of PP-IR in relation to figures 4.13 and 4.18 discussed above seems highly unlikely. This point of view is further borne out by the fact that neither NPY or PYY immunoreactive cells were found to be present in ligated tissue before day 3½ . If cross reactivity did exist between the antisera of PP and either NPY or PYY it would have been evident at an earlier stage as NPY/PYY antisera would have bound to PP located around normal islets and be visibly stained.

Although no evidence has been presented in this study, it would seem feasible to assume that the post-PDL events would of necessity be initiated and influenced by messengers/regulators of chemical and neural origin. The very early (day 2 post-PDL) and continued appearance of PYY until day 5½ and NPY for the full duration of the study, combined with the fact that unaffected (non-ligated) tissue adjacent to ligated tissue presented no immunoreactivity for NPY or PYY, could suggest a possible role for these two substances in the initial stages of tissue remodelling. Both have been implicated in wide ranging action on a large number of organs (Gehlert 1998, Goumain et al. 1998) other than the pancreas.

Whether there is an interchangeable relationship between cellular expression of PP/NPY/PYY is open to speculation. Whether or not cellular apparatus manufacturing either of the peptides could convert to either of the other during chemical interaction is also open to speculation. Organs have been shown to possess a multiplicity of receptors for NPY and PYY, some of which were responsive to either (Bischoff, Michel 1998a, 1998b; Matthews, Jansen, Lyerly, Cox et al. 1997; Mulder, Myrsen-Axcrona, Gebre-Medhin et al. 1997; Robidoux et al. 1998). Studies by and Bischoff and Michel (1998a, 1998b) report that NPY

receptors in the kidney could also be activated by PYY, and NPY lowering of blood glucose was mimicked by the same dose (2 μg/kg/min) of PYY. The finding by St-Pierre et al. (1998) that 70% of NPY binding sites were receptive to PYY and Yang, Li, Reeve, Rivier et al. (1998) that both peptides affected gastric secretion and function all lend credence to this viewpoint.

Neuropeptide tyrosine has been conclusively linked to islet innervation (Ding et al. 1997) in sympathetic neurons (Bischoff, Michel 1998a, 1998b) distributed throughout pancreatic parenchyma and particularly associated with pancreatic duct and vascular walls. It is considered a neuronal factor similar to PrP protein (Atouf et al. 1994). In contrast, PYY-IR has been found to be localised to islets only (Ding et al. 1997) although this was not observed in this study.

To summarise, up until day two, post ligation very little change occurred in the endocrine compartment of the ligated pancreas. At day 2½ post-ligation pancreatic tissue, unusually located islet PP-IR within the ß-cell core of islets is in close association with numbers of ST-IR cells, while in the PP-IR of the interstitium although ST-IR was absent from within the PP-IR cells.

The NPY/PYY presence represents one of the many catalysts that run/control/modulate the tissue morphogenesis process in much the same way as PYY has been shown to be involved in the establishment of other enteroendocrine cells (colonic endocrine differentiation). Both NPY and PYY all but disappear after birth and into adulthood (Upchurch et al. 1996).

5.2 ISLET STRUCTURE

5.2.1 Normal Profile

Stubbs et al. (1994) described rodent islet of Langerhans in terms of a ß-cell (insulin positive) core around which α-cells (glucagon positive) are arranged. This agrees in essence with the assessment of Fawcett and Bloom (1986) although these authors maintain that some α-cells (glucagon positive) are scattered along the capillaries which permeate the islet interior and δ-cells (somatostatin positive) occurring "anywhere" in the islet.

5.2.2 Sham Operated Controls and Unligated Tissue in Ligated Rats

Although the above might be true for humans and a variety of other species, on this point this study deviates from this pattern. No evidence of either glucagon Immunoreactivity (G-IR) or somatostatin immunoreactivity (ST-IR) could be found inside any of the islets in sham operated control or the unligated tissue of ligated rats studied, or in any other site than at the islet perimeter. Glucagon formed a prominent band of immunoreactive cells around only the perimeter of small, medium and large islets alike.

Although substantial amounts (up to 25% - Bouwens and Pipeleers 1998) of insulin secreting tissue (β-cells) have been shown to occur out of islet context in humans (and non-human primates - Louw 1995), these single cells and/or three to five cell clusters are not usually associated with any of the other of the pancreatic endocrine cell types, as is the case in conventional islets. This observation is of interest to this study as variations on these findings were observed in the rats in this study. Widespread evidence of extra islet insulin activity has already been reported in this study in ±15% of the rats. The reason and significance of this is open to speculation. One possible explanation for this phenomenon, could ascribed it to intra-species variation and/or anatomical or physiological anomalies. The reader is also referred to appendix "D" for additional comments regarding sham operated control animals.

5.2.3 Experimental Animals

A relatively consistent proportion of ß-cells, irrespective of islet size, is reported in the literature for normal Sprague Dawley rats (Colella et al. 1985). Endocrine tissue expressed as both a proportion of the total pancreas volume and as a proportion of the total endocrine volume varies substantially from this pattern. During the early stages (phase one – days zero to three) as depicted in the figures for day 2½ (figures 4.12 and 4.13 on page 138) both somatostatin and PP-cells were clearly evident within the insulin core in a number of islets. These islets were very obviously undergoing some sort of change. In the context of duct ligation tissue remodelling would appear to be a unique finding and will necessitate discussion at a later stage.

Fawcett (1986) placed an estimation for normal human islet ß-cell proportion (percentage of total endocrine tissue) at 60%, Fitzgerald and Morrison (1980) at 60 – 80% (human) while McEvoy and Madson (1980) place islet ß-cell content at day 18 gestation (in the laboratory rat) at 50%, 60% at birth and 80% at four months. Morphometric analysis of the tissue of sham operated controls and unaffected tissue in duct ligated rats has been reported in this study. The insulin content of pancreatic tissue under duct ligation conditions, for the most part, exceeded by some considerable margin (92.5% of the total endocrine area or 7.05 ±4.19% of the total area of the pancreas measured) even the highest estimate mentioned above throughout most of the ligation induced morphogenesis that occurred. Post duct ligation areas of endocrine neoformation, referred to as Ligation Induced Endocrine Formations (LIEF's) in this study, also did not conform to the given endocrine cell proportions thus far described, findings which concur with those of Edström et al. (1967).

In a number of specimens insulin/glucagon cell quantities, expressed as a percentage of the total area measured (normal values: insulin = 1.04%; glucagon = 0.18%; somatostatin = 0.09%; pancreatic polypeptide = 0.216%; NPY = 0.24%; PYY = 0%), did not conform to previously quoted findings of Edström et al. (1968), McEvoy et al. (1980), McEvoy (1981); Rosenberg (1998).

The number of factors that are thought to be involved in the induction of an increase in ductular activity and endocrine neoformation are presumed to "remain isolated in the exocrine interstitium" (Zenilman et al. 1996; Wang et al. 1995) and did not appear to come into contact with and affect pre-existing islets in close proximity. Although it has been shown that cytosol extracts from partially ligated (cellophane wrapped) pancreas does stabilise diabetes mellitus in streptozotocin diabetic rats (Rosenberg et al 1988), it remains an interesting issue which may justify further investigation, as the only noticeable evidence of change was detected in tissue distal to the ligation site.

Evidence has been presented in this study of areas of islet neoformation which suggest that newly formed endocrine tissue lying in close proximity to pre-existing islets eventually undergo amalgamation to form new islet tissue consisting of both pre-existing and LIEF's. This is of functional significance as newly formed islet tissue would need secretory access to the blood vascular system via the islet capillary network in order to be of any effect.

In a number of cases, pre-existing islets formed the core around which ligation-induced endocrine tissue formed. (pre-existing islets were characterised by a glucagon mantle while emerging islets had no association with any of the other endocrine cell types). In other cases, LIEF's linked up to adjacent existing islets, resulting in very large (>500 μm) and irregular structures. They were found to be (in figures 4.37 on page 144 and 4.41 on page 145) in the order of 600 μm x 400 μm (average diameter = 500 μm; volume = 162 812 μm^3) and fall far outside the scale for normal described by this study, and extend into the islet dimensions of Colella et al. (1985) and Ferrand et al. (1995). This phenomenon was also described by Edström (1967) who reported that the islets eventually broke up into smaller segments over the long term (5 months).

Both ST-IR and PP-IR in adjacent "normal islets" in sham operated control animals showed no deviation from the generally accepted pattern of distribution. NPY and PYY first made their appearance on day 3½ where after NYP persisted intermittently throughout while PYY disappeared after day 6½.

5.3 ISLET SIZE

5.3.1 Sham Operated Controls and Unligated Tissue in Ligated Rats

Although quoted values for islet size vary within a considerable margin, no apparent standardised criteria for their classification would seem to be in practice. Three independent studies (Colella et al. 1985; Ferrand et al. 1995; Reaven et al. 1981) all done on Sprague-Dawley rats report vast disparities in islet size in their experimental work. Some authors express islet size in diameter and radius terms (Ferrand et al. 1995), others (Wang et al. 1995, Rosenberg 1998) use islet surface area (μm^2) to express results, while another group (Edström et al. 1967) expressed total insulin content in terms of volume (μm^3). No indication could be found, in any of the studies, as to what proportion of islets were classified as either small, medium or large. One could perhaps expect equal relative proportions, either in terms of volume or numbers, to be grouped into each of the categories, especially as studies of this nature are based on endocrine neoformation.

For example, islet size for large islets varies from diameters of 80 – 100 μm (Colella et al. 1985), 753 – 1000 μm (Ferrand et al. 1995), 180 – 220 μm (Reaven et al. 1981) and 200 – 300 μm (this study). Islet size for small islets (with no proportions given) vary from 10 – 50 μm (Collela et al. 1985), 10 – 150 μm (Reaven et al. 1981), 10 – 140 μm (Ferrand et al. 1995). This lack of consistency therefore results in a disparity for the classification of especially small islets as one author would report an increase in small IRA's while referring to one set of criteria while another would report the same using a different set of criteria.

Islets of varying size, in sham operated controls, unaffected pancreas in duct ligated and duct ligated rat pancreas followed a pattern for small islets as described for Sprague-Dawley rats reported by Ferrand et al. (1995) and Reaven et al.(1981). For medium islets, similar to the description given by Colella et al. (1985) and Reaven et al. (1981). In this study, islets were however on average smaller and more numerous than those quoted by the above mentioned authors. For example, islet numbered one (1) in figure 4.30 has dimensions of 11 mm x 18 mm, which when converted, allowing for magnification, equal 122 μm x 200 μm. The average islet diameter would be 161 μm the radius 80.5 μm. The resulting surface area would be 20347.9 μm^2 (πr^2) and the eventual volume by the general mathematical formula for volume of a sphere = $4.189r^2$ = 27145.7 μm^3 (Diem, Lentner 1970). Classified by the criteria reported by Collella et al (1985) this would be a large islet while both Ferrand et al. (1995) and Reaven et al. (1981) would classify them as small, and this study as of medium size.

In the light of possible species variation and with the above disparities in mind and the lack of clarity on the relative proportion of islets falling into each category, I would like to propose (as was practised in this study) that a convenient standard, for medium islets, be established from the normal tissue of a particular laboratory animal species at the onset of a study. Islets therefore smaller than this norm would be classified as such, as would larger islets.

As will be discussed in the text ahead, a number (15% - 27 out of 184) of experimental animals (within the Sprague-Dawley strain used) presented with a preponderance of small ($d <$ 100 μm) islets, the remainder were medium islets with no large islets present. Contrasted with this was the balance of the group which conformed to the standard determined in this study. This variation would of necessity lead to a skewing of the results, especially when

dealing with ligation induced endocrine formations (LIEF's). No previously published record of this disparity within a particular strain of laboratory rat could be found.

In addition to the above classification, it became evident during the course of this study to add a further category applicable to LIEF's occurring in ligated tissue (Figure 4.20) designated islet clusters consisting of 3 - 5 by Bouwens, Pipeleers (1998). ICC positive cells, too small, irregular or scattered to be measured/classified as small islets.

5.3.2 Experimental Animals

The occurrence of islet tissue ranging from 10 – 100 μm in diameter, classified as small LIEF's, islet clusters and/or single cells with IIR (insulin rich area) has been a consistent finding in this study. Up until day 30 roughly 50% of the total number of insulin rich areas (IRA's) were seen to fall into this category. As these small structures are reported to be indicative of neogenesis (du Toit et al. 1998; Wang et al. 1995) it is of interest to note their persistent presence after 30 days in this study and beyond (Edström et al. 1967). Of special note was their isolation from the other endocrine cell types usually found in islet context.

5.4 ISLET FREQUENCY

5.4.1 Sham Operated Controls and Unligated Tissue in Ligated Rats

In the greater proportion of adjoining unligated pancreatic tissue in ligated rats as well as in the sham-operated control (SOC) group the islet tissue making up one percent the pancreas, is consistent with the findings of Hellerström (1984), Lucocq, Findlay (1981), and Bouwens (1998b). In rats the greater majority of endocrine tissue 99.1% (humans 85%) is situated in islet context (Bouwens 1998b) where the ratio of cells and their placement follow the pattern already described above. As already mentioned above, a number (±15%) of animals in the strain of rat used in this study presented with approximately 20% of normal of islets categorised as small ($d < 100$ μm), while they were absent from the remaining animals.

