DERMAL FIBROBLASTS:

A HISTOLOGICAL AND

TISSUE CULTURE STUDY

Postgraduate Student,

E. J. MAZYALA (D. D. S.)

Division of Anatomy and Histology,

Faculty of Health Sciences,

University of Stellenbosch.

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STUDY SUPERVISOR:

Prof. Benedict Page: BSc, MSc., PhD.

Division Head: Anatomy and Histology,

Department of Biomedical sciences,

Faculty of Health Sciences,

University of Stellenbosch.

MSc. STUDENT:

Dr. Erick John Mazyala D.D.S (University of Dar es salaam),

Department of Anatomy and Histology,

Bugando University College of Health Sciences (BUCHS),

Mwanza, Tanzania.

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“After climbing a great hill, one only finds that there are many more hills to climb.”

Nelson Mandela.

DECLARATION

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…… 1 December 2007

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GENERAL ABBREVIATIONS AND TERMINOLOGY

AR ……..Aortic regurgitation

AT(1)…… Angiotensin-1

AVE……Average

Bp……Base pair

CF ……...Cardiac fibroblasts

COPD……..Chronic obstructive pulmonary diseases

CPD …….... Cumulative population doubling

CTGF……...Connective tissue growth factor

DAB…….3,3-Diaminobenzidine tetrahydrochloride

DEJ…….Dermoepidermal junction

DNA ……Deoxyribonucleic acid

ECM …….Extra-cellular matrix

EM……Electron microscopy

ET-1……..Endothelin-1

Ex vivo culture …….Culture of dermal fibroblasts in laboratory

Fig……..Figure

FN……..Fibronectin

H&E ……..Haematoxylin and Eosin

IFN……Interferon

IL-4 ……...Interleukin-4

Kb …….Kilo base

LM…….Light-microscopy

LV……. Left ventricle

MEFs …….Mouse embryonic fibroblasts

MMPs ……Matrix metalloproteinases

MRC……...Medical Research Council

NF-kappa B……Necrotic factor kappa

NPR-A……Natriuretic peptide receptor A

NS ……..Not significant

SD ……..Standard deviation

PAS…….Periodic Acid-Schiff

PD……...Population doubling

PGE2……..Prostaglandin-E2

P-value……Statistical term used to express significance and power factor

RER……Rough endoplasmic reticulum

ROS ……...Reactive oxygen species

SE ……..Standard error of mean

TBS……Tris-buffered saline.

TEM…….. Transmission electron microscope

TGF-beta …….. Transforming growth factor-beta

TIMPs. ……...Inhibitors of matrix metalloproteinases

TNF-alpha …….Tumor necrotic factor alpha

UVA…….. Ultraviolet-alpha radiation

UVB ……..Ultraviolet-beta radiation

VEGF…….Vascular endothelial cell growth factor

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Dr. E. J. MAZYALA,

Division of Anatomy and Histology, Department of Biomedical Sciences, University of Stellenbosch,

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FORMAT OF MSc. THESIS

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2. **Index and contents of Chapters**
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4. **Clinical problem addressed**
5. **Historical Review and critical 2007 review update**
6. **Material and Methods, including applied statistics**
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8. **Laboratory Component:**
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CHAPTER 1: OVERVIEW OF CHAPTER CONTENTS.

Survey of various chapters

Chapter 2: Introductory remarks:

Definition;

- Fibroblasts, myofibroblasts, in relation to diseases like solar damage and photoaging, matrix-metalloproteinases, growth factors, and wound healing.

Definitions including;

- Characteristics of fibroblasts,
- Fibroblasts sites in organs of human body and their associated diseases,,
- Extracellular matrix (ECM), cell migration during wound healing,
- Collagen deposition, intergrin, keratinocytes, wound contraction during wound healing,
- Wound healing; maturation and remodelling phase,
- Fibroblasts in systemic diseases such as systemic sclerosis, keloids.

Chapter 3: Material and Methods.

This chapter covers the following topics.

- Hypothesis tested,
- Sampling,
- Definitions, fixation of tissues,
- Ethical approval of project,
- Statistical analysis,
- Embedding and tissue processing; sectioning,
- Stains and staining procedures:
  - ❖ Hematoxylene and eosin (H&E) staining,

- ❖ Masson trichrome staining,
- ❖ Von Gieson staining,
- ❖ Reticulum stain (Foot's Modification of Hortega's silver carbonate), and
- ❖ Verhoeff's staining.

- Tissue cultures of fibroblasts, culture media, analytical technique, cell proliferation, morphological assessment are included in chapter 7.

Chapter 4: Tissue culture of human fibroblasts

- Mediums,
- Technique,
- Infrastructure,
- End-point analysis,
  - ❖ Study groups.

Chapter 5: Results.

Included are;

- Cadaveric statistics, demographics and tabulation,
- Cell culture studies (exvivo) fibroblasts: monolayers, proliferation,
- Cell culture study illustrations,
- Histological illustrations:
  - ❖ Skin biopsies; dermis, epidermis,

Special stains; skin, heart, lung fibroblasts; collagen, elastin and reticulum.

Chapter 6: Discussion.

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- Sampling.

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Abstract

Fibroblasts are fully differentiated fibers-forming cells of adult connective tissues. They are the principal cells of connective tissue that elaborate the precursors of ECM components. These are most numerous and stable cells of connective tissues. As the name suggest, they are considered to be responsible for production of collagenous, reticular, and elastic fibers and for synthesis of glycosaminoglycans, (GAG) and glycoproteins of the amorphous intercellular substance. They however, synthesize MMPs, which are responsible for turnover of collagen and elastic fibers. Due to diverse synthetic functions, fibroblast activities need delicate control. Improper control at any step of any process in fibroblasts activities results in synthesis of either: quantitative excessive or inadequate, or qualitatively defective products that are reflected in diseases processes such as scleroderma, systemic or focal sclerosis, keloids, cardiac and pulmonary fibrosis, but also in wrinkles and premature aging of the skin.

Various factors diversely affect fibroblast, these includes UVA, UVB, and tobacco nicotine. These factors can easily play with the delicate balance of fibroblasts activities culminating to diseases processes. Connective tissue is found throughout the body. All body organs contain fibroblasts.

To study fibroblasts and photo damage, skin biopsies from; the glabella (sun-exposed) and upper third on both thighs medially (sun protected) of 36 human cadaver were used. Cadaver age ranged from 0 (fetuses) to 104 years, with mean age of 49.2. Routine and special histological coupled with computer assisted image analysis were used to assess dermal changes at light microscopic level. In sun-exposed skin, the collagen fiber architecture appeared disorganised after 3rd decade. Interestingly, collagen fiber architecture in sun-protected skin appeared to be organised throughout all age.

Human fibroblasts derived from the skin dermis were studied ex vivo within University of Stellenbosch approved ethical protocol 04/05 (Faculty of Health Sciences). The explant method of culture was adopted as this is the most satisfactory (Boss WK et al 2000 [I, II], Cristafalo VJ et al 1998, Matsuo M et al, 2004). Serum enriched and serum free Ham's F12 and DMEM mediums were used. Only 5% of cell growth could be affected in serum free medium compared to 85% in serum enriched medium ($p<0.05$). The result suggests that serum enriched medium renders better fibroblast proliferation over serum free medium. This however has clinical implication as serum rich media carry the possibility of transmitting zoonotic diseases in case fibroblast cell therapy is considered for human use (Boss et al 2000).

Keywords: Fibroblasts, Extracellular matrix, Photoaging, Explant.

Rationale

Studying fibroblasts is relevant as they are found through out the body in connective tissue. In present study, fibroblasts were demonstrated in all the organs studied (skin, heart and Lung). They are involved in various diseases processes of the human body as part of their normal or defective functions. As part of their normal reparative function to injured tissues fibroblasts replace fibrous tissues to parenchyma of irreversibly damaged organ such as in pulmonary fibrosis (lung fibroblasts) and myocardial fibrosis (cardiac fibroblasts). Abnormality (defects) in control of their synthetic and secretory function of fibroblasts results in diseases such as keloid, hyperplastic scars, sclerosis, wrinkles and premature aging, and other dermal fibrotic diseases including fibromatosis such as Dupuytren's contracture and retroperitoneal fibrosis. In the present study, replacements of some lung parenchyma by fibrous tissues were note.

In addition, the increasing demand for facial rejuvenation services necessitates looking for some safer serum free culture media.

CHAPTER 2: LITERATURE REVIEW

- Definitions
- Histology review
- Fibroblasts, myofibroblasts
- Disease processes

INTRODUCTION: THE HUMAN FIBROBLAST

Introduction:

Definition

Dorland's Medical Dictionary defines a fibroblast as the principal cell in the connective tissue; with characteristic flat and fuse form in shape having branching cytoplasmic processes at its periphery borders, its nucleus is also flat, oval in outline. It is also called fibrocyte and *desmocyte"*.

A **fibroblast** is a unique cell type, derived from mesodermal layer, involved heavily in synthesis and secretion of the extracellular matrix for maintenance of many animal tissues, this is possible by their constant secretion of precursors of the connective tissue matrix. Fibroblasts give a structural scaffold (stroma) for many tissues, and play a crucial role in wound healing. They are the most regular cell constituent of connective tissue in animals (Fanny D et al 2004).

The main function of fibroblasts is to preserve the structural integrity of connective tissue through constantly secreting precursors of the extracellular matrix. The secretory function of fibroblasts comprises of the precursors of the entire components of the extracellular matrix, principally, the ground substance, and an array of fibers, which includes collagen, reticulum and elastin. The composition of the extracellular matrix plays a key role in the physical properties of connective tissues (Fanny D et al 2004, Abraham L 2002, Wolfgang K 1997).

General histology

Fibroblasts are morphologically diverse, their location and activity determine their appearances. They have tendency to retain positional memory of the location and tissue context where they

had formerly resided, at least over a few generations (Abraham L 2002, Wolfgang K 1997, Fanny D et al 2004).

Fibroblasts do form flat monolayers in culture, otherwise, they do not show this feature in situ. Fibroblasts are not limited by means of polarity attachment to a basal lamina on one side; in the intestine, however, subepithelial myofibroblasts participate in formation of the basal lamina via their secretion of alpha-2 chain which incorporate the laminin protein. These intestine subepithelial myofibroblasts are absent in portions of epithelia that are associated with follicles. To distinguish it from epithelial cells, fibroblast demonstrates individual cell motility or migration, fills the gaps in the body of the organism, and therefore gives its shape by their ECM. (Wolfgang K 1997, Fanny D et al 2004).

Primitive mesenchymal cells are the predecessors for fibroblasts just like any other connective tissue cell from (Abraham L 2002). They therefore express vimentin, an intermediate filament protein, a marker for mesodermal origin. This marker, however, have been shown to be expressed by in vitro cultured epithelial cells on adherent substratum after some time. (Abraham L 2002, Wolfgang K 1997, Fanny D et al 2004).

Fibroblasts are large , flat, branching cells that appear spindle-shaped or fusiform in profile with an elliptical nucleus, thin cytoplasm, and usually not resolved under light microscope (L/M). The branching processes are slender and inconspicuous in most preparations under L/M. The nucleus is oval or elongated and has a delicate nuclear membrane, one or two distinct nucleoli, and a small amount of finely granular chromatin (Abraham L 2002, Wolfgang K 1997, Leeson L et al 1988, Bloom et al 1986, Midwood K S et al 2004, Alice S et al 2004).