The remaining 0.9% (humans 15%) of cells is said to occur as single IIR cells (Bouwens 1998, Bouwens, Pipeleers 1998) have been found as units of less than 20 μm (2 – 3 cells). They are not associated with any other types of immunereactive cells or in close association with ducts (Lucocq, Findlay 1981) in adult organs. In foetal and neonatal (Bouwens, Pipeleers 1998) organs, these single cells portray dual endocrine and ductal characteristics (cytokeratin expression) as portrayed in paragraph 2.5 of chapter 2 by Bouwens and Pipeleers (1998).

Whether the amount of extra islet IIR found to be scattered amongst normal acinar tissue in the animals used in this study amounts up to the estimates described above was an intriguing question. Ten random visual fields of SOC and unaffected pancreas in ligated rats were studied to determine the extra islet insulin immunoreactivity. Only areas of IIR of less than 30 μm (clusters of one to five cells) were counted. The comparison (Table 4.7) clearly shows the vast discrepancy existing between the number of IIR cells in the 2-5 cell cluster range between the various control groups cited and the experimental group. This finding is in concurrence with those of Rosenberg (1998); Bouwens (1998b); Bouwens, Pipeleers (1998).

5.4.2 Experimental Animals

As already mentioned, literature (and this study) support not only the concept of species variation influencing experimental results, but also the age of the animals and the type of experimental procedure carried out. It will however suffice to say at this stage that there are substantial differences between the results of this study and those quoted in literature.

One of the initial problems was thought to be the perception that the apparent increase in insulin rich areas and total percentage endocrine area was due to acinar deletion and resultant tissue shrinkage. Although this appeared to be the case in the short term, 30 day post ligation pancreatic tissue, although still devoid of acinar tissue, but significantly also any of the ductular activity that characterised earlier (day 3½) tissue sections (possibly taking up tissue space) appeared quite substantial in terms of total tissue volume (refer to figures 4.47 - 4.49 on page 146).

Studies have reported that the pancreas returned to its former volume after experimental intervention. Edström and Falkmer (1968) reported *marked* fatty involution (emphasis supplied). Watanabe et al. (1995) reported gradual deletion of acinar cells by apoptosis and pancreas exocrine infiltration by intralobular fat deposition reaching a volume approximating that of the pre-ligation pancreas by eight weeks post-PDL. No fatty infiltration was detected in any of the specimens in this study.

5.5 EXPERIMENTAL PROCEDURES

5.5.1 Introduction

The results of this study differed substantially from those obtained from similar studies into cell lineage development. Reconciling the results of a variety of experimental interventions performed on an even larger variety of experimental animals, ranging from prenatal through the various stages of development to adulthood with those obtained in this study has presented a major challenge. Hence the need to consider each of the various models individually.

The question which really should be answered first, before one decides to enter the debate about pancreatic endocrine/exocrine cell lineage development is which of the myriad of substances (40-or-so indicated in the special appendix at the end of the chapter), should be considered as co-expression factors and/or endocrine hormones, if at all. All of them play one or other role either in embryonic development, steady-state cell turnover or experimentally induced tissue remodelling. A large percentage of them have been reported to be either co-expressed and/or transiently expressed in various pancreatic cells as they go back and forth through various stages of development or in response to a stimulus.

This study proposes that only the main cell types that are traditionally accepted to be present in the mature pancreas (α-, β-, δ-, PP-cells, acinar and duct cells) serve as a starting point from which the cell lineage argument is developed. All other substances should then be considered factors/agents/catalysts/initiators and/or modulators providing the impetus, a vehicle or a pathway to reach a particular destination. If this assumption is sound, it then narrows down

the field considerably as to allow studies to focus on just the four primary endocrine cells, acinar and duct cells.

If this assumption is acceptable as a starting point for a discussion of pancreatic cell lineage (during embryonic development or under experimental conditions), the issue becomes less cluttered with peripheral detail and is it possible to predict a sequence of events with greater surety and accuracy. Bouwens (1998) has presented a convincing argument for back-and-forth re-, retro- and transdifferentiation of any of the pancreatic cells mentioned above. This concept is further reinforced by the work of Bertelli, Bendayan (1997) and Scharpelli, Rao (1994). This plasticity is also supported by a number of prominent research groups and amply supported in current literature as will become evident in the text ahead.

5.5.2 Streptozotocin (STZ)/Alloxan (ALX)

5.5.2.1 Adult Animals

Streptozotocin and Alloxan are both ß-cell toxins bringing about diabetes mellitus in varying severity depending on the dosage (McEvoy, Hegre 1977; Wang et al. 1994). The physiological vacuum formed by the resulting lowering of plasma insulin levels in adult rats, has been reported to induce a number of changes. The first is a slight to moderate, but insufficient, replacement of ß-cells involving ß-cell and islet hyperplasia. The second is replication by remaining ß-cells give rise to daughter cells, followed by the transdifferentiation of both endocrine and exocrine cells, termed neoplastic transformation, to form additional insulin secreting cells, and finally neogenesis (stem cell to ß-cell development – Bouwens, Klöppel 1996; Teitelman 1993; Guz et al. 1995).

McEvoy (1977) did however show that this recuperative capacity, in adult tissue, to be extremely limited. Pancreatic insulin mass declined from 928.8 ±70.2 insulin positive cells/mm to between 17.8 (±6.6) to 52.9 (±9.8) in experimental groups (other endocrine hormones remained relatively unaffected/unchanged with only one or two significant exceptions). One can deduce from these results that ß-cell deletion and/or insulin reduction alone are insufficient to induce the prolific development that has been shown to occur under a

variety of different experimental conditions. These results do however not apply to neonatal animals.

5.5.2.2 Neonates

Diabetes mellitus induced by STZ/ALX administration in neonatal rats, rapidly regressed due to the relative immaturity of the pancreas at birth (Ferrand et al. 1995). Ample evidence supports the view that the islet growth which continues after birth (McEvoy, Madson 1980) is due to the presence of two main types of terminally undifferentiated cells.

Firstly, those with endocrine and exocrine characteristics (Melmed 1972, 1978). Secondly, newly forming sub-populations of endocrine cells, different from α-, β-, δ- and PP-cells. They are characterised by morphological heterogeneity of secretory granuli-like organelles, insulin/glucagon co-expression and their relative abundance and proliferative ability. PEPS - polymorphic endocrine pancreatic cell sub-population cells (Ferrand et al. 1995) synonymous with intermediary cell of Melmed et al. 1972, Melmed 1978. In spite of STZ/ALX administration rat pups were normoglycaemic by day ten and grew normally, although slowly at first, into adulthood (Leahy, Bonner-Weir, Weir 1985; Wang et al. 1994).

Adult ß-cells then have been shown to have limited and poor regenerative capability as evidenced by the perpetuation of DM in STZ/ALX treated adult animals compared to the re-establishing of normoglycaemia by day 10 in neonatal rats treated with STZ/ALX on day 1.

This explains the inability of the IDDM patient to experience any long term relief from exogenous insulin use by experiencing spontaneous ß-cell regeneration as the case should be if ß-cells possessed high regenerative potential (Gu et al. 1994). Following this line of thinking, interesting results could be obtained from the next logical step in the investigation which would be to perform duct ligation experiments on adult animals with varying degrees of chemically induced DM. The results would enable one to ascertain the degree to which PDL is able to ameliorate hyperglycaemia and slow or reverse the insidious secondary complications of DM, if at all.

5.5.3 Cellophane Wrapping.

Cellophane wrapping (CW) represents a mechanism of achieving partial obstruction of the pancreatic duct and has been shown (Wolfe-Coote 1995; Rosenberg et al. 1988, 1990, 1998) to induce endocrine neoformation. The tissue transformation was characterised by ductular proliferation around the periphery of islets as well as in areas of exocrine/acinar occupation. Areas of ductular proliferation displayed immunoreactivity for predominantly pancreatic polypeptide and somatostatin. Dr Wolfe-Coote and her co-workers (1990a) showed, in the vervet monkey at least, that δ-cells (IR for ST) occurred earlier than either β-cells (IR for insulin) and α-cells (IR for glucagon) in the proliferating ductular tissue of CW experiments.

As her experimental group only consisted of five animals, no attention could be given to a more precise look at the day-to-day sequential changes that are thought to occur post-wrapping. The study thus makes no mention of the extent to which acinar deletion occurred by apoptosis and/or lymphocytic/macrophagal infiltration as described by Abe et al. 1995, Doi et al. 1997, Paraskevas et al. 1997, Scaglia et al. 1995, 1997, Wada et al. 1995; Walker 1987, and this study where it was found to be a prominent/important concept in the pre-neoformation phase of tissue remodelling. It also does not address the issue of acinar transdifferentiation/de-differentiation to cells with ductular characteristics (Fang, Hussong, Roebuck et al. 1997; Vay Liang et al. 1986) or of any other transitionary cells heretofore mentioned. Cellophane wrapping also gives the only thus far recorded account of adult pancreas remodelling occurring on both sides (duodenal and splenic), although mainly downstream of the wrap, and of the process being noticeably more prolific on the duodenal side/head portion of the pancreas (Wolfe-Coote 1995, Wolfe-coote et al. 1998; Rosenberg 1998). A further aspect of note in the study was the fact that endocrine addition seemed to occur at a much slower pace than in, for example, duct ligation already discussed in detail in the text.

5.5.4 Subtotal Pancreatectomy

As in the liver, which is capable of regeneration post partial hepatectomy, so also is the rat pancreas capable of the same after 90% pancreatectomy, although limited in the opinion of

Elsässer et al. (1986). Initial work on hemi-pancreatectomised animals took note of an increase in pancreas mass (Brockenborough et al. 1988; Bonner-Weir, Baxter, Schuppin et al. 1993) which included replacement of both endo- and exocrine components. The post pancreatectomy organ consisted of tissue within one to two mm of the main pancreatic duct extending along its length (tissue on either side of the 1-2 mm strip having been removed).

The studies concentrated on the ductular changes that occurred at pre-determined intervals (24, 48 and 72 hours), the results (histological and immunocytochemical) categorised according to duct size (paragraph 3.2.3 of chapter 3). Massive cellular activity, especially of ductular epithelium was shown using BrdU labelling. Anagenesis was characterised by replication of pre-existing endocrine and exocrine cells as well as the proliferation of duct epithelial cells which underwent differentiation to form additional/replace pancreatic lobules.

The procedure followed to induce pancreatic regeneration (termed partial pancreatectomy) differs vastly from the large-scale removal of diseased liver tissue or its subsequent regeneration. Liver tissue, where generation was first studied, developed so-called "oval cells" at or close to the interface of excision. These oval cells were responsible for regeneration of all the components of the removed tissue, a process which differs vastly from the selective experimental removal of portions of exocrine/endocrine pancreas around the ductular system (Radaeva, Steinberg 1995; Sarraf et al. 1994) which is a model of tissue development in response to injury and not necessarily the recapitulation of ontogeny (Rosenberg 1998).

More recent studies suggest that oval cells may be of haematopoietic stem cell (HSC) origin (from bone marrow). In an editorial published in the 01 May 1999 edition of New Scientist (page 22) mention is made of male (XY) HSC's transferred into a female hepatectomised animal colonising the liver as oval cells, giving rise to regenerating hepatocytes with XY chromosomes. If this is the case, it also raises interesting questions regarding our assumption that pancreatic pluripotent stem cells (DEC's) are responsible for the nesidioblastosis that has been shown to occur in tissue undergoing experimental intervention.

5.5.5 Pancreatic Duct Ligation Tissue Model

For the sake of convenience, the post duct ligation remodelling has been grouped into 3 phases. The first, phase 1, (days 0 – 3), the second, phase 2 (day 3 ½ - 6 ½) and the final stage (stage 3 – days 7 - 14). The character of the changes occurring during phase 1 was first described by Edström and Falkmer (1968). The sequence of changes, occurring within the first 14 days of their study, are repeated within 72 hours in this study. No explanation for this difference in time scale is forthcoming.

This study proposes that phase 1 could be considered to be a preparatory stage for phase 2. This is supported by the substantial alteration in the exocrine compartment which has been shown to occur during this time. When viewed in the light of embryogenesis, it is generally accepted to be true that the early embryonal rat pancreas is undifferentiated at E9.0 (Gittes, Rutter 1992, Teitelman et al. 1993, Øster et al 1998) and develops into a chiefly endocrine organ up to day 16 gestation after which exocrine (acinar) development occurs while endocrine development is only completed in the neonate (Wang et al. 1996, Pang et al. 1994, Scaglia et al 1997). I would like to propose that it is possible that phase 1 can be viewed as a regressionary phase, taking the pancreas back to an organ prior to endocrine differentiation similar to E9.0. At the end of day three the ligated pancreas resembles a similar state of undifferentiation (development) as the embryonal pancreas at day nine (excluding the presence of pre-existing endocrine tissue), consisting of pluripotent stem cells, stroma and ducts (Kaung 1994).