In connective tissue spreads, the nucleus appears pale, whereas in sectioned material it is usually appears shrunken and deeply stained with basic dyes. Under the electron microscope, these show the typical features of a protein-secreting cell; well developed RER and Golgi apparatus. Since the outlines of the cell are indistinct in most histological preparations, the nuclear characteristics are of considerable value in identification (Abraham L 2002, Wolfgang K 1997, Leeson L et al 1988, Bloom et al 1986, Midwood KS et al 2004, Alice S et al 2004).

Two stages of activity: active and quiescent, are observed in these cells. Cells with intense synthetic activity are morphologically distinct from the quiescent fibroblasts that are scattered within the matrix. Young fibroblasts, have an abundant and irregular branched cytoplasm, its nucleus is ovoid, large, and pale staining, with fine chromatin and prominent nucleolus (euchromatic nuclei), the cytoplasm is rich in RER, and Golgi complex is well developed and located near the nucleus. They appear relatively homogeneous and basophilic and are actively engaged in protein synthesis for production of intercellular substances. The mitochondria appear as slender rods, microtubules are also present and are thought to be required for the translocation of secretory vesicles (Abraham L 2002, Wolfgang K 1997, Leeson L et al 1988, Bloom et al 1986, Midwood KS et al 2004, Alice S et al 2004).

Old fibroblasts are relatively inactive fibroblasts, with heterochromatic nuclei, also referred to as fibrocytes. They are smaller than active fibroblast, tend to be spindle-shaped, have fewer processes, smaller, darker, elongated nucleus; sparse and only weakly basophilic or even acidophilic cytoplasm due to scanty granular ER (RER). However, if adequately stimulated, as in wound healing, fibrocytes may revert to the fibroblast state (Abraham L 2002, Wolfgang K 1997, Leeson L et al 1988, Bloom et al 1986, Midwood KS et al 2004, Alice S et al 2004).

In course of development, as in development of notocord and nephron, fibroblasts can transform into characteristic epithelial cell and vice versa by a processes known as mesenchymal to

epithelial (MET) and epithelial to mesenchymal transition (EMT) respectively (Abraham L 2002, Wolfgang K 1997, Fanny D et al 2004).

Fibroblasts have a branched cytoplasm surrounding an oval, dotted nucleus having mono- or bi-nucleoli (Abraham L 2002). Active or young fibroblasts are recognized by their numerous rough endoplasmic reticulums, a feature of protein secreting cells (Abraham L 2002, Midwood KS et al 2004). Inactive or old fibroblasts, **fibrocytes**, are heterochromatic, smaller and tapered. They possess less and condensed rough endoplasmic reticulum. They may be found scattered among the ECM or arranged parallel in clusters (Abraham L 2002).

Myofibroblasts

Myofibroblasts, cells with features of both fibroblasts and smooth muscle, are also observed during wound healing. It is now established that the myofibroblast can be found in normal tissue as well as in a wide variety of pathological processes (Oda D et al 1998).

The cell has morphologic characteristics of fibroblasts but contains increased amounts of actin microfilaments and myosin and behaves like smooth muscle cells. Their activity is responsible for wound closure after tissue injury, a process called wound contraction (Midwood KS et al 2004, Abraham L 2002).

Myofibroblasts are characterised by high expression of alpha-smooth muscle actin (alpha-SMA), are important and transient cells in normal wound healing but are found in increased number in various pathological conditions of the lung including asthma and pulmonary fibrosis (Gu L et al 2004). The mechanisms that regulate the myofibroblasts phenotype are unknown but are likely to involve signals from the extracellular matrix transmitted via specific integrins. Fibronectin is a glycoprotein released during inflammation and has been shown to regulate the phenotype of

vascular smooth muscle cells via alpha v and beta 1 integrins (Gu L et al 2004, Scaffidi AK et al 2001). TGF-beta1 induced fibroblast-myofibroblast differentiation in a Smad proteins-dependent manner (Gu L et al 2004). IFN-gamma could block this process but it was not mediated by interrupting smad2/3 phosphorylation and their nuclear translocation and DEX played a synergism with TGF-beta1.Differentiated myofibroblasts, however, are resistant to both Interferon-gamma and DEX (Gu L et al 2004).

Myofibroblasts disclose irregular, often stellate cellular outline with numerous and long cytoplasmic extensions, and are connected by intermediate or adherens and by gap junctions, the latter considered as low-resistance pathways for intercellular communications (Gabbiani G et al, 1978). In addition, myofibroblasts that is associated with a basal lamina; posses junction complexies, dense patched or dense bands, and pinocytotic vesicles. They are also connected to extracellular matrix by cell-to-stromal attachment sites through the fibronexus, which is transmembrane complex of intracellular microfilament bundles in apparent continuity with extra cellular fibronectin fibers (Singer II et al 1984). These cell-to-stromal attachment sites are well developed and numerous in myofibroblasts compared to their attenuated appearance in smooth muscle cells. At the surface of myofibroblasts, three types of fibronexus are observed; plaque-like, track-like and tandem associations (Singer II et al, 1979). The cytoplasm of myofibroblasts have abundant bundles of microfilaments also called stress fibers, these fibers are normally parallel organised to the long axis of the cell. Also, within the cytoplasm are interspersed several dense bodies. As in vascular smooth muscle cells, these structures may be in continuity with dense bands or plasmalemmal attachment plaques. Rough endoplasmic reticulum and Golgi area are well developed. The nucleus displays deep indentations, an ultrastructural feature that has been correlated with cellular contraction in several systems (Bloom S et al 1969, Frank WW et al 1969, Lane BP et al 1965, Majno G et al 1969). Several nuclear bodies are usually present and nucleoli are conspicuous.

Although morphologically best defined with electron microscope, myofibroblasts disclose several typical histological traits that allow their recognition in routine paraffin sections. They are usually large, spindle shaped, often stellate (spider-like) cells. In addition, they have numerous elongated branching cytoplasmic processes, and discrete fibrillar and acidophilic cytoplasm with cable-like condensations (stress fibers) coursing parallel to the long axis through the subplasmalemmal cytoplasm. The nuclei are frequently show constriction or reveal strangulations of nuclear portions, a feature reflecting cellular contraction. The chromatin is delicately granular, uniformly distributed, and nucleoli are prominent. Well differentiated myofibroblasts are observed in collagen poor and oedematous areas. In heavy collagenized zones, myofibroblasts are difficult to recognise with the light microscope since they correspond ultrastructurally to poorly developed myofibroblasts of fibroblasts.

Even though most of myofibroblasts are derived from fibroblasts, a certain proportion of them are derived from vascular smooth muscle cells, and only a low proportion form pericytes (Ronnov-Jessen L et al 1995). Other mesenchymal cells with possible derivation for myofibroblasts are; perisinusoidal stellate cells in hepatic fibrosis and cirrhosis (Friedman SL et al 1993, Ramadori G et al 1990, Schmitt-Graff A et al 1991 and Blazejewski S et al 1995), and glomerular mesengial cells (Johnson RJ et al 1991, Diamond JR et al 1995, Goumenos DS et al 1994, Boukhalfa G et al 1996).

Fibroblast release factors

Fibroblasts secrete all ECM proteins, which include; collagens, glycosaminoglycans, reticular and elastic fibers, and glycoproteins (Abraham L 2002, Kohyama T et al 2002). They also secrete growth factors. The activity and composition of fibroblasts varies with the age of an individual. In young individuals, most of fibroblasts are young actively dividing and

synthesizing ground ECM substance. Tissue injury stimulates fibrocytes of elder individuals to revert to young active fibroblast form which then undergo mitotic division to increase their number and start the synthetic activities for repairing the damage. Fibroblast production of extracellular matrix is crucial not only for normal tissue development and maintenance of tissue structure but also for the repair and remodelling processes after injury (Kohyama T et al 2002).

Cardiac fibroblasts

Cardiac fibroblasts constitute greater than 90% of non-myocyte cells in the heart. Because they are responsible for synthesis of components of the extracellular matrix, growth factors and cytokines in the myocardium, they play an important role in normal and pathologic performance of the heart (Agocha AE et al 1997, Neuss M et al 1996). Cardiac fibroblasts are the source of extracellular matrix, growth factors and cytokines in the heart and their interactions with cardiac myocytes are recognized (Zhao L et al 2001). Their effects on biological responses of endothelial cells are observed in angiogenesis in the heart (Zhao L et al 2001).

Cardiac remodelling involves the accumulation of extracellular matrix (ECM) proteins including fibronectin (FN) released by cardiac fibroblasts (Huntley BK, 2006). FN contains RGD motifs that bind integrins at DDX sequences allowing signalling from the ECM to the nucleus. It has been noted that the natriuretic peptide receptor A (NPR-A) sequence contains both RGD and DDX sequences. Modulation of extracellular matrix degradation in the human heart is done by the gp130 ligand oncostatin M that regulates tissue inhibitor of metalloproteinases-1 through ERK1/2 and p38 in adult human heart myocytes and fibroblasts (Weiss TW et al 2005).

Fibroblasts are hypothesized to play a role in cellular communication between the endothelium and epithelium and are positioned to provide leukocytes a surface on which they may migrate through the interstitium (Sirianni FE et al 2006). They are also capable of secreting a diverse

repertoire of cytokines and are able to be activated by pro-inflammatory cytokines and cell-cell contact (Fitzgerald SM et al 2004, Sato E et al 2002).

Contrarily to shapes of many cells that normally correspond to their function performed, fibroblasts can perform different function while retaining the same morphology. Fibroblass movement involves the production of filopodia. During moulding of the ECM, fibroblasts contracts, pulling others cells and components of ECM with them (Fanny D et al 2004, Kohyama T et al 2002). Fibroblasts can differentiate into other cells of mesenchymal origin, these includes; osteoblasts, adipocytes, and myocytes (Abraham L 2002, Sirianni FE et al 2006).

The fibroblasts can thrive easily in culture, this feature single them out as favourable cells in biological cell culture research (Fanny D et al 2004). Most cell culture studies however, have been done entirely on zoonotic fibroblasts cells or partly on human fibroblasts but utilising zoonotic serum (Boss et al 2000, Fanny D et al 2004).

Fibroblasts and disease processes:

Strict control of fibroblast activity is important for the integrity of extra cellular matrix. Weiss TW et al 2005, show that alterations in the balance between matrix deposition and matrix degradation brought about by changes in the respective activities of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) contribute significantly to cardiac dysfunction and disease. Lerman OZ et al 2003, also, show that impairment of fibroblast functions result in impediments of normal wound repair, thereby contributing to the chronic and non-healing wounds. Fibroblasts contribute to structural alteration processes as in chronic inflammatory and chronic obstructive pulmonary diseases, COPD, like asthma. Matthiesen S et al 2007, reported expression of multiple muscarinic receptors in human lung fibroblasts and

demonstrated muscarinic receptor-induced, G(1)-mediated proliferation in these cells. Fitzgerald SM et al 2004, Sato E et al 2002, showed that fibroblasts play a sentinel role in asthmatic and other diseases like graft-versus host disease. They are the main constituents of connective tissue and are increased in number in the asthmatic lung. They are also capable of secreting a diverse repertoire of cytokines and are able to be activated by pro-inflammatory cytokines and cell-cell contact. Allergic asthma and allergic dermatitis are chronic inflammatory diseases and are characterized by an accumulation of eosinophils at sites of inflammation. Eotaxin-1/CCL11 and eotaxin-3/CCL26 are members of the CC chemokine family, which are known to be potent chemoattractants for eosinophils. Rokudai A et al 2006, Antonelli A et al, 2005, observed that a human lung fibroblast, HFL-1 produces eotaxin-1 and -3 in response to TNF-alpha plus IL-4 stimulation, accompanied with NF-kappa-B and STAT6 activation. Fibroblast/myofibroblast expansion is critical in the pathogenesis of pulmonary fibrosis (Ramos C et al 2006).