There is wide disagreement on the sequence of morphogenesis, mainly as a result of the interpretation of experimental results. A number of study groups maintain rodent embryonic development of the main components (insulin, glucagon, somatostatin and pancreatic polypeptide) to be preceded by either NPY (Teitelman 1993) or PYY (Upchurch et al. 1994, Jackerott et al. 1997, Larsson 1998) in individual cells or co-expressed with insulin and glucagon and eventually also somatostatin and pancreatic polypeptide before culminating in cells with individual character.

Under the conditions of the various experimental interventions considered in this study, namely cellophane wrapping (Wolfe-Coote et al. 1998), 90% pancreatectomy (Brochenborough, Weir, Bonner-Weir 1988), ß-cell destruction by streptozotocin (Gu et al.

1997), pancreatic duct ligation (Walker 1987, Watanabe et al. 1995, Yamaguchi et al 1993, Idezuki et al 1969) however, it would appear that tissue remodelling in a backward direction (retrodifferentiation) occurs parallel to and concurrently with a change in the phenotype of existing cells (transdifferentiation) and nesidioblastosis. Neogenesis is preceded by the proliferation of cells, thought to be the pluripotent stem cells, namely duct epithelial cells (Teitelman 1993).

This would explain the presence of PP and ST positive cells amongst acinar cells, either as individual cells or as cells with dual (endocrine and exocrine) characteristics. We have thus far assumed that experimental intervention recapitulates (repeats) embryogenesis. Although this may or may not be the case, it is the opinion of this author that phase one is a preparatory stage of cellular events (days 0 – 3) involving acinar deletion and retro- and/or transdifferentiation not to be confused with phase two events (neogenesis). During phase one the groundwork is being laid for the recapitulation of embryogenesis and phase two is embryogenesis. The "groundwork" includes acinar deletion by apoptosis and/or necrosis, acinar retro- and/or transdifferentiation to either pluripotent stem cells or cells able to contribute to the endocrine proliferation shown to occur, the formation of pluripotent stem cells (ductular proliferation?), ductular proliferation and the transformation of existing islets (endocrine transformation), the percentage and how wide spread it is, is open to speculation.

By the weight of evidence, and depending on the species (the rat in this study) the predominant (first?) endocrine hormone to appear during apparent true neogenesis (day 3½) is insulin (11.5%) accompanied simultaneously by very small amounts of glucagon (0.4 – 2.29%), somatostatin (0 – 4.27%), pancreatic polypeptide (0 – 0.7%). Over the duration of the study insulin maintains its dominant position. As islets reconstitute glucagon once again becomes exclusively linked to the islet mantle while pancreatic polypeptide and somatostatin disappear from ligated tissue.

5.6 PROLIFERATION

Morphometric quantification was able to identify endocrine tissue forming "blobs" between 500 and 1000 μm^2 in diameter, around which were closely associated smaller (<500 μm^2)

areas designated "parent" and "child" areas respectively. Although ligation studies thus far reviewed have recognised this phenomenon, it has received little or no attention to date. The formation of so called "child" is presumed to be indicative of the endocrine proliferation quoted under similar conditions by Walker 1987; Walker et al. 1992; Wang et al. 1994, 1995, 1996; Watanabe et al. 1995.

These areas have been shown to eventually amalgamate by cellular migration (Rosenberg 1998), some of which form islets of exceptional size and shape. Not all insulin positive tissue however amalgamates or takes on true islet character. Day 30 post duct ligation specimens viewed in this study show the persistence of large numbers of small insulin positive areas, devoid of glucagon, somatostatin and pancreatic polypeptide. No satisfactory explanation for this finding could be found.

5.7 CELL TRANSITION THEORY

The observation that acinar deletion (disappearance) can be ascribed to apoptosis/necrosis only, needs to be reconsidered in this model. Various previous studies (Yamaguchi et al. 1993; Bouwens et al. 1994; Wang et al. 1995) have indicated the presence of transitional cells with either ductal and/or acinar together with endocrine characteristics. Bertelli and Bendayan (1997) subsequently proved beyond any doubt that transitional cells with both acinar and endocrine characteristics (amylase and insulin-IR) to be present both within islets as well as the pancreatic interstitium in post duct ligation pancreatic tissue. Bouwens and Rooman (1997); Arias and Bendayan (1993) have also illustrated (although *in vitro*) that acinar cells were able to transdifferentiate into cells with ductular characteristics.

The factors that initiate and regulate regeneration are not necessarily the same as those that regulate embryonic development (Bouwens 1998b). The issue could appropriately be summarised in the sketch adapted from Bouwens and Klöppel (1996) illustrated in figure 5.4.

The increase in endocrine tissue, especially insulin rich areas (IRA's), in response to experimental intervention (obstruction of the pancreatic duct in this study), has been reported to derive from either the budding of precursor cells situated in the pancreatic ducts (Rosenberg

1998; Wang et al. 1995) and/or transdifferentiated acinar cells (Bertelli and Bendayan 1997; Walker 1987) and/or hyperplasia and replication of existing endocrine tissue (Waguri, Yamoto, Miyagawa et al. 1997). Their close proximity to and association with areas of increased ductular activity, could lead to the hypothesis that the ligation induced insulin formations (LIEF's) are likely derived from pluripotent stem cells within ducts and/or duct epithelial cells as suggested by the above quoted authors.

Initially, much of the especially IRA's are in the form of single cells or clusters of three to five cells ($d < 30$ µm) as already reported. Some of the IRA's would appear to undergo some sort of amalgamation and or change. Many of them have been shown (mainly during phase two in this study) to disappear (as small entities) giving rise to LIEF's which vary in shape and size from the parameters already discussed in the preceding text. Rosenberg (1998) is of the opinion that these insulin rich areas "migrate out from the epithelium....to organise themselves into new islet-like structures." If this is the case, then it is equally possible for these IRA's to join up with islets already in existence. Hence the observation of islet-like structures that deviate from what can be considered to be the profile (in terms of size and shape, not endocrine cell composition) of normal islets, as has been reported to be the case in this study.

Both pancreatic polypeptide and somatostatin positive cells within the ß-cell core of islets, very early on in the ligation induced morphological change, has been reported here. Many authors have reported finding cells at various sites (including islets) within the pancreas with multiple characteristics. These vary from cells with dual and even triple endocrine expression, cells with dual endocrine/exocrine expression, cells with endocrine/ductular and those with duct/acinar characteristics. This lends credence to the hypothesis that firstly, some insulin cells are capable of transdifferentiating into cells with PP and/or ST secretory characteristics; secondly, that dormant stem cells, reported to be present inside the islet core (Rosenberg 1998), are able to undergo transformation into PP- and/or ST-positive cells.

Ligation induced endocrine formations, ICC positive for insulin are evident in the examples cited. In a number of instances an insulin positive so called "halo" could be seen to surround such formations. Insulin immunoreactive cells, either unable to migrate, as suggested by Rosenberg (1998), to reconstitute islets or join up with established islets, would in all likelihood not have access to the blood capillary glomerular-like network known to occupy the

centre of an islet. These cells would then be forced to secrete their endocrine contents into the surrounding tissue, hence the appearance of the insulin positive "halo".

5.8 THE RECAPITULATION THEORY

Early endocrine activity was shown to take place in this study. It involved the appearance of all four major endocrine hormones simultaneously although in varying proportions. This places a question mark behind the assumption that pancreas morphological change, originating as a result of any of the various experimental procedures (ligation, cellophane wrapping, ß-cell destruction by STZ, 90% pancreatectomy), can indeed be compared with one another.

The question as to whether the induction of morphological change from experimental intervention can be compared with the embryonal development described in the chick embryo by Rawdon (1998); rat (Kaung 1994; Bouwens and de Blay 1996; Bouwens et al. 1994; Bouwens et al. 1995; Kanaka-Gantenbein et al. 1995a, 1995b; Min et al. 1998), vervet monkey (Louw 1995, Wolfe-Coote et al. 1990a, 1998), mouse (Gittes and Rutter 1992; Gittes et al. 1996, Guz et al. 1995; Herrera et al 1991; Teitelman et al. 1993), transgenic (IFN-g) mice (Gu and Sarvetnick 1993; Gu, Molony, Krahl, Sarvetnick 1995; Gu et al. 1994; Upchurch et al. 1994, 1996), baboon (Wolfe-Coote et al 1987) needs asking, as they all show substantial differences. This question was recently asked by Rosenberg (1998) who maintains that the two processes (organogenesis and neogenesis) are fundamentally different and suggests that a number of the experimental procedures did not stimulate the reiteration foetal ontogeny.

The first issue that places this assumption in jeopardy is the predominance of NPY found in this study and supported by Teitelman et al (1993) not PYY as described to take place during rodent embryonal development by Jackerott and Larsson (1997), Jackerott et al. (1996), Upchurch et al. (1994, 1996); Larsson (1998). It is perhaps prudent to re-emphasise at this point that neither NPY or PYY were evident in normal tissue adjoining to duct ligated tissue or the tissue of sham-operated controls, and equally prudent to place on record that the NPY and PYY antibodies used for ICC has not been characterised for rat.

Secondly, the spontaneous and simultaneous appearance of immunoreactive cells for I, G, ST and PP is a setback to the generally accepted view of a cell lineage development recapitulating embryogenesis. This concept was put forth by Susan Bonner-Weir (Bonner-Weir et al. 1993) who suggested that it was *possible* (emphasis supplied) that regeneration was a recapitulation of embryonic development. In this case, cells either went through the lineage development very rapidly or in fact appeared concurrently. A further possibility could be that the metamorphosis found to occur in the endocrine compartment differs from that taking place in the exocrine compartment. In the former, both PP- and ST-positive cells formed, indicating that insulin cells underwent some sort of change (transdifferentiation?) within the ß-cell core of islets at day 2½ (Figure 4.13). Simultaneously glucagon immunoreactive cells in the mantle also underwent metamorphosis/change. These two processes are best illustrated in figure 5.1 and 5.2 in the special appendix at the end of this chapter.

5.9 DUCT LIGATION INDUCED TISSUE REMODELLING THEORY

Figure 5.15 (special appendix at end of chapter) represents a proposed pathway of tissue remodelling occurring during occlusive duct ligation. Aspects of the proposal are confirmed by findings on pancreatic endocrine cell lineage development expressed by especially Melmed et al. (1972), Melmed (1978), Herrera et al. (1991) and Wolfe-Coote et al. (1998). The proposed remodelling involves two processes. The first is proposed to entail the retrodifferentiation of existing endocrine cells (insulin, glucagon and somatostatin) to either pancreatic polypeptide or acinar tissue, the last of which in turn is thought to transdifferentiate into duct epithelial cells and/or oval cells and/or pluripotent stem cells. This process is indicative of phase one (days zero to three) where both somatostatin and pancreatic polypeptide are derived from insulin secreting cells, glucagon positive cells convert into acinar cells and acinar cells convert into both PP-cells and stem cells.

The apparent increase in IRA's, as reflected in figures 4.1.1(a) and D1 (in appendix D), lend credence to the hypothesis that insulin immunoreactivity is the first to appear in any significant amount. Whether or not the issue is clouded by tissue shrinkage, and the results "skewed" as suggested earlier, would appear to be of little consequence. On day 3.5 insulin forms 84.6% of the total endocrine presence, with near insignificant amounts of any of the

other endocrine hormones except glucagon. This trend is continued for most of the duration of the experiment.

5.10 CELL PROLIFERATION INDICATORS

Repeat immunocytochemical staining for the cell proliferation markers BrdU, MIB5 and/or Ki67 was performed on four separate series of histological slides from four of the six experimental groups in this study as described by Githens et al. 1976 and Guesdon et al. 1979. Due to unknown reasons, all but one slide (day 13) in one of the series tested negatively for both BrdU and MIB5. Various possible reasons for the failure to detect cell proliferation using these techniques have been discussed with the project advisors and no suitable answer is forthcoming on this issue.

Special Appendix to Chapter 5

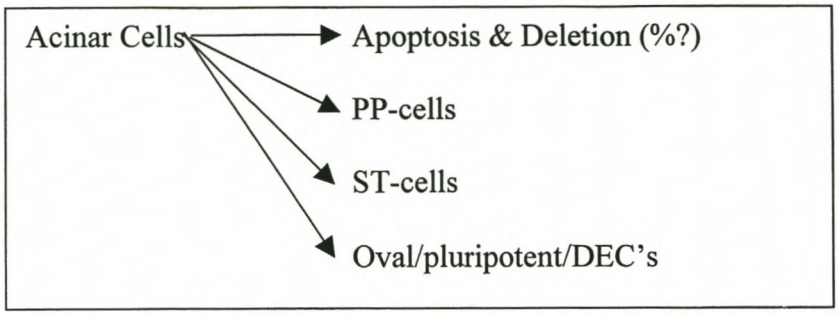


Figure 5.1: Schematic representation of proposed events early post duct ligation in the acinar compartment during this study.

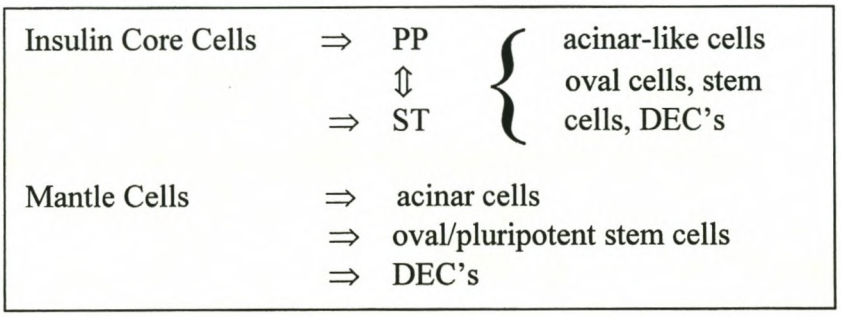


Figure 5.2: Schematic representation of the islet events early post-duct ligation, while at the same time, acinar cells are undergoing change as illustrated in figure 5.1 above.