Recently, Baglole CJ et al, 2006, observed the development of emphysema to be associated with the loss of alveolar fibroblasts. Cigarette smoke increase oxidative stress which may injure fibroblasts resulting to their death by apoptosis. Of the spectrum of cigarrate smocher, only some sufer chronic obstructive lung diseases (COPD), giving the impression that different human fibroblasts strains exist with varied adoptability to the unprecedented oxidative stress situations. Sirianni FE et al 2006 also concluded that the endothelial/fibroblast/epithelial linkage is disrupted in emphysematous human lungs and postulate this disruption may disturb leukocyte migration and account for their accumulation in the alveolar interstitium of emphysematous lung tissue.

Myocardial fibrosis has been identified in biopsy specimens from catheterization and valve replacement surgery in patients with severe chronic aortic regurgitation (AR). Characterization of these extracellular matrix (ECM) alterations has been performed in humans. Fibrosis also has

been identified in chronic severe experimentally created AR, in which ECM composition features abnormal fibronectin/ glycoprotein production, with normal collagen content. Virtually identical ECM variations have been induced when normal rabbit cardiac fibroblasts (CF) are subjected in culture to cyclic mechanical strain mimicking that found in the left ventricle (LV) in severe AR (Gupta A et al 2006). It has been showed that Endothelin-1 (ET-1), is a trophic agent in the human heart, has the ability to influence the development of cardiac fibrosis by eliciting a potent collagen synthesis response in cardiac fibroblasts similar to those of transforming growth factor-beta, TGF-beta (Kawano H et al 2000, Tsutsumi Y et al 1998, Hou M et al 2000, Hafizi S et al 2004, Villarreal FJ et al 1998). Other trophic agents in cardiac fibroblasts and myocytes includes; angiotensin I and II peptides, and chronic beta2- adrenergic receptor stimulation. Angiotensin I exert its trophic effect by its direct influence without involving AT (1) receptor while angiotensin II acts on AT (1) receptor (Hafizi S et al 2004, Villarreal FJ et al 1998).

Chronic beta2-adrenergic receptor stimulation exerts its trophic effect through an autocrine mechanism (Turner NA et al 2003, Villarreal FJ et al 1996). Hypoxia also has trophic effect in human cardiac fibroblasts: the process mediated by various growth factors and hormones including; angiotensin II, transforming growth factor-beta 1, basic fibroblast growth factor and thyroid hormone (Agocha A et al 1997).

Fibroblast in solar damage and skin aging

Skin aging is the reflection of pathophysiological processes carried out at cellular level. The central cell that is involved during premature aging is the dermal fibroblasts, (Meinhard Wlaschek et al 2003). It has been demonstrated by Meinhard Wlaschek et al 2003 and Saunders et al 2001, that fibroblasts cellular senescence can be induced artificially by exposure of sub lethal irradiation dose as that used in treating dermatological diseases. Gary J Fisher et al 1997

showed that long term exposure to background ultraviolet irradiation from sunlight causes premature (photo) aging. They went further, to grossly characterize the photodamaged skin as having wrinkles, altered skin pigmentation and loss of skin tone. Du Toit et al 2007 and Bernstein Ef et al 1996 histopathologically characterised the photo damaged skin as having: deposition of abnormal elastic fiber, the so called elastosis, basophilic degeneration of collagen and elastic fibers, increased deposition of glycosaminoglycans, the ECM ground substances, in the site formerly occupied by collagen type I, increased number of melanocytes and uneven distribution of melanin, collagen disappear proportionately also perivascular infiltration of lymphocytes, histiocytes and mast cells. DNA mutation secondary to sun exposure is the agreed cause of all the histological and clinical observed dermal changes (Berneburg M et al 1997). The Dermis lies beneath the epidermis and providing mechanical support for the later (Abraham L 2002). The main structural component of the dermis is type I collagen and type III collagen to the lesser amount (Abraham L 2002, Gary J Fisher et al 1997). The principal cell for the dermal extracellular matrix synthesis is the dermal fibroblast (Abraham L 2002, Gary J Fisher et al 1997). Meinhard Wlaschek et al 2001 implicated the accumulation reactive oxygen species, ROS, generated by the exposure to ambient sun light are responsible for dermal fibroblasts DNA damage. According to Meinhard Wlaschek et al 2001, ROS activates cytoplasmic signal transduction pathways in resident dermal fibroblasts that are related to growth differentiation senescence and connective tissue matrix degradation. These pathways involve the activation of matrix metalloproteinases, MMPs a family of zinc-dependent proteases secreted as latent precursors (zymogens) and proteolytically activated in the ECM (Meinhard Wlaschek et al 2003 Abraham L 2002, Gary J Fisher et al 1997). Apart from fibroblasts, MMPs are also synthesised by chondrocytes, keratinocytes, monocytes, macrophages, hepatocytes and tumour cells (Abraham L 2002). To date, about four members of MMPs family are known which includes: family collagenases 1, 2 and 3 in which 1 can degrade type I, II, III, and type V collagen, 2

stored in cytoplasmic granule of polymorphonuclear leucocytes and released in response to a stimuli, collagenase 3 can degrade type I, II, III, IV, IX, X, and XI, but also laminin, and fibronectin and other ECM components. Family Stylomelysins (1, 2, and metalloelastase) can degrade basement membrane component (type IV collagen and fibronectin) and elastin, family Gelatinases A and B can degrade type I collagen and normally produced by alveolar macrophages, family matrilysin, and family membrane-type matrix metalloproteinases which are produced by tumour cells (Abraham L 2002, Remacle AG 2006 et al, Gary J Fisher et al 1997, Meinhard Wlaschek et al 2003, Bernstein EF et al 1996). The normal inhibition mechanism of MMPs in extracellular space by tissue inhibitors of metalloproteinases (TIMPs) is disrupted in photodamaged fibroblasts (Abraham L 2002). The preferential degradation of type I collagen can be attributed to MMP1.

Fibroblasts in Wound healing

Wound healing is the body's natural process of regenerating injured or damaged tissue. When tissue is injured some growth factors, wound hormones, are released by injured cells which will lead to cell division and migration so that damage is repaired (Midwood KS et al 2004, Abraham L et al 2002). Healing can occur in one or both of the two ways: by regeneration which involve the replacement of injured tissue by the same kind of tissues or by fibrosis in which fibrous connective tissues proliferates to replace the injured tissue with the scar tissue (Chang HY et al 2004, Abraham L et al 2002). Regeneration is limited to some few tissues, like epithelium (epidermis and mucous membranes), bone and fibrous connective tissues. Other tissue heals by scar formation due to their limited regeneration capacity (Abraham L et al 2002). It is the fibroblasts which take the role of healing whenever the injured tissue has limited regenerative capacity. An array of events takes place in a sequential manner to repair the damage. These

events merge in time (Abraham L et al 2002, Stadelmann WK et al 1998, Iba Y et al 2004). Events involved are basically divided into three stages which include: inflammation stage, organisation stage and lastly is the regeneration and or fibrosis stage which affects a permanent repair. Assuming the injury occurred involving the skin (epidermis and dermis), the three events will happen as follow:

Inflammation stage is set by inflammatory mediators which aim at mobilising inflammatory cells (leucocytes, platelets, macrophages, mast cells) and clotting factors, antibodies and other substances to seep into the injured area so that bleeding is stopped and the wound is isolated, hence preventing bacteria, toxins or other harmful substances from spreading to the surrounding tissues. Formation of scab ends this stage (Kuwahara RT et al 2006, Chang HY et al 2004, Abraham L et al 2002).

Fibroblasts are set into action during the organisation stage, which aims at restoring the blood supply (Quinn, JV 1998). Here temporary blood clot is replaced by granulation tissue, a delicate pink tissue composed of several elements most importantly are fine reticular fibers which form a delicate meshwork across the wound (DiPietro LA et al 2003). Later fibroblasts replace the fine reticular fibers by further synthesising new collagen type I fibers to permanently bridge the gap (Lansdown ABG et al 2001). At the same time, macrophages digesting and removing the original blood clot (Kuwahara RT et al 2006). The granulation tissue that formed is going to become a scar tissue, a fibrous tissue patch highly resistant to infection because it incorporates bacterial-inhibiting substances (Romo T et al 2006, Greenhalgh DG 1998).

Regeneration and or fibrosis stage effects permanent repair. While organisation is still going on, the surface epithelium stars to regenerate and migrate across the granulation tissue (Falanga V 2005, Mercandetti M et al 2005). After maturation of granulation tissue, fibroblasts, under the influence of transforming growth factor beta, assume the myofibroblastic form and start wound

contractions that further reduce the size of the scar (Eichler MJ et al 2005, Lorenz HP et al 2003, DiPietro L A et al 2003). Remodelling is simultaneously taking place where by the excess collagen is degraded to resume the normal shape and arrangemrnt of the original site (Greenhalgh DG 1998).

Disruption in various processes of wound healing may result to either to complete failure in healing as in chronic ulcers or inappropriate healing of the wound with exaggerated scar tissue formation as in hyperplastic scar, keloid scar (Midwood K S et al 2004 , O'Leary R et al 2002, Desmouliere A et al 2005).

Fibroblasts in systemic sclerosis

Systemic sclerosis (SSc) is a chronic debilitating disease characterized by an excessive production and accumulation of collagen and other extracellular matrix components in the skin and systemic organs by fibroblasts (Sonnylal S et al 2007).

It is widely accepted that fibroblasts excessive collagen fiber production leading to fibrosis is induced by transforming growth factor (TGF)-beta in the early stage and is subsequently maintained by connective tissue growth factor (CTGF). CTGF is a cysteine-rich mitogenic peptide that has been involved in various fibrotic disorders and can be induced in fibroblasts by activation with TGF-beta, (Xiao R et al 2006).

Its symptoms and signs includes; binding down of skin, Raynaud's phenomenon, pigmentary change, hand contractures, fingertip ulcers, dyspnoea, restricted mouth opening, telangiectasia, fingertip resorption, joint complaints, dysphasia, and gangrene (Krishna Sumanth M et al 2007).

Various immunosuppressive drugs have been used to treat it with limited success, among them are; Mycophenolate mofetil (MMF) which is used to prevent organ rejection after transplantation. MMF inhibits COL1A1 and COL1A2 mRNA at the level of transcription, via repression of their promoters, (Roos N et al 2007). Cyclophosphamide is reasonably effective in ameliorating and/or stabilizing lung function from scleroderma interstitial lung disease, (Antoniu SA 2007, Beretta L et al 2006).