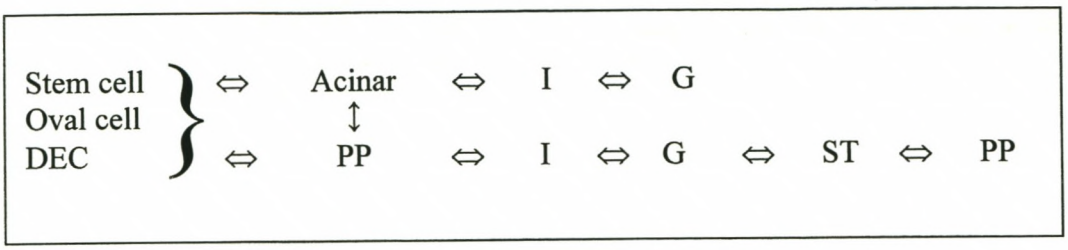


Figure 5.3: Scheme of a proposed cell lineage interrelationship between the various cells of the pancreas based on the results and observations in this study.

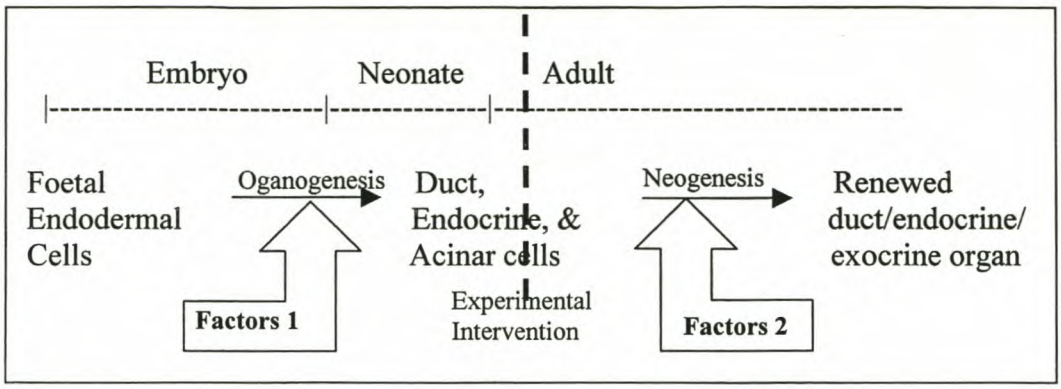


Figure 5.4: Schematic representation of islet cell neogenesis and its regulation. The duct cell compartment contains stem cells that feed the new endocrine cells into the islet compartment (neogenesis). Known growth and differentiation factors that have been proposed as being involved in this differentiation axis are indicated under factors 1 and 2 below. (Adapted from Bouwens and Klöppel 1996).

Factors 1. – Have been shown to be involved during embryogenesis.

1. Clusterin (Min et al. 1998).
2. Glut-2 – Glucose Transporter Type 2 (Pang et al. 1994; Stubbs et al. 1994).
3. Growth Hormone – (Scharfmann et al. 1994).
4. HGF – Hepatocyte Growth Factor (Jeffers et al. 1996; Lefebvre et al. 1998).
5. IAPP – Islet Amyloid Polypeptide (Mulder et al. 1997, 1998).
6. IGF – Insulin-like Growth Factor (Hogg and Hill 1994; Schofield 1992; Smith et al. 1991).
7. Mesenchymal Growth Factor (Kramer, Andrew, Rawdon, Becker 1987).
8. NGF – Nerve Growth Factor (Kanaka-Gautenbein et al. 1995a, 1995b).
9. NPY/PYY Neuropeptide Tyrosine (Jackerott et al. 1996, Upchurch et al. 1994, 1996, Jackerott, Larsson 1997, Larsson 1998). Considered to be neuroendocrine markers by O'Reilly et al. (1997).
10. Pdx-1; Nkx-6.1 – Homebox-gene proteins (Øster, Gu, Sarvetnick et al. 1998).
11. TGF-ß1 – Transforming Growth Factor-ß1 (Herrera et al. 1995; Hill and Hogg 1991).
12. VEGF – Vascular Endothelial Growth Factor (Rooman et al. 1997).

Factors 2. – Have been shown to be involved in neonatal/adult pancreas development and under experimental conditions.

1. Adrenomedullin (Martinez, Cuttitta, Teitelman et al. 1998).
2. Bcl-2/PCNA – Proto-oncogene Product-Bcl-2 (Wada et al. 1997, Bouwens and de Blay 1996).
3. BDNF – Brain Derived Neurotropic Factor (Corbett et al. 1997).
4. Cytokeratins 7, 19 and 20 – Bouwens et al. 1994).
5. Cytokines (Gu and Sarvetnick 1993).
6. Dopa Decarboxylase (Teitelman and Lee 1987b).
7. Endothelin-1 (Kakugawa, Paraskevas, Metrakos et al. 1996).
8. Gastrin (Wang et al. 1993).
9. GLP-1 – Glucagon –like Peptide-1 (Moens et al. 1998).
10. Glucose (Colella et al. 1985; Ling et al. 1996a, 1996b, 1998).
11. HLF-E47 – Helix Loop Factor E47 (Peers et al. 1994).
12. INGAP – Islet Neogenesis Gene Associated Protein (Rafaeloff et al. 1997).
13. IFN-γ - Interferon-γ (Gu and Sarvetnick 1993).
14. IGF – Insulin-like Growth Factor (Hill and Hogg 1991).
15. IGF-BP – IGF Binding Protein (Hill and Hogg 1992).
16. IGF-II - Insulin-like Growth Factor II (Zhang et al. 1997b).
17. Matrix Metalloproteinase (Barro et al. 1998).
18. Mesenchyme (Dudek, Lawrence, Hill, Johnson 1991).
19. Neurotensin & Secretin (Lopez et al. 1995).
20. Neurotrophin (Teitelman et al. 1998).
21. NGF – Nerve Growth Factor (Editorial in Endocrinology, 34(6):2319-22; Corbett et al. 1997).
22. NRSF/REST – Neuron-restrictive Silencing Factor/Repressor Element Silencing Transcription Factor (Atouf et al. 1997).
23. NT3 – Neurotrophin-3 (Corbett et al. 1997).
24. Prolactin (Brelje et al. 1994; Scharfmann 1994).
25. PrP - Prion-related Protein (Atouf et al. 1994).
26. Reg Gene (Zenilman et al. 1996).

27. Soyabean Oil – (Brandhorst, Hering, Brandhorst et al. 1994).
28. STF-1 – Somatostatin Transactivating Factor-1 (Guz et al. 1995, Peers et al 1994).
29. TGF-α - Transforming Growth Factor Alpha (Wang et al. 1997).
30. TH - Tyrosine Hydroxylase (Teitelman and Lee 1987b).
31. TNF-α - Tumor Necrosis Factor Alpha.
32. Vimentin (Bouwens and de Blay 1996).

COLLECTIVE VIEWPOINTS

Here below is set out in flow diagram format the viewpoints of the main participants in the debate around pancreas development, change, adaptation under normal and experimental conditions.

Summary of pancreatic cell lineage development proposed by Upchurch et al. (1994). Mouse Embryo's. + indicates positive; therefore PYY+ indicates cells that are positive for PYY.

Gestational Age	Cell Type
E9.5	PYY+ & G+
	PYY+
E10.5	PYY+/G-
	PYY+/I+ → declined as gestation progressed, after birth only I+
	PYY+
E15.5	PYY+
	G+
	I+
	PYY+/ST+
	PYY+/G+
	PYY+/I+
P2	PYY+/PP+
	PYY+/G+
	I+
	G+
	ST+
	PYY+

Figure 5.5: Summary of pancreatic embryonic cell lineage development proposed by Upchurch et al. (1994).

Teitelman et al (1993). Mouse Embryo's

E9.5	NPY+/I-/CP+ (CP = C-peptide) NPY+/G+
E11.5-13.5	NPY+/I+
E15.5	NPY+/ST+ NPY+/PP+ I+ G+
P1	I+ G+ PP+ ST+ NPY+

Figure 5.6: Foetal pancreas endocrine expression as proposed by Teitelmann et al. 1993.

Larsson (1998).

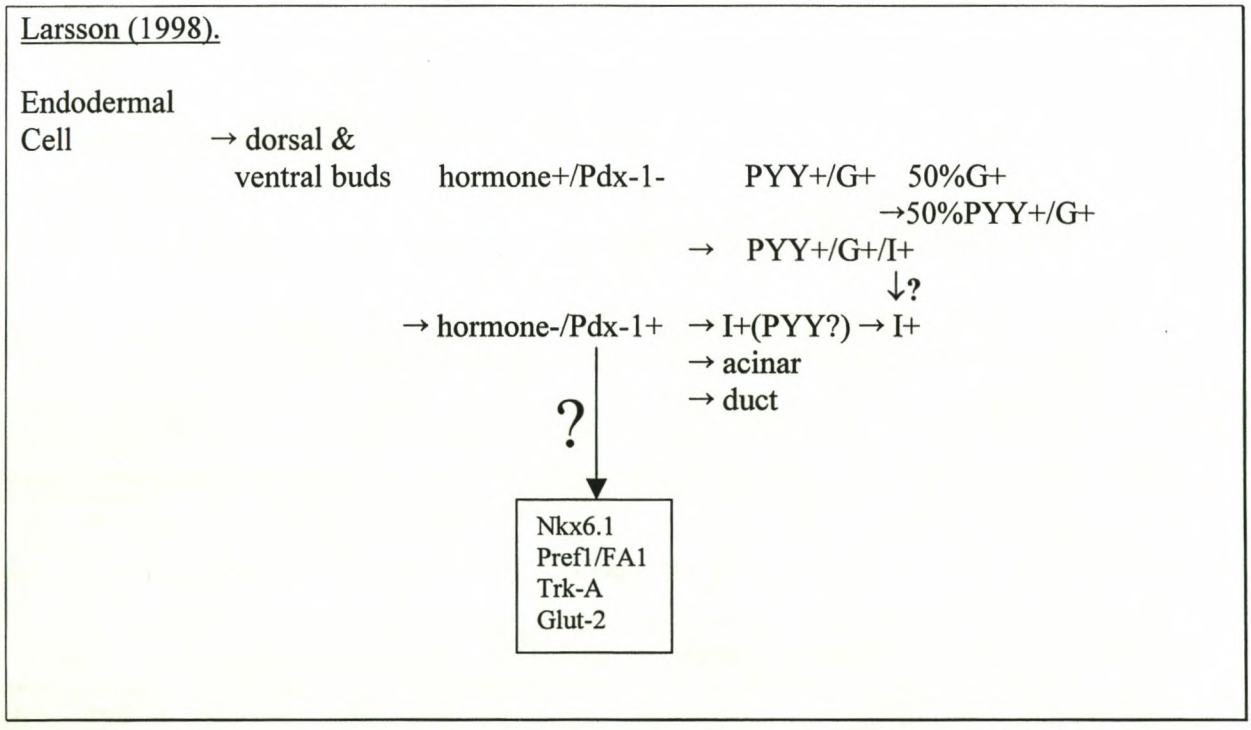


Figure 5.7: Foetal pancreatic development pattern proposed by Larsson 1998.

Øster et al. (1998) Murine Embryonal Development.

E11.0 (22 somite) → All epithelial cells of dorsal and Ventral buds Pdx-1/Nkx6.1+
→ G+/Pdx-1 & Nkx6.1-

E11.5 (30 somite) → I+/G+ (Pdx-1/Nkx6.1 slightly +) → found in G+ clusters
→ G+; 10%Pdx-1/Nkx6.1+

E11.5 - 13.5 → increase in no of I+ & G+ cells
→ I+/G+ or I+ (I+ cells Pdx-1/Nkx6.1+) up to E15.5

E15.5 – 18.5 → increase in I+ with Pdx-1/Nkx6.1 as single cells
→ decrease in I+/G+ → rare @ E18.5

E16.5 → ST+/Pdx-1+/Nkx6.1- (some co-storing I; no ST/G detected).

E18.5 → Pdx-1 & Nkx6.1 only in DEC's and I+ cells
→ increase in ST+ cells (very few ST+/I+) – 90%ST+ also Pdx-1+
↓
decrease to 10 – 20% in adult

Figure 5.8: Murine foetal development proposed by Øster et al. (1998)

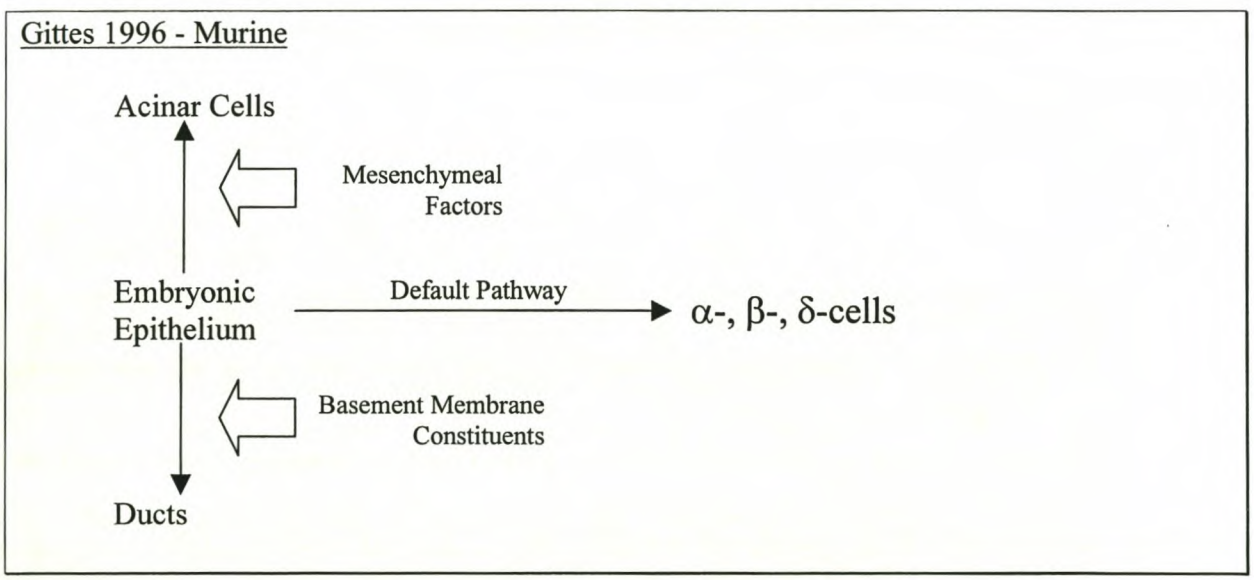


Figure 5.9: Embryonic epithelium/stem/endocrine/acinar cell interaction proposed by Gittes 1996.

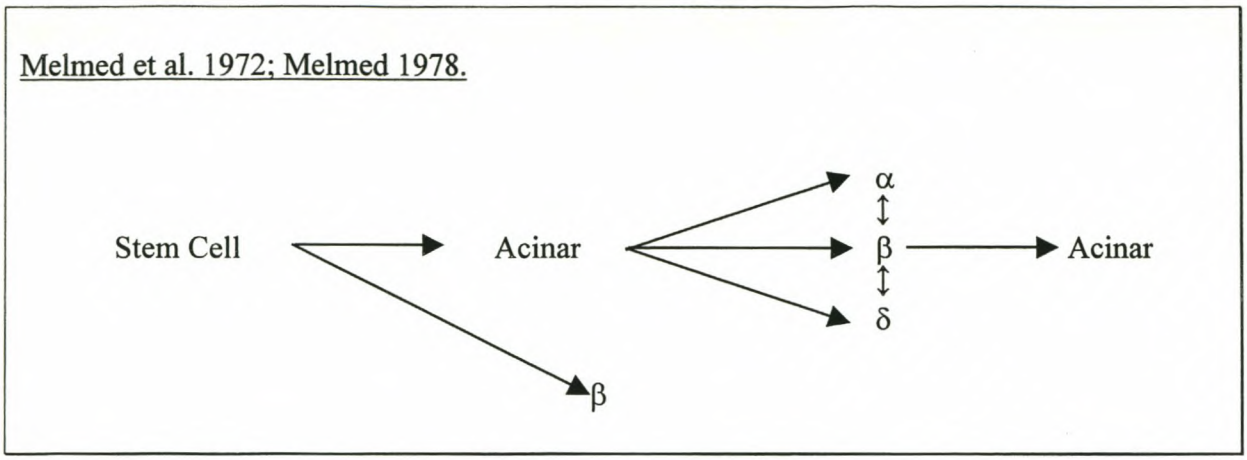


Figure 5.10: The pathway for the development of pancreatic cells follow a course of transitional development and a plasticity enabling retrodifferentiation.

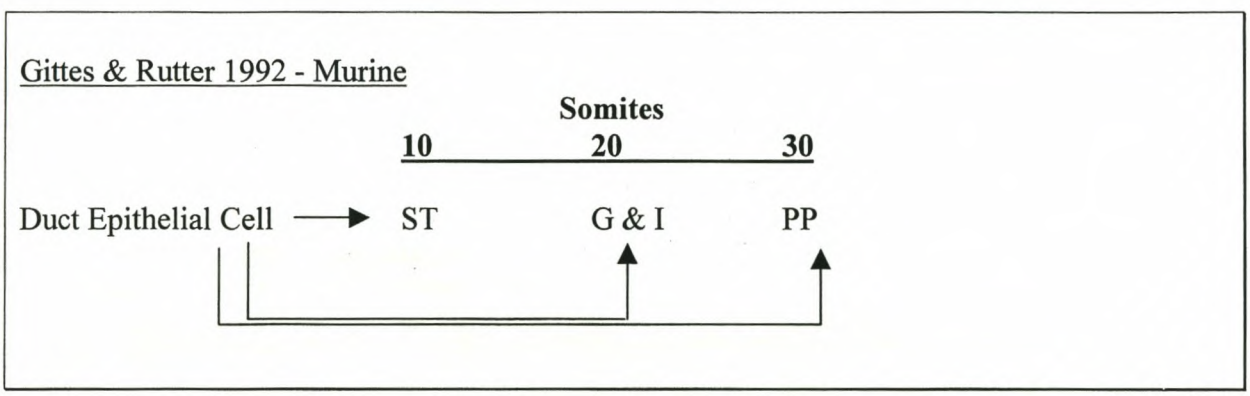


Figure 5. 11: Endocrine differentiation proposed by Gittes and Rutter in 1992.

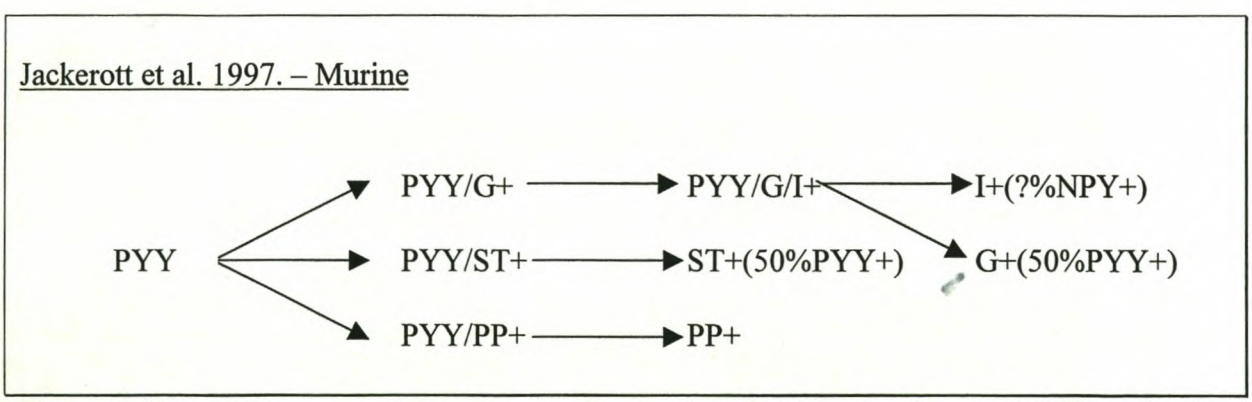


Figure 5.12: PYY expressing stem cell dominates the development of endocrine differentiation proposed by Jackerott et al. 1997.

Wolfe-Coote et al. (1998) – Cellophane Wrapped Vervet Monkey Pancreas.

- Increase in DEC proliferation.
- Increase in endocrine volume.
- Proliferation seldom (to never) seen in islets.
- DEC proliferation produce PP+ and ST+ cells.

Figure 5.13: Post cellophane wrapping events occurring in the Cape Vervet Monkey.

Wolfe-Coote et al. (1998) – Developing Monkey Embryo.

WEEK	BUD 1	BUD 2	ISLETS
5	PP+ ST+		
7	PP+ ST+ C-Pep+ G+		
8	PP+ ST+ C-Pep+ G+	ST+ PP+ C-Pep+ G+	ST+ PP+ C-Pep + G+
9	PP+ ST+ G+ I+/C-Pep+		ST+ PP+ G+
10-14			I/G/PP/ST+

Figure 5.14: Foetal pancreas development in the Cape Vervet Monkey observed by Wolfe-Coote et al. 1998.

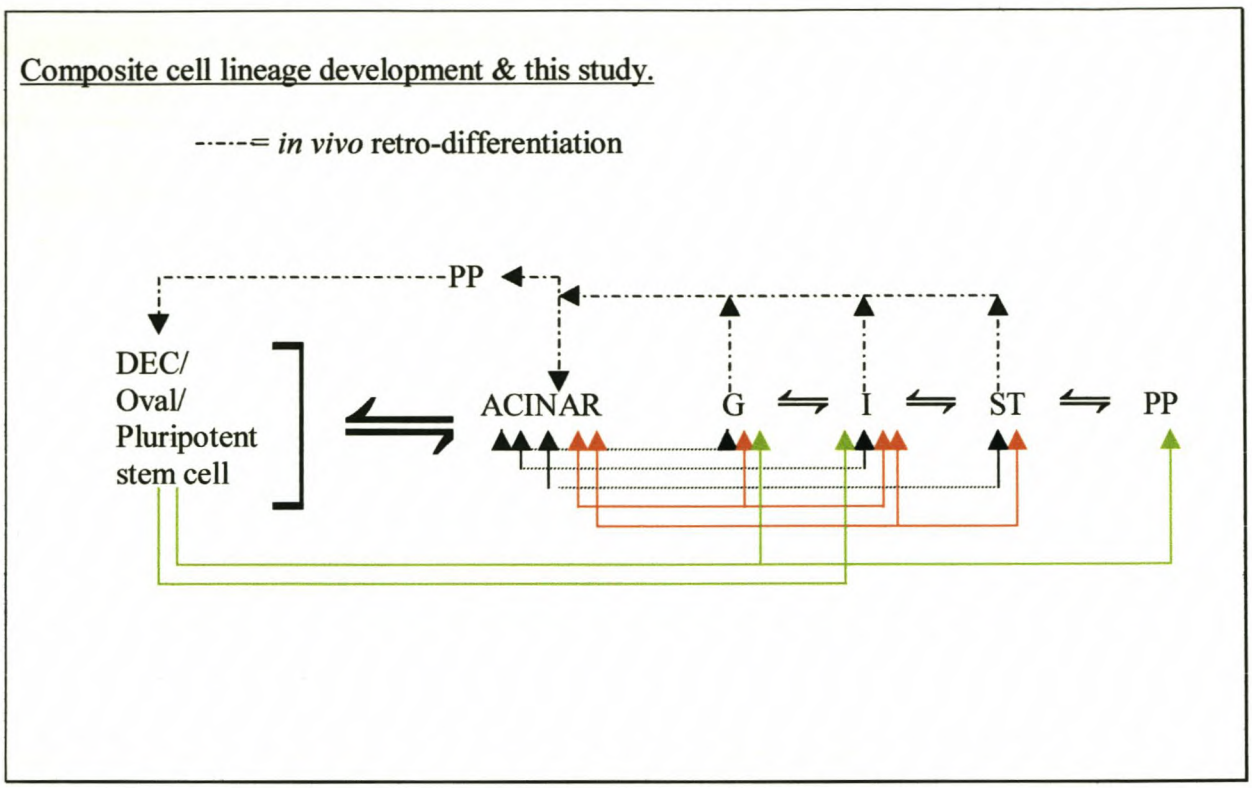


Figure 5.15: Proposed pathway of transdifferentiation during total occlusive PDL in a laboratory rat model during this study. Orange lines indicate pathways consisting of cell lines with multiple expression proposed by Melmed et al. (1972), Melmed (1978). Green indicates cell lines with dual expression proposed by Herrera at al. (1991), Wolfe-Coote et al. (1998). Broken lines (---------) represent probable *in vivo* transdifferentiation and dash-dot lines (-·-·-·-·) probable retro-differentiation.