Fibroblasts in keloids

Keloids are fibrotic lesions that result from an abnormal wound-healing process that lacks control of the mechanisms that regulate tissue repair and regeneration (Sandulache VC et al 2007).

The proliferation of normal tissue-healing processes results in scarring that enlarges well beyond the original wound margins (Ong CT et al 2007). Represent the most extreme example of cutaneous scarring that uniquely afflicts humans as a pathological response to wound healing. It is characterized by excessive deposition of collagen and other extracellular matrix components by dermal fibroblasts. Upon cutaneous injury, cocktails of chemokines, cytokines and growth factors are secreted temporally and spatially to direct appropriate responses from neutrophils, macrophages, keratinocytes and fibroblasts to facilitate normal wound healing. Keloid formation has been linked to aberrant fibroblast activity, exacerbated by growth factors and inflammatory mediators. Lu F et al 2007 showed that there is a loss of gap functional intercellular communication and connexion expression in fibroblasts derived form keloid and hypertrophy scar tissue which could then affect intercellular recognition and thus break the proliferation and aptosis balance. The molecular mechanism(s) behind keloid pathogenesis remains unclear

IL-6 signalling may play an integral role in keloid pathogenesis and provide clues for development of IL-6 receptor blocking strategies for therapy or prophylaxis of keloid scars (Ghazizadeh M et al 2007, Kim JY 2006).

It is thought that epidermal VEGF exerts significant paracrine control over the dynamics and expression profile of underlying dermal fibroblasts, (Ong CT et al 2007).

Impaired fibroblast PGE2 production due to aberrant paracrine fibroblast signalling has been linked to lower airway fibrosis and recently to keloid formation, (Sandulache VC et al 2007). Enhanced expression and phosphorylation of Stat3 in keloid scar tissue, and in cultured keloid fibroblasts (Lim CP et al 2006), the over expression of three genes not previously reported as being up-regulated in keloids (annexin A2, Transgelin, and RPS18) (Satish L et al 2006).

Many treatment modalities for keloids have been tried with variable amounts of success: Surgical excision, compressive therapy, silicon dressings, corticosteroid injections, radiation, cryotherapy, interferon therapy, and laser therapy have all been used alone or in combination. Despite this wide range of available treatments, recurrence rates typically remains in the 50%-70% range.

Study aim:

Since fibroblasts are such important cell populations as they involve in many diseases processes some of them highlighted above, it is obvious worth studying. This study aimed at utilising cadaver tissue biopsy specimen to histological studying behaviour of human fibroblasts from different organs by simple routine histological techniques, also using both serum-enriched medium, and serum free medium to cultural study the human dermal fibroblasts.

CHAPTER 3: MATERIAL AND METHODS

1. Sampling
2. Definitions
3. Ethical analysis
4. Statistical analysis
5. Tissue preparation for light-microscopy and special stains

MATERIALS AND METHODS

Hypotheses tested

1. Fibroblasts can be demonstrated in different organs in the body using routine and special stains for light microscopy alone, (Haematoxylin and eosin, van Gieson, Verhoeff's and silver stains).
2. Dermal fibroblast morphology remains the same regardless of age, sex and race of an individual, as demonstrated by the application of routine stains alone at light microscopic level.
3. Masson Trichrome staining can enhance tissue collagen identification at light microscopic level in the lung, heart and skin.
4. There is questionable difference in collagen morphology of the skin regardless of; age, sex, site and race of an individual.
5. Elastic fiber content of skin shows degradations in sun-exposed versus sun-protected skin of an individual.
6. Special stains at light microscopic level are useful in demonstrating quantitatively and qualitatively the collagen and elastic fiber content of the skin.

Materials

Sample size:

Skin biopsies were obtained from 36 embalmed human cadavers.

Selection criteria:

Cadavers with normal looking skin were included.

Exclusion criteria:

All cadavers with damaged skin and underlying dermatological diseases were excluded.

Definitions of terms

Tissue processing; refers to any treatment of tissue necessary to impregnate them within a solid medium (paraffin wax) to facilitate the production of sections for microscopy

Fixation is a complex process involving a series of chemical events that aim at prevention of autolysis and bacterial attack to the tissue specimen and hence preserves cellular structure and maintains the distribution of organelles as close to the living state as possible while allowing subsequent procedures like staining, to be carried out clearly. **Formaldehyde** and **glutaraldehyd**e are the most commonly used chemical fixatives. Formaldehyde in solution is referred to as formalin.

Labelling of tissues; refers to giving identification number/tag to tissue to ensure that there is little danger of incorrect reporting due to errors or exchanges of tissue identity. The Tissue Tek system was used in which tissue identity was written on the cassette and retained as a permanent record during sectioning and storage of tissue blocks.

Methods

Skin biopsies from 36 cadavers and biopsies from five human heart and lungs, for light microscopic study were collected in the dissection laboratory at Stellenbosch University,

Tygerberg campus. The sex composition was for skin biopsies were; 21 males and 14 females. Age ranged from 0 to 104 years, a mean age 49.2 years.

Ethical approval:

The University of Stellenbosch is a registered tertiary institutional and medical school and resorts under the Health Act, which provides for the use of human cadavers. Cadavers were always treated with respect. All the information concerning cadavers was used for academic purpose alone and at high confidentiality. Tag numbers instead of actual names of cadaver were used to increase degree of confidentiality.

Statistical analysis:

The study used descriptive statistics that included mean, median, average data for comparison of samples and small numbers. Non-parametric testing with the Mann-Whitney U test was applied. Results were expressed as mean ± SD. Significant results referred to $p<0.05$.

Procedures:

From each of 36 cadavers, skin biopsy specimens were cut from the face (glabella) and upper third on both thighs medially. Site selection was based on the perception degree of sun exposure of the area during lifetime, the face being exposed to the sun and the medial aspect of the thighs as sun protected areas. The demographic data were obtained from the database by retrospective search using the tagged cadaver number. Skin specimens were cut deeply to involve the subdermal layer of about 20 x 25 mm surface areas using dissection scalpels and tissue forceps.

Fixation:

Tissue specimens were immediately in 10% formalin in small containers pre-labelled with the corresponding cadaver number and site. Upon arrival in the histology laboratory, all specimens were registered in a record book. Specimens were then removed from the fixative containers, trimmed and inserted into processing cassettes bearing specific serial number of the appropriate tissue as registered in the record book. The pencil labels on processing cassette accompanied the specimen through all stages for identification purpose.

Dehydration, infiltration, and embedding

The specimen blocks were immersed in 98% formic acid (Merck) for an hour to decontaminate. The blocks were then washed in running tape water for 20 minutes and returned to fixative prior tissue processing.

Tissue water is not readily mixed with the embedding solutions and must be replaced using a series of alcohol at increasingly higher concentrations. This was done by immersing the specimen cassettes in series of increasing alcohol concentrations.

This step followed by alcohol replacement with an intermediate solvent that is miscible with both alcohol and the embedding solutions.

Tissue specimen blocks were then loaded in an autoprocessor with the schedule as follows;

Table 1: The overnight processing schedule of tissue specimens at room temperature.

Position	Reagent	Duration(hour)
1	10% formalin (fixative)	2
2	70% alcohol	2
3	96% alcohol	2
4	96% alcohol	1
5	100% alcohol	1.5
6	100% alcohol	1.5
7	100% alcohol	1.5
8	Xylene	1
9	Xylene	1
10	Paraffin wax	1
11	Paraffin wax	1
12	Paraffin wax	1

The process was normally commenced at 1400 and completed by 0800 the following day. The time taken for the arrived specimen to stay in fixative containers before processing started varied from 1 to 72 hours depending on the availability of autoprocessor and whether the tissue arrived at the beginning of the weekend.

Infiltration and Embedding

The blocks were taken out of the processor, the tissue specimens removed from processing cassette. The liquid form of the embedding compound, **paraffin wax**, replaced the intermediate solvent. The liquid embedding medium was allowed to solidify, thereby provided rigidity to the tissue for sectioning. Molten paraffin wax, at temperature 60ºC (two degrees above the melting point) was used as embedding medium. The wax was poured into the corresponding labelled plastic embedding cassette mould to a depth enough to cover the thickest tissue block. As soon as a thin film of semi-sold wax has formed on the base of the mould, the tissue was introduced with warmed forceps, gently pressing the correctly oriented tissue into the semi-solid wax. The mould and wax were placed into an embedding machine at -5°C to hasten solidification of wax forming a block. The purpose of this was to reduce the tendency of large crystal formation in the wax. Blocks were trimmed once the ax cooled completely.

Sectioning

Excessive wax was trimmed prior to placing the block on the microtome.

Sections were made by slicing at 3-5 µm thickness. Each slice was immediately floated on warm water at 40° C, in water bath, and put on a clean slide. Of each tissue block, slides were made for the following five stains; (H & E, von Gieson, Masson trichrome, Verhoeff and silver impregnation). Slides were warmed at 60°C in an incubator for 2 hours prior to staining.

STAINING TECHNIQUES

Haematoxylin and Eosin (H&E):

This is the most regular routine histology and pathology laboratories staining technique. It employ a basic dye (Haematoxylin), and acidic dye (Eosin). Haematoxylin stains blue to purple intracellular acidic molecules (Nucleic acid and rough endoplasmic reticulum, RER). Eosin stains rddish to pink basic intracellular molecules, these includes most of the cytoplasmic protein. In general, Haematoxylin & Eosin will stain nuclei blue to purple and cytoplasm red to pink.

Haematoxylin and Eosin (H &E): Histological Technique

Harris 1990, Mallory 1938 technique was used. (Please, refer to appendix 2)

Identification:

Positive identification of the fibroblasts was based on seeing under the light microscope a purple blue spindle-shaped cell or fusiform-shape cell with an elliptical nucleus with or without a thin pinkish cytoplasm, associated with collagen fibers.

Distinguishing active fibroblasts from fibrocytes under the light microscope was based on the density of staining on nuclei, where as big pale (euchromatic) stained spindle shaped nucleus surrounded with relative less collagen fibers or bundles identified as **active fibroblast,** and small deeply stained (heterochromatic) spindle shaped nucleus cell surrounded with abundant collagen bundles or fibers identified as **fibrocytes.**

Positive identification of collagen fiber was based on seeing under the light microscope red or pink wavy fibers.

Identification of elastic fiber was based on seeing thin straight branching red lines on tissue section.

Positive identification of ground substance was based on seeing under the light microscope an empty space that is not a result of tissue preparation and staining procedures.

Masson Trichrome Staining

Principle: Three dyes with different molecular sizes are used. The permeability of molecules depends on the porosity of the tissue concerned. Erythrocytes, muscle, fibrins and collagen fibers are stained differently as deterrmined by the degree of their permeability to the staining molecules. Thus small molecules will stain red less permeable structures red while the more permeable structures such as collagen fibers will be stained blue after binding to larger molecular size aniline blue.

Fixative: buffered formalin **(**10%) was used.

Technique: 4 -5**µ**m thick paraffin sections were cut.

Equipment: glasses were rinsed in distilled water. Coplin jars, 60°c oven or water bath, microwave were used.

Reagents used:

Sigma accustain trichrome stain kit (catalog# HT15) contains:

Biebrich scarlet-acid fuchsin solution (#Ht15- 1, 0.9% Biebrich scarlet, 0.1% acid fuchsin, 1% acetic acid),

Phosphotungstic acid solution (#Ht15-2, 10% phosphotungstic acid),

Phosphotungstic acid solution (#Ht15-3, 10% phosphotungstic acid), and

Aniline blue solution (#HT 15-4, 2.4% acetic acid),

Bouin's solution (#HT10132- 1L or HT101128-4L),

Wegert's solution (Sigma catalog#HT10-79).

For the detail of procedure please refer to appendix 2

Identification:

Positive identification of the fibroblasts was based on seeing under the light microscope a black spindle-shaped cell or fusiform-shape cell with an elliptical nucleus with or without a thin red cytoplasm, associated with collagen fibers.

Distinguishing active fibroblasts from fibrocytes under the light microscope was based on the density of staining on nuclei, where as big pale (euchromatic) stained spindle shaped nucleus surrounded with relative less collagen fibers or bundles identified as **active fibroblast,** and small darkly stained (heterochromatic) spindle shaped nucleus cell surrounded with abundant collagen bundles or fibers identified as **fibrocytes.**

Positive identification of collagen fiber was based on seeing under the light microscope blue wavy fibers.

Identification of elastic fiber was based on seeing thin straight branching red lines on tissue section.

Positive identification of reticulum fiber was based on seeing under the light microscope delicate meshwork of fine fibers.

Positive identification of ground substance was based on seeing under the light microscope an empty space that is not a result of tissue preparation and staining procedures.

Van Gieson Staining

Principle: Van Gieson staining technique is very handy for demonstration of collagen from smooth muscles in neoplasia. Its mechanism of staining is dependent on the molecular size. The smaller molecular size of picric acid stains less porous muscles and red blood cells yellow. Ponceau S has larger molecular size and hence will stain more porous collagen fibers bright red collagen fibres, which have larger pores, and allow the larger molecules to enter.

Preparation of solutions

1. Celestin Blue:

   0.5 g Celestin Blue was put in 100mls of 5% ammonium ferric sulphate (iron alum) and boiled for 3 minutes. The preparation was left to cool. It was then filtered and kept refrigerated.

2. Curtis stains:

   Was prepared by mixing the following solutions, 90.0 mls of saturated aqueous picric acid, 10.0 ml of 1% ponceau S and 10.0 mls glacial acetic acid.

3. 1% Ponceau S:

   Was prepared by mixing 1gm of Ponceau S in 100 mls of distilled water.

   For the detailed procedure, please refer appendix 2

Identification:

Positive identification of the fibroblasts was based on seeing under the light microscope a blue spindle-shaped cell or fusiform-shape cell with an elliptical nucleus with or without a thin yellow cytoplasm, associated with collagen fibers.

Distinguishing active fibroblasts from fibrocytes under the light microscope was based on the density of staining on nuclei, where as big pale (euchromatic) stained spindle shaped nucleus surrounded with relative less collagen fibers or bundles identified as **active fibroblast,** and small darkly stained (heterochromatic) spindle shaped nucleus cell surrounded with abundant collagen bundles or fibers identified as **fibrocytes.**

Positive identification of collagen fiber was based on seeing under the light microscope bright red wavy fibers.

Identification of elastic fiber was based on seeing thin straight branching lines on tissue section

Positive identification of reticulum fiber was based on seeing under the light microscope delicate meshwork of fine fibers.

Positive identification of ground substance was based on seeing under the light microscope an empty space that is not a result of tissue preparation and staining procedures.

Foot's Modification Of Hortega's Silver Carbonate Method For Reticulum.

The silver carbonate solution of Hortega was obtained by precipitating the silver from the silver nitrate solution with lithium carbonate and then redissolving that precipitate with ammonia. After treatment in that solution, the tissue was reduced in formalin.

Indications: Staining reticulum in sections.

Fixation: 10% formalin.

Technique: Paraffin.

Staining solutions:

Silver nitrate solution.

(Foot's Modification of silver Ammonium Carbonate Solution)

All glassware were chemically clean. For optimum results, the purest reagents available were used. This solution was always be freshly prepared.

10 ml of a 10% aqueous solution of silver nitrate was placed in a 100ml. capacity graduated glass cylinder. 10ml.of saturated (1.25%) aqueous solution of lithium carbonate was added. The white precipitate were washed three or more times with distilled water. This was done by adding approximately 30 to 40 ml. of distilled water to the silver carbonate mixture in the cylinder. Parawax was stretched over top of cylinder and the cylinder shaken vigorously. Precipitate were allowed to settle to the bottom. The supernatant fluid were careful decanted. This was done by letting to settle 3 to 5 times. After completely washed, the precipitate were settled in a small compact mound of fine particles of slightly green-gray colour. 25 ml. of distilled water was then added to the cylinder. 28% ammonia water was added drop by drop (approximately 6 to 15 drops) while shaking the container vigorously to almost dissolve the precipitateed. To avoid adding too much ammonia water; a few grains of precipitate were left. The solution was then made up to 100ml. with 95% ethyl alcohol. Solution was poured into a small flask (250ml. capacity) for easier handling. A precipitate was again formed which was dissolved by adding a

few more drops of ammonia water. This alcoholic solution was filtered, covered, and then placed in screw-cap Coplin jar in the hot water floatation bath, warmed at 43°C., for 20 to 30 minutes.

Note: 28% ammonia water is same strength as 58% ammonium hydroxide.

0.25 % potassium permanganate

was made by placing 0.25 gm Potassium permanganate in 100 mls in distilled water

5% oxalic acid solution

Was made by placing 5 gm oxalic acid in 100 ml distilled water.

20% neutral formalin

Was made by mixing 20mls of neutral formalin and 80 mls of distilled water

0.2% gold chrolide solution

Was prepared by adding 1gm of Gold chrolide in 500ml of distilled water

For the detailed procedure please refer appendix 2.

Identification:

Positive identification of the fibroblasts was based on seeing under the light microscope a black spindle-shaped cell or fusiform-shape cell with an elliptical nucleus with or without a thin cytoplasm, associated with collagen fibers.

Distinguishing active fibroblasts from fibrocytes under the light microscope was based on the density of staining on nuclei, where as big pale (euchromatic) stained spindle shaped nucleus surrounded with relative less collagen fibers or bundles identified as **active fibroblast,** and small darkly stained (heterochromatic) spindle shaped nucleus cell surrounded with abundant collagen bundles or fibers identified as **fibrocytes.**

Positive identification of reticulum fiber was based on seeing under the light microscope black to dark violet delicate meshwork of fine fibers.

Positive identification of collagen fiber was based on seeing under the light microscope brown-pink wavy fibers.

Identification of elastic fiber was based on seeing brown-pink thin straight branching lines on tissue section.

Positive identification of ground substance was based on seeing under the light microscope an empty space that is not a result of tissue preparation and staining procedures.

Verhoeff's Method

This classical method for elastic fibers works well after all routine fixatives. Coarse fibers are intensely stained, but the staining of fine fibers may be lass than satisfactory. The differentiation step is critical to the success of this method, some expert is necessary to achieve reproducible results; it is very easy to over-differentiate (and lose) the fine fibers.

Although some older texts state that the prepared working solution has a usable life of only 2-3 hours, satisfactory results has been obtained using up to 48 hours old.

Verhoeff's method for elastic fibers (1990)

Preparation of stain

a. 5 g of Haematoxylin was added into 100 cm^3 of absolute alcohol

b. 10g of Ferric chloride was added into 100 cm^3 of distilled water

c. Lugol's iodine solution

   Was prepared by 1gm of Iodine adding and 2gm of Potassium iodide into 100cm^3 of distilled water

- Working solution

  Was prepared by mixing 20 cm^3 of solution (a), 8 cm^3 of solution (b), and 8 cm^3 of solution (c)

Addition was in the above order and there were mixing between subsequent additions.

For detailed staining procedures, please refer appendix 2.

Identification:

Positive identification of the fibroblasts was based on seeing under the light microscope a black spindle-shaped cell or fusiform-shape cell with an elliptical nucleus with or without a thin cytoplasm, associated with collagen fibers.

Distinguishing active fibroblasts from fibrocytes under the light microscope was based on the density of staining on nuclei, where as big pale (euchromatic) stained spindle shaped nucleus surrounded with relative less collagen fibers or bundles identified as **active fibroblast,** and small darkly stained (heterochromatic) spindle shaped nucleus cell surrounded with abundant collagen bundles or fibers identified as **fibrocytes.**

Positive identification of reticulum fiber was based on seeing under the light microscope delicate meshwork of fine fibers.

Positive identification of elastic fiber was based on seeing black thin straight branching lines on tissue section.

Positive identification of collagen fiber was based on seeing under the light microscope red wavy fibers or bundles.

Positive identification of ground substance was based on seeing under the light microscope an empty space that is not a result of tissue preparation and staining procedures.

Immunocytochemistry:

Preview:

Monoclonal Mouse Anti-Human Smooth Muscle Actin, Clone 1A4, was used for immunocytochemistry labelling of myofibroblasts in the skin, lung and heart. The antibody also can be used in labelling other cells that have actin microfilament protein such as smooth muscle and myoepithelial cells. These cytoplasmic actin proteins consists of six isoforms differing in amino acid sequence but equal 42kDa molecular mass. They have about 90% sequence homology. The antigenic portion is mainly the 18 N-terminal with a sequence of 50-60%.

Procedures

The whole processes were automated, please, refer appendix 3 for detailed automated immunocytochemical processing schedule.

Paraffin-embedded blocks, pre-fixed with formalin, of normal human skin, lung and heart tissues were sent to Histopathology laboratory for immunocytochemistry staining of myofibrobalasts. The tissue was pre-treated with epitope retrieval induced by heating for 20 minutes. 10mmol/L. Tris buffer, 1 mmol/L EDTA, pH 9.0 were used to optimise the results. The 20 minutes heat-induced epitope retrival in 10mmol/L. Tris buffer, 1 mmol/L EDTA, pH 9.0 was used to apply 1:75 diluted Monoclonal Mouse Anti-Human Smooth Muscle Actin, code No. M0851 on formalin-fixed, paraffin-embedded sections of normal human skin, lung and heart tissues and incubated with primary antibody for 30 minutes at room temperature. 1:75 diluted DakoCytomation Mouse IgG2a, code No. X0943, was used as negative control. In both cases, dilutions were performed immediately prior to their application.

Weakly stained non-cadaver tissues were used as positive control. Validation of positive staining depended on successful staining of positive control specimen while specificity in staining depended on successful staining of intact cells in negative control specimen. For details of the immunocytochemistry staining protocol, please refer appendix 4.

CODING:

The relative amount of dermal collagen in different sites of skin was coded as follows:

Abundant referred to presence of more collagen fibers and bundles relative to ground substances (empty spaces) as apparent when looking under light microscope.

Moderate referred to relative equal amount of collagen fibers and ground substances (empty spaces) as apparent when looking under light microscope.

Scanty referred to less collagen fibers relatively to more ground substances (empty spaces) as apparent when looking under light microscope.

Relative arrangement of dermal elastin was coded as follows:

The collagen bundles were considered **organised** most bundles are arranged parallel to the dermoepidermal junction as apparent when looking under light microscope.

The collagen bundles were considered **less organised** if there is a mixture of some bundles arranged parallel to the dermoepidermal junction and some other bundle without specific orientation as apparent when looking under light microscope.