CHAPTER 6

Conclusions

Prospective Avenues for further Exploration

6. CONCLUSIONS

6.1 Pancreatic duct ligation results in:

6.1.1 transient (day one)oedema in the pancreas causing intra-acinar swelling, and

6.1.2 ductular distension of longer duration (days one to 14),

6.1.3 transient lymphocyte infiltration in a minority of experimental animals and a general absence of the pathological changes of acute pancreatitis or fat necrosis,

6.1.4 total deletion of the acinar compartment by day 3½,

6.1.5 an increase in duct dilatation/visibility (by day 3½) in the compartment where acinar deletion had occurred

6.1.6 the formation of a notable quantities of insulin rich areas in amongst the areas of increased duct activity termed ligation induced endocrine formations (LIEF's),

6.1.7 a transient pancreatic polypeptide presence developing in the acinar compartment on day 2½ prior to acinar deletion,

6.1.8 glucagon deletion from the mantle zone of islets from day 2½ onwards,

6.1.9 the appearance of PP- and ST positive cells within the insulin core of selected islets by day 2½,

6.1.10 endocrine formations (LIEF's) consisting of an insulin positive core surrounded by a thin broken glucagon positive mantle, devoid of either PP or ST by day 30 post-PDL,

6.1.11 circular and/or irregular medium ($d = 100 - 200$ μm) to large ($d > 250$ μm) islet-like structures with a thin broken glucagon positive mantle;

6.1.12 an increase in the number of small ($d < 100$ μm) islet-like structures devoid of any association with pancreatic polypeptide, somatostatin or glucagon. Although insulin producing cells have been morphologically identified by ICC, it is not sure if the islet deposits are functional,

6.1.13 the intermittent and transient presence of both NPY and PYY in ligated tissue, but not in adjacent unligated tissue in ligated rats or in sham operated controls. NPY occurred in both islets and ductules while PYY occurred in ductules only,

6.1.14 no NPY or PYY presence in the early (days one to 3½) stages post-PDL,

6.1.15 no PYY presence after day 5½ post-PDL,

6.2 A lineage sequence, for the ligation induced cellular change occurring in the pancreas, cannot be determined, with any degree of accuracy, from the results of this study.

6.3 No distinction can be made between pre-existing islets and LIEF's after day 10 post-PDL.

6.4 Acinar reformation, after day 10, occurred in 33% of experimental animals.

6.5 No signs of the formation of hepatocytes as reported by Rao et al. (1995), Wolfe-Coote et al. (1997) could be found in this study.

6.6 It was not possible to detect the oval cell proliferation thought to serve as precursors for either hepatocytes or pancreatic duct cells, resulting from acinar cell loss as proposed by Sarraff et al. (1994).

6.7 Limited intra-species variation in islet size may be found in laboratory rats. Approximately 15% of the Sprague-Dawley strain of laboratory rat used presented with a preponderance of small islets ($d < 100$ μm). A possible explanation could be that the phenomenon is an intra-species anomaly.

6.8 The relative increase in endocrine tissue (especially insulin) is most probably due to tissue shrinkage resulting from acinar compartment deletion and LIEF's (endocrine neoformation).

6.9 The relative increase in endocrine tissue (insulin, glucagon, pancreatic polypeptide and somatostatin), over the 14 day period of the study appeared to follow a cyclic pattern.

6.10 Ligation induced pancreatic morphological change (in the Sprague-Dawley strain of laboratory rat) does not recapitulate the same embryological cell lineage as proposed for other species of laboratory animals, i.e., the pattern of cell lineage development is not fixed but varies with experimental conditions and type (species) of animal used.

6.2 PROSPECTIVE AVENUES OF FUTURE EXPLORATION

6.2.1 An alternative to the harvesting of foetal tissue is desirable. Pancreatic duct could possibly be used as an alternative source of pancreatic endocrine tissue for the amelioration of chemically induced diabetes mellitus in foetal rat pancreas transplantation studies conducted in our laboratory.

6.2.2 Pancreatic duct ligation induced acinar deletion and apparent increase in the endocrine component occurred by day 3½, as described in this study. The organ distal to the ligature has been shown to consist primarily of endocrine, duct and connective tissue components, as is the case in the developing foetal rat pancreas between E10 – 15. It should therefore be possible to remove a segment of the tail portion and culture it in a suitable environment. It would be interesting to discover what would become of such tissue as it may have potential clinical applicability in the treatment of NIDDM.

6.2.3 The main problem with transplanted tissue is early post-transplantaion ischaemia and immune rejection. Hypothetically a diabetic animal could undergo PDL, a section of the pancreas removed and cultured until sufficient endocrine tissue was present for transplantation into any of a number of potential transplantation sites (eg., renal subcapsularly - du Toit et al. 1998) and metabolic studies performed (IVGTT, urinalysis) to determine graft success. In vitro, pancreatic acinar cells have been shown to retrodifferentiate to form cells with duct-like characteristics (Githens 1994).

6.2.4 The effects of the β-cell toxin streptozotocin/alloxan have been proven. Pancreatic duct ligation has been shown to bring about an increase in pancreatic endocrine components. This study has focused on the tissue changes occurring in normal (non-diabetic) animals. An interesting question arrises as to whether the same or similar sequence of events would take place in experimental animals which were first made diabetogenic by STZ/ALX. If, as this study proposes, endocrine development under experimental conditions, is not dependent on the pre-existence of β-cells, then it is possible that STZ/ALX destroyed insulin secreting cells could be replaced by tissue originating from duct ligation induced morphogenesis. If this were so, euglycaemia could be restored in experimentally induced diabetic animals.

The potential transferal of this line of thinking to the clinical setting on humans would then become an issue for consideration, bearing in mind that PDL is a minor surgical procedure performed with a laparoscope.

6.2.5 It would appear (from literature) that the cells of the pancreas possess a certain plasticity, which could enable cells of any of the primary types to undergo transdifferentiation. It should be an interesting study to compare various cell populations in NIDDM and IDDM patients to determine whether ...

- insulin cells are able to differentiate from other cell types,
- whether they are destroyed by the autoimmune response thought to characterise IDDM,
- and/or inhibited from producing insulin,
- and/or prevented from secreting insulin,
- and/or develop a cell membrane incapable of recognising hyperglycaemia,
- and/or the aetiology of insulin resistance in the face of exogenous insulin therapy.

6.2.4 Why does some acinar tissue reform after day 10 in some PDL's and again by day 15 in others? Could a particular lobe/lobule have alternative passage for enzyme secretion into the duodenum and/or does the stitch dissolve within this time period to allow drainage access which in turn is the stimulus for acinar reformation.

6.2.5 Near total pancreatectomised (90%) laboratory animals have been shown to completely regenerate both exocrine and endocrine excised components. Could this procedure be applied to diabetic animals?

6.2.6 If adenocarcinomas are pancreatic duct epithelial cells (Githens 1994) then why do they not transdifferentiate into endocrine tissue *in vivo* or *in vitro?*.

6.2.7 What is the relationship of LIEF's to the blood vascular glomerular-like network known to occupy the core of an islet, and do all LIEF's eventually obtain access to a physiologically active islet?

APPENDICES

APPENDIX A – Study Protocol

APPENDIX B – Treatment of Laboratory animals

APPENDIX C – Expression of individual endocrine cell quantities as a percentage of the total endocrine presence

APPENDIX D – Sham Operated Control Animals

CURRENT RESEARCH PROTOCOL FOR EXPERIMENTAL RESEARCH FACILITY

RESEARCH DETAILS:

Title of Research:

Endocrine and Ductular Proliferation in the Adult Rat pancreas using an Occlusive Pancreatic Duct Ligation Model: A Histological and Morphometric Assessment.

Statement of Originality:

This study is based is on the comparison of the different stages of cell development between PDL and other experimental procedures viz., duct ligated, and cellophane wrapping, 90% partial pancreatectomy with embryogenesis. The controversy that exists as to the exact sequence of development is evident from the difference of opinion in the literature of Githens, Tietelman and Wolfe-Coote.

STATISTICAL PLANNING:

Values will be expressed as "s.e.m". A two-tailed paired or unpaired Student's t-test will be used to test the significance of the results. $P < 0.05$ will be used to indicate the levels of significance. Each phase of PDL development will be represented by the results from 4 individuals and two controls as mentioned in materials and methods (below). The sample size, although small is sufficient to give a meaningful result. The project design and methods are appropriate to the questions being addressed and the results will be valid. Quality control will also be applied. The results obtained will be analysed appropriately and interpretation of the results from the data obtained will be possible.

ETHICAL ASPECTS:

Infrastructure:

Histology, laboratory and animal housing and facilities has been provided at the Department of Biology at the University of the North-West (UNW) by kind favour of Mr S.D. Phalatse (Acting Head of Department) and the School of Agriculture (UNW) by kind favour of Prof. M. Funnah (Dean of School). Technical expertise will be supplied by the Chief Technical Officer at the Department of Biology Mr David Kgwadi & Mr D.O. Monnye.

Immunocytochemistry will be done at the MRC per kind favour of Dr. S.A. Wolfe-Coote, Mrs Charna Chapman & Dr. Johan Louw. UNW has not had the opportunity to develop suitable laboratory/research facilities due to it being a Historically Black University (HBU). The project attempts to do something to correct the problem and develop the necessary infrastructure to enable us to do meaningful research.

Literature Overview:

Studies to date (6, 11, 13, 20, 21, 23, 29) show that both exocrine and endocrine tissue regenerates within 12 days in animals undergoing ligation or cellophane wrapping of the tail portion of the pancreas. Beta cells and beta cell clusters increase with a margin of up to 1.7X that found in non-ligated control animals. BrdU (Bromodeoxy- uridine) pulse labelling indicates a 5X increase in beta-cells and a 3X increase in alpha-cells. Double immunostaining showed transitional cytodifferentiation forms varying between duct cells expressing cytokeratin 20(CK 20), beta-cells expressing insulin and alpha cells expressing glucagon. The proliferation and differentiation of exocrine cells represents a major mechanism of endocrine beta-cell neogenesis.

Wang, Kloppel and Bouwens (23) also showed that beta-cell volume doubled within seven days post-ligation in the ligated portion of the pancreas there was also an increase in small islets, islet cell clusters and scattered single cells suggesting islet neogenesis. Wang et al (23) and Scott et al (16) suspect a paracrine or autocrine regulation of tissue remodelling. The trophic response could be affected by an as yet undefined or unidentified growth factor - perhaps from recruited macrophages which phagocytose the PDL cells, plays a role in cell proliferation. Whatever the cause of the regeneration, there seems to be divided opinion as to the sequence of development. A number of authors (1, 2, 6, 20) are of the opinion that the regeneration sequence follows embryonal development, whereas others found glucagon secreting cells appearing first (18) and then glucagon cells co-expressing insulin (19) and eventually all insulin secreting cells co-expressing glucagon.

Wolfe-Coote et al (29) however found that the co-expression of insulin with glucagon also occurred with other peptides namely NPY and PYY, which although rare, were preceded by the secretion of somatostatin and PP. Lukinius (1992) as quoted by Wolfe-Coote (29) as also Herreira (12) found PP secretion to occur first, followed by somatostatin. This

view is contested by Tietelman (19) who found PP secreted with NPY. Wolfe-Coote et al (26, 24, 28, 29) and Herreira (12) have concluded that PP and glucagon secreting cells originate from a common stem cell.

Hypothesis:

Studies have found that both endocrine and exocrine pancreatic tissue regenerates after partial obstruction of the pancreatic duct (pancreatic duct ligation, PDL) or cellophane wrapping of the head portion of the pancreas. Current evidence suggests that this regeneration originates from the cells forming the ducts of the pancreas and seems to go through a developmental sequence that mimics the events occurring during embryonic development.

Project Aims and Objectives:

This study aims to:

1. follow the sequence of cellular changes that are presumed to occur in both the endocrine and exocrine compartments of the pancreas in duct ligated laboratory rats,
2. compare the sequence of cellular events especially in the endocrine compartment with those quoted in literature for similar and/or alternative experimental models,
3. to establish whether any part of the sequence of cellular change is the same as during embryonic development, and in which respects it is so, and
4 propose a model for the evaluation of the sequence of cellular change occurring in the pancreas after occlusive duct ligation.

RESEARCH PROGRAMME:

TIME SCHEDULE RESEARCH PROGRAMME

Phase 1: Training at MRC - December 1995

Obtaining breeding pairs - December 1995

Commence Breeding Programme - January 1996

Obtaining necessary chemicals and equipment January - April 1996

Commence with PDL - May 1996

Histology - June/July 1996 (MRC)

Assessment and recording June 1996 - June 1997

January 1997 - December 1997

Histology and assessment - June 1997 - December 1997

Comparison and Recording of data January 1998 - September 1999

Submission - October 1999

Final Paper for publication - November 1999

Materials and Methods: Stage 1 - Group 1 . Pancreatic Duct Ligation (PDL).

One hundred and eighty four Wistar rats, housed in standard housing facilities with free access to water and balanced diet, will be bred from 5 breeding pairs obtained from the Medical Research Council (MRC).

The treatment of experimental animals will be according to the Helsinki Protocol and under the supervision of Dr L. Phiri (B.Vet.Sc) from the Dept of Agriculture of the North-West provincial government. When the rats are 3-4 months old (240-280 g) they will be anaesthetised by a single intraperitoneal dose of sodium-pentobarbitone general anaesthesia (40 mg/kg 6% m/v) using Saggital (Maybaker) after initial induction in an di-ethyl ether environment. The level of anaesthesia will be confirmed by pinching a hind leg with a forceps and the suspension of the plantar reflex.

Anaesthetised rats are prepared for surgery by shaving the abdominal surface and thereafter cleaning it with a hibitane solution. (made up from 2 l of a 5 % hibitane solution, 3 l of water and 17 l of iso-propyl alcohol). A laparotomy is perfromed through a first abdominal incision made in the midline, cutting through the skin from ±1,0 cm above the genitalia to the xiphisternum. The second incision is made slightly off centre on

the side of the pancreas and duodenum. After exposing the pancreas, it is then prized away from the surrounding tissue with a blunt forceps. The ligation is made from the point where the pancreas is attached to the first portion of the duodenum to the point just before its attachment to the transverse colon. The posterior (tail) portion of the pancreas is tied off with a single stitch of suture material.