The collagen bundles were considered **disorganised** if bundles lacked specific orientation as apparent when looking under light microscope.

CHAPTER 4: TISSUE CULTURE OF HUMAN FIBROBLASTS

Contents:

Introduction:

Human fibroblasts derived from the skin dermis were studied ex vivo within University of Stellenbosch approved ethical protocol 04/05 (Faculty of Health Sciences). The explant method of culture was adopted as this is the most satisfactory (Boss WK et al 2000 [I, II], Cristafalo VJ et al 1998, Matsuo M et al, 2004)

Analytical methods

1. Culture facility: Tissue culture laboratory, Division of Anatomy and Histology, Biomedical Sciences, Faculty of Health Sciences, University of Stellenbosch.
2. Material and infrastructure:
    a. Laminar flowhood (Labotech; horizontal flow)
    b. CO2 incubator (Cell Laboratory) 37ºC. 5% carbon dioxide and 95% air.
    c. Room temperature: 17 degrees centigrade and sprayed daily with 75% ethyl alcohol (anti-septic effect).
    d. Ultraviolet light: To prevent cell contamination and cross infection.
    e. Working surface; air conditioned room; clear room environment

3. Chemicals and culture equipment
    1. BD Biocoat™ : 6-well Petri dishes (30mm diameter)
    2. Culture mediums:

a. Ham's F12 medium (Ham, R.G. 1965 Proc. Nath. Acad. Sci 53, 288) enriched with L-glutamine (Highveld Biological PTY Ltd): or

b. DMEM (Dulbecco's modified Eagle's medium: Dulbecco R et al, 1959 Virology 8, 39: 4.5g/l glucose with 0.110/l Sodium pyruvate with glutamine (Highveld Biologicals PTY Ltd). Both mediums were enriched with 10% fetal calf serum and contained, penicillin, streptomycin and fungizone.

Tissue preparations

1. Skin biopsy was placed in sterile Hams F12 medium at 4 degrees centigrade (carrier medium)
2. Under a 10x (dissecting) Olympus microscope the epithelium was separated and cleared (aseptically) from the dermis under sharp dissection. Hypodermal part was excised from the deep dermis.
3. Small explants (0.2x 0.2cm) were cut from the prepared dermis.
4. Six explants were placed and dried per well. Thereafter the explant (oriented with the dermis upwards) were covered with a drop of either culture medium (Ham's or DMEM) for 24-hours. Thereafter the medium was aspirated and the explant was covered with fresh medium. Medium changes were affected Monday, Wednesday, and Friday (2ml DMEM or Ham's medium).
5. Cultures were inspected daily (Olympus Inverted microscope) for signs of cell proliferation and contamination. Medium exchanges were performed in a

laminar flow cabinet. Incubation of cell cultures was performed at 37 degrees centigrade, and gas flow rate of 95% oxygen and 5% carbon dioxide..

6. When necessary cells were released by trypsin- versene digestion and cell viability was assessed by trypan blue stains.

End-point analysis: Measured outcomes were the identification of

1. Fibroblast proliferation
2. Monolayer formation
3. Presence and absence of cell crawling, lamello and filopodia formation.

Study Groups

Group 1: 48 explants in either serum enriched Ham's or DMEM culture medium (previous studies from our laboratories demonstrate equivalent results).

Group 2: Control: 48 explants in serum –free medium,

(Group1 and 2 biopsies were of the same age, and site: mean 48.6 years)

CHAPTER 5: RESULTS

1. Cadaveric: Histological findings
2. Tissue culture results.

Table2. The demographic characteristics of biopsed specimen.

Slide Number	**Cadaver Number**	**Site**	**Age**	**Sex**	**Race**	**Cause of death**
50/07	K63/05	Glabella	104	M	Coloured	Natural (advanced age)
58/07	K63/05	Thigh	104	M	Coloured	Natural (advanced age)
78/07	K5/07	Thigh	48	M	Coloured	DTB
84/07	K5/07	Glabella	48	M	Coloured	DTB
98/07	K67/05	Glabella	41	F	Coloured	ARC/TB
101/07	K67/05	Thigh	41	F	Coloured	ARC/TB
112/07	K 42/06	Glabella	71	M	Coloured	CVA
115/07	K 42/06	Thigh	71	M	Coloured	CVA
116/07	K 50/06	Thigh	35	F	Coloured	ARC/TB
120/07	K 50/06	Glabella	35	F	Coloured	ARC/TB
123/07	K 48/06	Thigh	38	M	Black	PTB
129/07	K 48/06	Glabella	38	M	Black	PTB
240/07	K 32/06	Glabella	85	M	White	MI
143/07	K 32/06	Thigh	85	M	White	MI
235/07	Heart					
236/07	Heart					
237/07	Heart					
238/07	Heart					
239/07	Heart					

240/07	K 40/05	Glabella	40	F	Coloured	Intracranial infection
244/07	K 40/05	Thigh	40	F	Coloured	Intracranial infection
245/07	K 1/07	Glabella	85	F	White	MI/RF
249/07	K 01/07	Thigh	85	F	White	MI/RF

288/07	K25/07	Glabella	63	M	Coloured	ARC/PTB
289/07	K25/07	Thigh	63	M	Coloured	ARC/PTB
290/07	K21/07	Glabella	55	M	Coloured	PTB
291/07	K21/07	Thigh	55	M	Coloured	PTB
292/07	K30/07	Glabella	50	M	Coloured	Respiratory failure
293/07	K30/07	Thigh	50	M	Coloured	Respiratory failure
294/07	Zenele 22029946	Glabella	Fetus	M	?	Still born`
295/07	Zenele 22029946	Thigh	Fetus	M	?	Still born`
296/07	-	Glabella	Fetus	F	?	Still born`
297/07	-	Thigh	Fetus	F	?	Still born`
298/07	-	Glabella	Fetus	M	?	Still born`
299	-	Thigh	Fetus	M	?	Still born`
314/07	K24/07	Glabella	34	F	Coloured	ARC/PTB
315/07	K24/07	Thigh	34	F	Coloured	ARC/PTB

316/07	K26/07	Glabella	-	F	Black	ARC/PTB
317/07	K26/07	Thigh	-	F	Black	ARC/PTB
318/07	K10/07	Glabella	30	M	Black	ARC
319/07	K10/07	Thigh	30	M	Black	ARC
320/07	K31/07	Glabella	70	M	Coloured	Septic shock
321/07	K31/07	Thigh	70	M	Coloured	Septic shock
322/07	K28/07	Glabella	-	M	Coloured	PTB
323/07	K28/07	Thigh	-	M	Coloured	PTB
324/07	K50/05	Glabella	50	M	Coloured	Intracerebral bleeding
325/07	K50/05	Thigh	50	M	Coloured	Intracerebral bleeding
326/07	K70/05	Glabella	-	F	Coloured	-
327/07	K70/05	Thigh	-	F	Coloured	-
328/07	K66/07	Glabella	34	F	Coloured	ARC
329/07	K66/07	Thigh	34	F	Coloured	ARC
330/07	K43/07	Glabella	32	M	Black	Hypoglyceamia/ PTB
331/07	K43/07	Thigh	32	M	Black	Hypoglyceamia/ PTB
332/07	K05/05	Glabella	50	M	Coloured	Cerebral infarction
333/07	K05/05	Thigh	50	M	Coloured	Cerebral infarction
334/07	K34/07	Glabella	70	M	White	Pnemonia/ leg

						amputaion
335/07	K34/07	Thigh	70	M	White	Pneumonia/ Leg amputation
336/07	K38/07	Glabella	83	M	Black	Natural
337/07	K38/07	Thigh	83	M	Black	Natural
338/07	K62/05	Glabella	46	F	Black	Pneumonia/ TB
339/07	K62/05	Thigh	46	F	Black	Pneumonia/TB
340/07	K60/05	Glabella	39	M	Coloured	PTB
341/07	K60/05	Thigh	39	M	Coloured	PTB
343/07	K54/05	Glabella	69	M	Black	Coronary artery disease
344/07	K54/05	Thigh	69	M	Black	Coronary artery disease
345/07	K01/06	Glabella	56	F	Coloured	ARC
346/07	K01/06	Thigh	56	F	Coloured	ARC
347/07	K69/05	Glabella	41	M	Coloured	Pneumonia
348/07	K69/05	Thigh	41	M	Coloured	Pneumonia
349/07	K73/05	Glabella	46	F	Coloured	PTB
350/07	K73/05	Thigh	46	F	Coloured	PTB
351/07	K72/05	Glabella	67	F	Coloured	Lung cancer
352/07	K72/05	Thigh	67	F	Coloured	Lung cancer
353/07	K61/05	Glabella	43	M	Coloured	Haemoptysis/TB
354/07	K61/05	Thigh	43	M	Coloured	Haemoptysis/TB

Age: (Mean 49.2, SD=24, 95% CI is 40.5-57.9), 98% CI is 39.0- 63, Median (47.0)

Table3.The age and sex distribution of skin cadaveric biopsies.

Age (years)	**Sex**		
	Male	**Female**	**Total**
0	2	1	3
21-30	1	-	1
31-40	3	4	7
41-50	6	3	9
51-60	1	1	2
61-70	4	1	5
71+	4	1	5
Total	21	11	**32**

There was no cadaver between ages 1-20 years, and female between 1-30 years. One (1) male and two (2) female cadavers had no age records, not included in the table.

70% were male and 30% were female.

Table 5: Relative staining of dermal tissue components in different types of routine stains.

Tissue component	**Type of stain**				
	H & E	**van Gieson**	**Masson trichrome**	**Verhoeff**	**Silver impregnation**
Fibroblasts	++++	++++	++	++	+
Collagen fibers	+++	++++	++++	++++	+++
Elastic fibers	++	+++	+++	+++++	+
Reticular fibers	+	+	+	+	++++

Key: ++++= clearly stained, +++= fairly stained, ++ stained but indistinctive, +unstained.

Under the light microscope, Verhoeff staining clearly stained elastic fibers but

Table 6: Relative staining of lung tissue components in different types of routine stains.

Tissue component	**Type of stain**				
	H & E	**van Gieson**	**Masson trichrome**	**Verhoeff**	**Silver impregnation**
Fibroblasts	++++	++++	++	++	+
Collagen fibers	+++	++++	++++	+++	+++
Elastic fibers	++	+++	+++	++++	++
Reticular fibers	+	+	+	+	+

Key: + ++++= clearly stained, ++++= fairly stained, +++ stained but indistinctive++= poorly stained, + unstained

Table 7. Relative staining of heart tissue components in different types of routine stains.

Tissue component	**Type of stain**				
	H & E	**van Gieson**	**Masson trichrome**	***Verhoeff**	**Silver impregnation**
Fibroblasts	++++	++++	+++	++++	+
Collagen fibers	+++	++++	++++	++++	++++
Elastic fibers	+++	+++	+++	++++	+
Reticular fibers	+	+	+	+	+

Key: + ++++= clearly stained, ++++= fairly stained, +++ stained but indistinctive++= poorly stained, + unstained. *Only verhoeff stain demonstrated intercalated disc in cardiocytes.

Table 8.The relative distribution of human fibroblasts in different organs (heart, skin, and lungs).

Fibroblasts presence	**Organ**		
	Heart	**Lung**	**Skin**
Present	2	1	3
Absent	-	-	-

Key: 3= present abundantly, 2=moderately present, 1=scantly present,

Table 9. Relative amount of dermal fibroblasts in the face according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0	2			2	1			1	3
21-30		1		1				-	1
31-40		1	2	3		1	3	4	7
41-50		1	5	6			3	3	9
51-60			1	1			1	1	2
61-70			4	4			1	1	5
71+			4	4			1	1	5
Total	2	3	16	21	1	1	9	11	32

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80

Table 10: Relative amount of dermal fibroblasts in the thigh according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0	2	0	0	**2**	1	0	0	**1**	3
21-30	0	1	0	**1**	0	0	0	**0**	1
31-40	0	2	1	**3**	0	3	1	**4**	7
41-50	0	1	5	**6**	0	0	3	**3**	9
51-60	0	0	1	**1**	0	0	1	**1**	2
61-70	0	0	4	**4**	0	0	1	**1**	5
71+	0	0	4	**4**	0	0	1	**1**	4
Total	2	4	15	**21**	1	2	9	**11**	32

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80

Comparing the relative amount of dermal fibroblasts between sun exposed table 9 and sun protected skin show significant difference, more fibroblasts in sun protected skin (p-value < o.5) Refer appendix 1.

Table 11: Relative amount of dermal fibroblasts in the face according to age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0													
21-30		1		1									1
31-40		1	1	2		1	4	5					7
41-50			1	1		1	7	8					9
51-60				0			2	2					2
61-70			1	1			3	3			1	1	5
71+			1	1			2	2			2	2	5
Total		2	4	6		2	18	20			3	3	29

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80. The races of three (3) fetuses were not available.

Reject the null hypothesis because p value <0.05

Table 12. Relative amount of dermal fibroblasts in the thigh according age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0													
21-30		1		1									1
31-40		1	1	2		4	1	5					7
41-50		1		1		1	7	8					9
51-60						1	1	2					2
61-70			1	1			3	3			1	1	5
71+			1	1			2	2			2	2	5
Total		3	3	6		6	14	20			3	3	29

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80. The races of three (3) fetuses were not available.

Table 13: Relative amount of dermal collagen in the face according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0	2			2	1			1	3
21-30		1		1				-	1
31-40		1	2	3		1	3	4	7
41-50		3	3	6		2	1	3	9
51-60			1	1			1	1	2
61-70		2	2	4			1	1	5
71+		1	3	4			1	1	5
Total	2	8	11	21	1	3	7	11	32

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80

.

Table 14: Relative amount of dermal collagen in the thigh according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0	2			2	1			1	3
21-30		1		1				-	1
31-40		2	1	3		3	1	4	7
41-50		5	1	6		2	1	3	9
51-60			1	1			1	1	2
61-70		2	2	4			1	1	5
71+		1	3	4			1	1	5
Total	2	11	8	21	1	5	5	11	32

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80

.

Table 15: Relative amount of dermal collagen in the face according age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0													
21-30		1		1									1
31-40		1	1	2		4	1	5					7
41-50		1		1		1	7	8					9
51-60						1	1	2					2
61-70			1	1			3	3			1	1	5
71+			1	1			2	2			2	2	5
Total		3	3	6		6	14	20			3	3	29

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80. The races of three (3) fetuses were not available.

Table 16: Relative amount of dermal collagen in the thigh according to age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0													
21-30		1		1									1
31-40		2		2		4	1	5					7
41-50		1		1		6	2	8					9
51-60						1	1	2					2
61-70		1		1		1	2	3			1	1	5
71+			1	1			2	2		1	1	2	5
Total		5	1	6		12	8	20		1	2	3	29

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80. The races of three (3) fetuses were not available.

Table 17: Relative arrangement of dermal collagen in the face according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Or	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	
0	2			2	1			1	3
21-30		1		1				-	1
31-40		1	2	3		1	3	4	7
41-50		1	5	6			3	3	9
51-60			1	1			1	1	2
61-70			4	4			1	1	5
71+			4	4			1	1	5
Total	2	3	16	21	1	1	9	11	32

Key: Or= organised, Lo= less organised, Do=disorganised.

Table 18: Relative arrangement of dermal collagen in the thigh according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Or	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	
0	2	0	0	**2**	1	0	0	**1**	3
21-30	1		0	**1**	0	0	0	**0**	1
31-40	3			**3**	4			**4**	7
41-50	6			**6**	3	0		**3**	9
51-60	1	0		**1**	1	0		**1**	2
61-70	2	2		**4**	0	1		**1**	5
71+	2	2		**4**	0	1		**1**	4
Total	17	4	0	**21**	9	2	0	**11**	32

Key: 3= organised, 2= less organised, 1=disorganised.

Table 19: Relative arrangement of dermal collagen in the face according to age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Or	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	
0													
21-30		1		1									1
31-40		1	1	2		1	4	5					7
41-50			1	1		1	7	8					9
51-60							2	2					2
61-70			1	1			3	3			1	1	5
71+			1	1			2	2			2	2	5
Total		2	4	6		2	18	20		0	3	3	29

Key: Or= organised, Lo= less organised, Do=disorganised. Races of three (3) fetuses were not available.

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Table 20 Relative arrangement of dermal collagen in the thigh according to age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Or	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	
0													
21-30	1			1									1
31-40	2			2	5			5					7
41-50	1			1	8			8					9
51-60					2			2					2
61-70	1			1	1	2		3		1		1	5
71+		1		1		2		2	1	1		2	5
Total	5	1		6	16	4	0	20	1	2		3	29

Key: Or= organised, Lo= less organised, Do=disorganised. Races of three (3) fetuses were not available.

Table 21. Relative amount of dermal elastin in the face according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0		2		2		2		1	3
21-30		1		1					1
31-40	1	2		3	1	3		4	7
41-50	4	2		6	2	1		3	9
51-60	1			1	1			1	2
61-70	4			4	1			1	5
71+	4			4	1			1	5
Total	14	7		21	6	5		11	32

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80

.

Table 22: Relative amount of dermal elastin in the thigh according to age and sex.

Age	**Sex**								
	Male				**Female**				**Total**
	Ab	**Mo**	**Sc**	**Total**	**Ab**	**Mo**	**Sc**	**Total**	
0		2		2		1		1	3
21-30		1		1					1
31-40		1	2	3		2	2	4	7
41-50		1	5	6		1	2	3	9
51-60			1	1			1	1	2
61-70			4	4			1	1	5
71+			4	4			1	1	5
Total		5	16	21		4	7	11	32

Key: Ab=abundant, Mo=moderate, Sc=scanty. Refer Fig 8-80

.

Table 23: Relative arrangement of dermal elastin in the face according to age and race.

Age	Skin colour												
	Black				**Mixed race**				**Caucasian**				**Total**
	Or	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	
0													
21-30		1		1									1
31-40		1	1	2		1	4	5					7
41-50			1	1		1	7	8					9
51-60							2	2					2
61-70			1	1			3	3			1	1	5
71+			1	1			2	2			2	2	5
Total		2	4	6		2	18	20		0	3	3	29

Key: Or= organised, Lo= less organised, Do=disorganised. The races of three (3) fetuses were not available.

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Table 24: Relative arrangement of dermal elastin in the thigh according to age and race.

Age	**Skin colour**												
	Black				**Mixed race**				**Caucasian**				**Total**
	Or	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	**Or**	**Lo**	**Do**	**Total**	
0													
21-30	1			1									1
31-40	2			2	5			5					7
41-50	1			1	8			8					9
51-60					2			2					2
61-70	1			1	1	2		3		1		1	5
71+		1		1		2		2	1	1		2	5
Total	5	1		6	16	4	0	20	1	2		3	29

Key: Or= organised, Lo= less organised, Do=disorganised. The races of three (3) fetuses were not available.

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Generally, there were epidermal thinning and flattening of dermo-epidermal junction with increasing age of the subjects Fig. 8-80

TISSUE CULTURE OF HUMAN FIBROBLASTS

Results:

In the control group (**group 2**) fibroblast proliferation was detected at a mean of 7 days, but cell growth was inconsistent. Only 25/48 showed fibroblast proliferation at 3 weeks (50.9%). In the proliferation group, monolayers to the edge of the dish occurred at 4 weeks. A second passage was needed then to maintain proliferation and to prevent folding of the cells with cell death. On second passage, onto fresh plates, only 5% of cell growth could be affected in serum free medium.

In **group 1**, 85% of dermal explants showed fibroblast proliferation ($p < 0.05$, group 1 versus group 2) and 75% reached the edge of the plate ($p < 0.05$) showing that serum enriched medium renders better fibroblast proliferation over serum free medium.

In both groups, lamello- and filopodia could be identified, but no differences were apparent in the two groups. Figure1-7 demonstrate morphogical features of fibroblasts in tissue culture.