After surgical exploration the peritoneum and abdominal layers are sutured separately with Dexon (4.0) and the skin closed with Prolene or similar synthetic non-absorbable suture material (4.0 - 6.0) using a taper-cut needle. Surgical Chromic catgut will be soaked in saline for ±10 minutes prior to use. The wound is then swabbed with hibitane and alcohol solution, powdered with dry penicillin and sprayed with silicon spray. Rats also receive prophylactic penicillin and streptomycin intramuscularly to prevent infection. Postoperatively animals are kept under a 60 W electric lamp for 48 hours, and restricted to fluids only for this period to prevent ileus. Post-operatively the animals will be housed in standard laboratory cages a suitable analgesic will be administered every 6 hours under the supervision of a qualified vet from the Dept of Agriculture at this University. On the days post PDL, as described below, animals will be sacrificed after induction as described above and a single lethal dose of Saggital, the pancreas removed using the following sequence:

Group	Days Post Duct Ligation	Experi-mental Group (No of animals)	Number of SOC's	Total number per group
1	1	6	2	8
2	1.5	6	2	8
3	2	6	2	8
4	2.5	6	2	8
5	3	6	2	8
6	3.5	6	2	8
7	4	6	2	8
8	4.5	6	2	8
9	5	6	2	8
10	5.5	6	2	8
11	6	6	2	8
12	6.5	6	2	8
13	7	6	2	8

14	7.5	6	2	8
15	8	6	2	8
16	8.5	6	2	8
17	9	6	2	8
18	9.5	6	2	8
19	10	6	2	8
20	11	6	2	8
21	12	6	2	8
22	13	6	2	8
23	14	6	2	8
	Total	138	46	184

The whole pancreas will be fixated in Bouin's fixative and prepared for histological processing. One hour prior to sacrificing animals will receive 50 mg/kg body weight BrdU intravenously via a tail vein. Double immuno-cytochemical staining will be applied. Two series of 15, 4 micron paraffin sections at equidistant levels will be prepared on marked, frosted, APES coated microscope slides. The first section of each sequence will be stained with H& E and consecutive sections double immunostained for BrdU and Insulin, Glucagon, Pancreatic Polypeptide, PYY, NPY and Somatostatin using the ABC technique described by Bouwens et al (23).

PILOT STUDY

Partial duct ligation (as described above) has been performed on 8 bio-bred laboratory rats to test and establish work procedure. Animals were sacrificed on days 3, 6, 9, 12 respectively post-operatively. The whole pancreas was removed and histologically processed for light microscope with H & E.

Light-microscopy confirms findings of degeneration of the ligated portion (days 3 and 6) followed by tissue regeneration from ductal tissue (days 6, 9 and 12) **confirms the findings** of studies quoted in this protocol. Due to expense, immuno-staining was not used to ascertain the distribution and morphology of endocrine tissue. *Experimental animals remained normoglycaemic (BGC 3-6 mmol/l) throughout the study!!*

POTENTIAL CLINICAL APPLICABILITY IN SOUTHERN AFRICA

Diabetes mellitus (DM) has been identified by the MRC as one of the so-called Chronic Diseases of Life-Style (CDL) and the recorded incidence is on the increase especially in rural areas where medical care is inadequate. Together with other CDL's diabetes mellitus costs the country millions of rands on medi-care spending annually and accounts for a staggering proportion of potential life-years lost, resulting in a drain on the labour force and the subsequent drop in productivity due to work stay-aways.

If research confirms that pancreatic duct ligation can give rise to the regeneration of sufficient endocrine tissue in diabetic patients to replace the dysfunctional beta cells, it could mean a major breakthrough in an alternative to the traditional exogenous treatment of DM as PDL is a relatively simple process not requiring major surgical intervention and can be performed via laparoscopy.

At this stage science does not know enough about the processes, mechanisms and factors involved in the regeneration of pancreatic tissue from duct cells, and until we know more about this exciting new avenue and the potential it might offer clinicians in the amelioration of DM, research must continue.

STATEMENT ON THE RECONSTRUCTION AND DEVELOPMENT PROGRAMME

The University of the North-West is situated in a rural environment and is able to make a meaningful contribution to the advancement of science and the upliftment of the impoverished rural inhabitants through constructive interaction on as many levels of society as possible. In this regard the Department of Nursing Science is constructively and actively involved in designing projects that will assist in uplifting the peoples of the region so that their quality of life is improved. The primary objective is to establish an experimental research facility at this University, which will be used to further research in the biological and related sciences and create opportunity for HBU-students to be exposed to experimental research and advanced techniques in histology and tissue culture. As and when the need arises, this facility will be expanded to include other scientific research facilities, offer employment and be involved in the training of laboratory technologists.

In practice, establishing a histology processing and tissue culture facility is the first step in creating experimental research capacity at this University.

ABSTRACT

There is an urgent need to develop research capacity at UNW. To this end the Departments of Nursing Science, Biology and Agriculture and involved to a lesser or greater degree in planning, implementing and logistical support for the development of an Experimental Biology Facility. After consultation with interested parties and promoters, it was decided to develop facilities that can be expanded. Hence this project of studying endocrine tissue regeneration in laboratory rats. Not only is the topic contentious but attainable within the limited budget available.

MATERIALS

STAGE 1 *items acquired on own budget

*Experimental animals - Breeding pairs from MRC

Animal housing facilities - University of the North-West (UNW)- Department of Biology

*Micro-dissecting kit - Carolina Biological Supply Co @$75-00

*Tissue forceps 4" straight 62-4590 @ $6-50 ea

*Rochester Pean Hemostatic Forceps 62" 62-5321 @ $6-74 ea

*Mayo Hegar Needle holder 62-5500 @ $7-15 ea

*Saggital (Maybaker) - UNW

*Penicillin & Streptomycin - UNW

*Suture material (taper-cut needles) - UNW

*Surgical gloves - UNW

Microscope with camera and dissection microscope - UNW

Ether, Bouin's fixative, ethanol, xylene/xylol - UNW

*Lymph-angiocaths for penetration of tail vein

Microtome - UNW

Paraplast paraffin wax and heating facilities - UNW

Tissue-Tek moulds - MRC

*Microscope slides 63-2100 @ $7-30/box X 7

*Cover slips 25 mm 63-3017 @ $11-95/100 X 3

*DePeX mounting medium

*3-Aminopropyltriethoxsaline (APES)

*Artists paintbrush (fine)

Incubator - UNW

H & E staining - UNW

Staining bowls and racks - UNW

*Colour film (slides and prints)

Immunocytochemical stains/Vectastain - MRC

*BrdU - Sigma Corp/MRC 5g @ $173.25

Tissue processor

Embedding Centre

Microtome (additional)

Automated staining centre

Incubator

Slide dryer

BUDGET

*1994/5 Carolina Biological Supply Company Catalogue		
Rats(8) - Breeding pairs @ R8-00 ea		64-00
Microdisecting Set *62-1340 @ $65-00		260-00
Tissue Forceps 4" straight *62-4590 @ $6-50		26-00
Rochester Pean Haemostatic Forceps 62" Curved *62-5321 @ $6-75 ea		27-00
Microscope slides (frosted ends) *63-2100 @ $7-30/box x10		292-00
Cover Glasses 25mm *63-3017 @ $11.95/100 x7		334.50
Mayo Hegar needle holder *62-5500 @ $7-50		30-00
Peripherals/Consumables		400-00
Photographic Film		567-00
Disposable Microtome blades (Feather Safety Razor Co., Japan)		2 500-00
Microtome Blade Holder		2 800-00
BrdU 5g		1649-68
Immunohistohemistry Kits	(BrdU, Insulin, Glucagon, Somatostatin, PP, NPY, PYY)	15 000-00
Vectastain Kits X 2		4 000-00
	Sub-total	27 950-18
	GST @ 14%	3 913-02
	Inflation @ 15%	4 779-48
	TOTAL	**R36 642-68**

APPENDIX B

Guidelines on Ethics for Medical Research

Revised edition, 1993. Based with permission extensively on Reports from the Royal College of Physicians of London

USE OF ANIMALS IN BIOMEDICAL RESEARCH

1. Introduction

The optimal care of animals used in biomedical experimentation is vital in the interests of both the animals and the research itself. There are two main reasons for this statement. Firstly, it is fitting for a civilised community to consider the humane aspects. Secondly, it must be remembered that trials involving animals are generally very expensive, and consequently the use of animals which are housed in poor facilities, which suffer from diseases or are infested with parasites, and which are badly cared for, will lead to results which are unreliable and/or unrepeatable. Good care improves the welfare of the animals and makes a large contribution to the attainment of high ethical and humane standards.

The MRC therefore wishes to:

emphasise that the use of animals has made a large contribution to the welfare of both humans and animals in the past, and that future use of animals is necessary for further progress;

acknowledge that humans have an ethical duty to treat all animals with great care and to be aware of their susceptibility to pain and suffering; emphasise that experimentation with a wide variety of animal species is necessary for the advancement of knowledge in biology, as well as the development of methods for prevention, diagnosis and treatment of diseases of humans and animals and the advancement of their welfare and productivity; support the responsible use of animals for experimentation and other scientific purposes, but encourage the use of valid alternative methods, where possible;

insist that complete care be taken to protect animals from pain, suffering, discomfort and permanent injury, and will ensure that in cases where it is unavoidable, it will be kept to a minimum;

ensure compliance with all relevant South African legislation, the National Code for the use of animals in research and international regulations. These include the following:

the Animal Protection Act (Act No. 71 of 1962);
the Animal Diseases Act (Act No. 35 of 1984);
the National Parks Act (Act No. 57 of 1976);
the Nature Conservation Ordinances of the four provinces (Cape Province - Ordinance No. 19 of 1974; Orange Free State - Ordinance No. 8 of 1969; Natal - Ordinance No. 15 of 1974; Transvaal - Ordinance No. 12 of 1983); the National Code for the Handling and Use of Animals in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa; and Convention on International Trade in Endangered Species (CITES).

2. Definitions

`Experimental animal' means any living non-human vertebrate, non-human vertebrate fetus, or any other animal species which in the opinion of the Ethics Committee for Research on Animals (ECRA), has a nervous system which is so sophisticated that it might be capable of experiencing pain in much the same way that any vertebrate might experience it.

An `animal experiment' is any procedure involving the use of live animals in which the aim is to test a hypothesis, collect information and advance, impart or demonstrate knowledge, test or collect a product, or register the effect of a certain procedure on an animal.

3. Prerequisites for the use of animals in research

The main purpose of any animal experimentation must be to gain usable results and scientific information of high quality to the benefit of humans and animals. The experimentation must therefore not be purposeless or unnecessary.

The experiment must be carefully and scientifically planned, based on the available knowledge of the disease or problem being studied and designed so that the expected results, wherever possible, will justify the experiment. A written protocol should therefore be prepared before each experiment which clearly indicates the purpose of the experiment as well as all procedures to be carried out. No experiments on animals must be allowed to

start before the research protocol has been passed by the Research Committee of an institution and the ECRA has separately evaluated and passed the experiments.

All attempts should be made to keep the use of animals to a minimum. Care should be taken to ensure that the correct species is chosen and that animals with all the necessary genetic attributes and microbiological qualities are chosen to ensure reliable results.

The application of in vitro biological systems, statistical analyses and mathematical models should be considered as alternatives to supplement or replace animal experimentation. These alternatives must not only be considered on the grounds of humane principles but also because they generally demand less time, space, equipment and funding. Where the experiment inflicts inescapable pain or more pain than the use of anaesthetics would cause, the proper use of analgesics or the administration of anaesthesia according to recognised veterinary practice is obligatory until the procedure is completed. The only exception to this principle is in cases where the administration of anaesthesia would nullify the aims of the experiment and the results could not be obtained by any other more humane method. If approved, the Committee must then appoint a veterinarian to take responsibility for the welfare of the animals subjected to those procedures. As a general guide, researchers should accept that any procedure which will cause pain to humans will also cause pain to other vertebrates.

The scientist in charge of any animal experiment must be prepared to terminate it if it becomes clear that the continuation thereof will cause unwarranted pain and suffering. If the procedure causes serious injury, the animal should be killed before recovery from anaesthesia. If it becomes clear that an animal will suffer unwarranted pain or discomfort after an experimental procedure, it should be killed in a humane manner. The veterinarian must have an overriding discretion as to when animals shall be killed or withdrawn from an experiment for humane reasons.

The clinical care of experimental animals before, after and during the application of the experimental procedure must be of high standard and according to accepted veterinary practice so that pain, discomfort and any detrimental effects, caused by the procedure, can be eliminated. If it becomes necessary to kill an experimental animal it must be done in a humane manner and according to accepted principles which ensure immediate death. It is preferable that only personnel with experience in the application of euthanasia are responsible for this task.

No animal may be disposed of before there is absolute certainty that it is dead.

Animals already used in an experimental procedure should not be subjected to it for a second time, unless the first procedure was harmless or non-invasive and left the animal in good health.

No animal should be subjected to more than one procedure that causes significant pain.

4. Care

The care of experimental animals should be under the direct control and supervision of a veterinarian, preferably one with experience in animal experimentation, and a qualified laboratory animal technologist. The supervisory personnel should also include the services of technical staff and workers with experience and/or training in laboratory animal science.

High standards should be maintained in the daily care of experimental animals. Special consideration should be given to regular feeding, adequate and clean water, hygienic surroundings, adequate ventilation and the elimination of excessive heat, cold or noise in the animals' environment.

Care should be taken to eliminate disease, injury, overpopulation and stress factors and to safeguard the animals from endo- and ectoparasites. Careful and thorough supervision of the welfare of all animals is absolutely essential.

Experimental animals should be kept under optimal conditions at all times. This includes good housing, correct environmental conditions with acceptable space for movement and opportunity for social interaction except where non-compatibility or the requirements of the experiment prevent it. The animal holding cages as well as the premises in which the cages are housed should therefore meet with accepted minimum standards.

Wild animals which are captured in their natural habitat, for research purposes, should be trapped in cages that meet with the standards of the nature conservation authorities. The traps should be visited regularly, preferably daily, to prevent animals being left without food and water for long periods.

It is the duty of the head of the research institute to ensure that all researchers who use experimental animals have the necessary training and experience to do so correctly. In-service training programmes should be established at research institutes to ensure that this requirement can be met.

5. Transport

Experimental animals must be transported according to the recognised minimum standards and regulations for the transportation of animals.

During transportation special care should be taken for the provision of good ventilation, the elimination of discomfort, excessive cold or heat and the spread of diseases.

Provision must be made during long journeys for regular provision of food and water.

Animals must be unloaded as quickly as possible on arrival at an airport, harbour or railway station. At their ultimate destination they must be removed from their transport cages immediately and placed in suitable permanent accommodation.

Appropriate veterinary care must be given to animals found to be diseased, injured or in a poor state during travel or on arrival.

6. Ethics Committee for Research on Animals (ECRA)

All institutions using animals in experimentation must establish an ECRA. No animal experiment may be permitted to start before the research protocol has been passed by both the ECRA and the Research Committee of the institution.

The objective of an ECRA is to control the use and care of experimental animals. The ECRA must be properly constituted, ensure that these guidelines are followed and applied, keep records of the type and number of animals used in each experiment and ensure that quantifiable norms and standards are adhered to.

An ECRA must be constituted by and shall report to the management of the research institution.

The ECRA shall preferably include persons from each of the following categories as members:

Established researchers who have experience of the use of animals in experiments.

A registered veterinarian.

Persons not engaged in research representing recognised animal welfare organisations and appointed by mutual agreement between the institution and the organisation.

Persons not engaged in research and not associated with the institution or a recognised animal welfare organisation.

The responsibilities of an ECRA are:

to establish an institutional code of practice for the use and care of experimental animals and to ensure that all proposed procedures comply with this code and meet accepted ethical, legal and scientific requirements;
to maintain a register with particulars of the species, number and origin of the animals used as well as the type of experiments done;
to inspect the animal facilities regularly and without warning;
to only approve an animal experiment if convinced that it is justified and feasible, if not, the ECRA must refer the matter back to the Research Committee or to subject-matter experts who are not members of the ECRA;
to preview and review all animal experiments in the institution;
to ensure that the species chosen is the best model and that no valid alternative method can be applied;
to ensure that pain, discomfort, stress or distress is minimised or eliminated;
to ensure that experiments do not exceed preset time limits, and that avoidable delays do not occur;
to ensure that all experimental procedures and related activities done on animals are performed by persons with the necessary experience and skills; and
to ensure that its decisions are carried out, because the final responsibility for the welfare of experimental animals lies with the Committee.

The duties of the ECRA shall be to create mechanisms that provide for:

regular meetings;
the stringent review of experiments;
acceptable standards of clinical care of animals;
acceptable standards of husbandry care of animals;
the immediate termination of experiments in which the animal is suffering unacceptable or uncontrollable pain, in the view of the veterinarian; and
to report regularly to the management of the institution.

7. Special provisions in respect of standards and norms

Organisations using experimental animals should accept and apply quantifiable norms and standards. For this purpose, the proposals of recognised national and international authorities, advisory bodies and other authorised organisations should be accepted, in order to make provision for:

the appointment of ECRAs;
the procurement of experimental animals;
correct transportation of animals;
adequate accommodation and animal care;
optimal environmental conditions;
correct feeding;
thorough veterinary care and treatment;
the keeping of thorough records; and
euthanasia.

APPENDIX C

ALTERNATIVE COMPUTATION OF DATA OBTAINED FROM DUCT LIGATED ANIMALS

INTRODUCTION

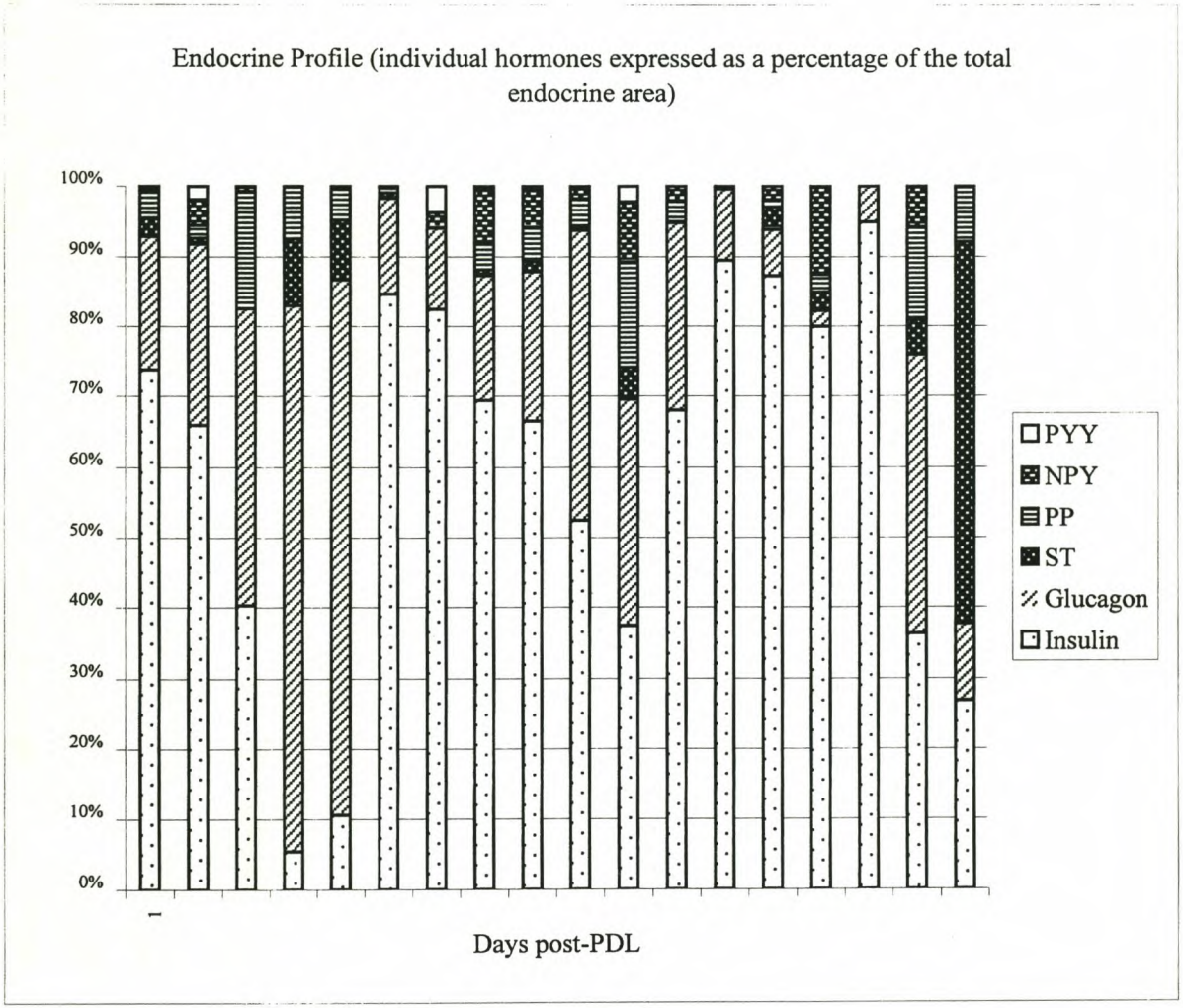
The bulk of the data obtained from studies on pancreatic duct ligated rats was subjected to analysis as indicated in materials and methods (chapter 3). Literature however supports various alternatives for calculation and interpretation of data obtained from studies of this nature. In order to ensure that both methods are addressed it was deemed necessary to include the results of data manipulation described by those studies mentioned in chapter three.

Table C1: Data for endocrine content in duct ligated rat pancreas tissue on the various days as prescribed in the study (n = 6).

		PDL	PDL	PDL	PDL	PDL	PDL	Total
DAY		% IIR	% GIR	% ST-IR	% PPIR	% NPY	% PYY	Endocr %
1		3.0612	0.7965	0.1065	0.1558	0.0208	0.0109	4.1516
1.5		4.0896	1.6104	0.0516	0.1118	0.2232	0.1203	6.2069
2		2.8847	3.0215	0	1.2008	0.05	0	7.157
2.5		1.0966	15.769	1.9589	1.5056	0	0	20.33
3		0.653	4.7006	0.5281	0.2779	0.0187	0	6.1783
3.5		11.536	1.8577	0.1071	0.0969	0	0.0335	13.631
4		10.879	1.5312	0	0.013	0.277	0.492	13.192
4.5		7.1589	1.8518	0.0872	0.389	0.7898	0.0463	10.323
5.5		6.8462	2.1975	0.1552	0.5052	0.5484	0.0492	10.302
6.5		4.1992	3.3044	0.037	0.3204	0.1243	0.0201	8.0054
7.5		0.5897	0.5056	0.0712	0.2394	0.1348	0.035	1.5757
8		3.6806	1.453	0.009	0.1588	0.1117	0	5.4131
9		8.7186	0.9896	0	0.0371	0	0	9.7453
10		11.992	0.9122	0.4345	0.1403	0.2666	0	13.746
11		14.549	0.4136	0.4894	0.4615	2.2885	0	18.202
12		25.864	1.377	0	0	0	0	27.241
13		2.1067	2.2933	0.291	0.7775	0.3342	0	5.8027
14		2.1067	0.8447	4.2728	0.6169	0	0	7.841

Table C1: Continued

Mean		6.7784	2.5239	0.4777	0.3893	0.2882	0.0448
Standard Error		1.5118	0.8198	0.2481	0.0976	0.1282	0.0273
Median		4.1444	1.5708	0.0969	0.2587	0.118	0
Mode		2.1067	#N/A	0	#N/A	0	0
Standard Deviation		6.4141	3.478	1.0528	0.4141	0.5437	0.1157
Sample Variance		41.141	12.097	1.1083	0.1715	0.2956	0.0134
Kurtosis		3.4869	14.071	10.9	2.3578	11.858	15.09
Skewness		1.6842	3.6083	3.2292	1.6308	3.2783	3.7925
Range		25.274	15.355	4.2728	1.5056	2.2885	0.492
Minimum		0.5897	0.4136	0	0	0	0
Maximum		25.864	15.769	4.2728	1.5056	2.2885	0.492
Sum		122.01	45.43	8.5995	7.008	5.1881	0.8073
Count		18	18	18	18	18	18



APPENDIX D

INTRODUCTION

The classic ebb and flow phase related to the physiologic and metabolic response to surgery, trauma and injury is well documented. Sympatho-adrenal, neuro-endocrine and cytokine responses are partially responsible for this phenomenon.

AIM

The effects of hyperglycaemia in initiating ß-cell growth has been established. The apparent divergence in results obtained from this group of experimental animals prompted us to look into the short term changes in sham operated animals after laparotomy only and after pancreatic duct ligation (PDL) only in two popular strains of inbred laboratory rats (WAG and Sprague Dawley).

METHODS

Two strains of inbred conditioned rats (n = 5) as indicated above underwent laparotomy alone (Group 1) and PDL (Group 2) under general anaesthesia. Whole Blood Glucose (WBG) determinations (Haemoglucotest®) were recorded before, during and within two hours of laparotomy or PDL at 15 minute intervals.

RESULTS

Group 1 recorded a basal WBG of 5.6 ±0.2 mmol/l and 5.8 ±0.7 mmol/l after two hours (P: not significant). Group 2 recorded a basal WBG of 6.3 ±0.15 mmol/l and 12 ±.18 after two hours ($P < 0.05$)

CONCLUSION

1. Mild and early hyperglycaemia are detected after PDL and not after sham operated laparotomy controls.
2. Laparotomy alone, in the short term, did not elevate WBG.
3. Manipulation of the pancreas is needed to elevate WBG.

4. These findings might be relevant, if early ICC or histology is performed after PDL.

COMMENTS

Islet, especially ß-cell hyperplasia has been conclusively linked to hyperglycaemia. Glucose perfusion studies performed on laboratory rats has been shown to induce ß-cell proliferation and an increase in insulin positive tissue volume. Whether or not this has any bearing on this study in open to conjecture. It is however interesting that early PDL induced hyperglycaemia in these animals. One could therefore not exclude the possibility that ligation induces hyperglycaemia which is the trigger for the increase in endocrine tissue observed in this study.

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