Immunocytochemistry **Illustrations:**

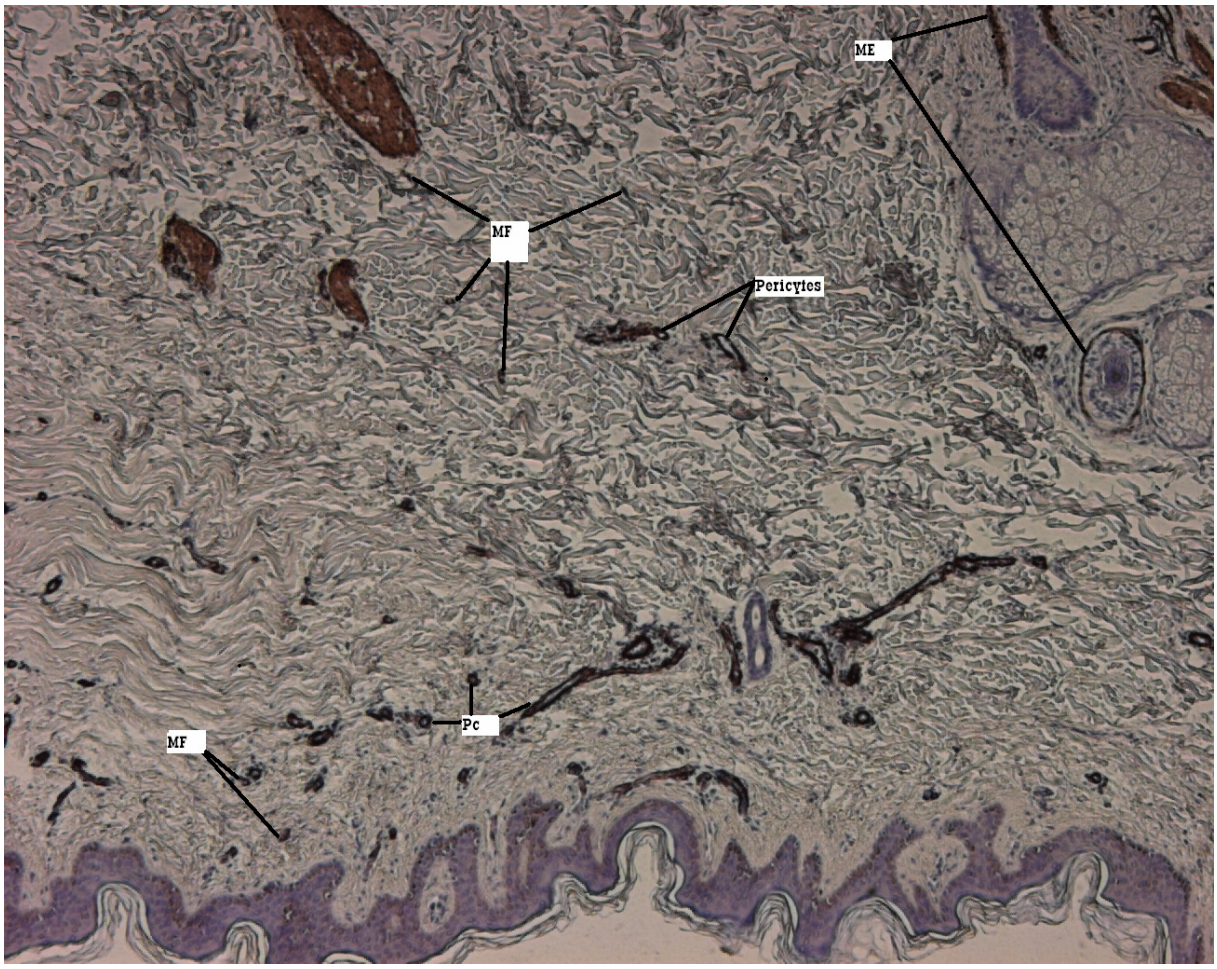

Figure 82: Human skin, α-smooth muscle actin immunostain (Avidin-biotin-complex-peroxidase) note myofibroblasts (MF), pericytes (PC) and myoepithelial cells are stained with this antibody. 50x Myofibroblasts are derived from fibroblasts under the influence of TGF-β, 50x.

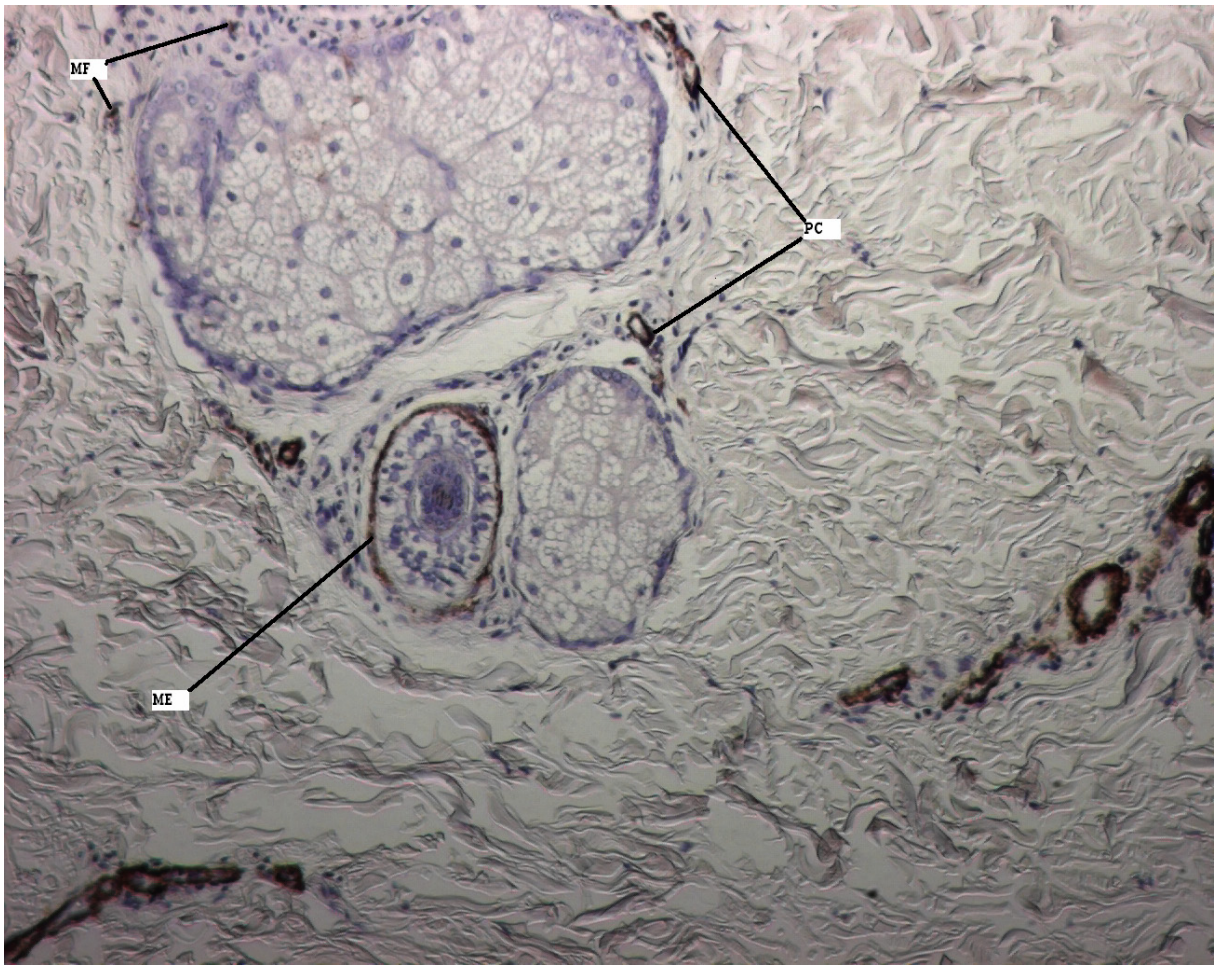

Figure 83: Human dermis, α-smooth muscle actin immunostain (Avidin-biotin-complex-peroxidase); note myofibroblasts (MF) pericytes (PC) and myoepithelial (ME) cells. Just few myofibroblasts are demonstrated due to abundance of collagen fiber bundles which correspond pale fiber bundles, 100x.

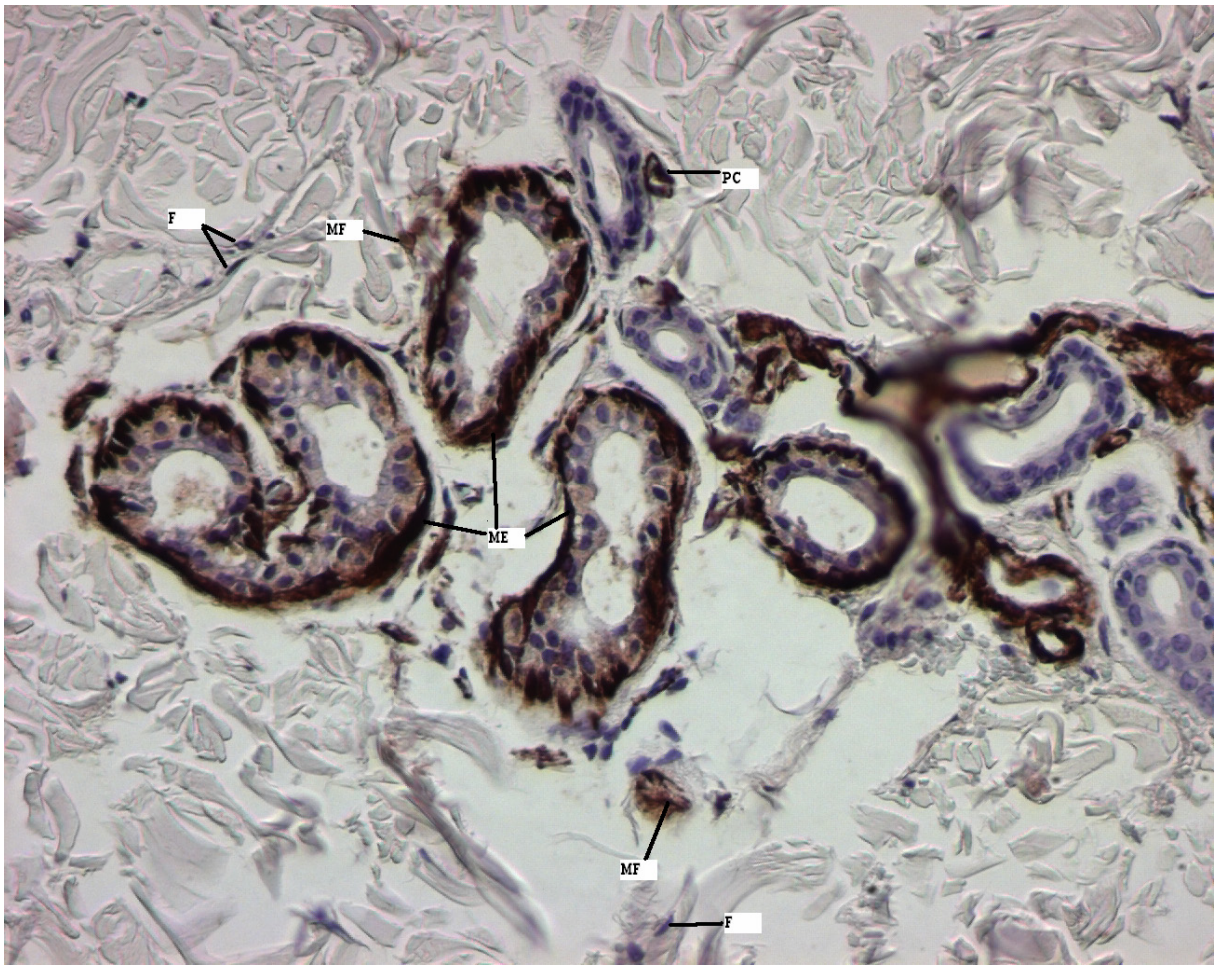

Figure 84: Human dermis, α-smooth muscle actin immunostain (Avidin-biotin-complex-peroxidase); note myofibroblasts (MF), pericytes (PC) and myoepithelial cells (ME). Fibroblasts (F) are not stained with this antibody, 200x.

CHAPTER 6: DISCUSSION

1. Light microscopy
2. Sampling
3. Fibroblast tissue culture study

DISCUSSION

Premature aging is characterised by skin wrinkles, pulmonary and cardiac fibrosis, impaired wound healing, scleroderma and other fibrotic diseases are relevant hallmarks of impaired fibroblast functions. Complicated techniques and methods have been employed to study dermal fibroblasts and their associated products in relation to physiological and premature or photo induced aging of the skin, as well as other fibrotic diseases(Harley BA et al 2007). This inspired me to carry out the study on fibroblasts in relation to photodamage of skin using simple routine histological methods and techniques as indicated on methodology part. In this study, however, there were some pitfalls as explained in the following paragraphs.

Delay in fixation:

It normally takes some days before dead bodies are fixed, this can have some influence in the outcome of my study as the autolysis and bacterial infections change the tissue far away from the living state.

Accurate quantification of collagen and elastin could not be achieved in the present, this limited statistical comparison.

Only available cadavers in dissection laboratory were used. This limits generalisation. In addition, the subject were not matched in sex and race, hence limited comparison. Regardless of the above downfall, the following were obvious.

In this study, both active, spindle euchromatic, and quiescent, spindle heterochromatic nuclei, dermal fibroblasts were found in all ages as demonstrated by Haematoxylin and Eosin and von Gieson stains. Other stains, Masson Trichrome, Verhoeff's and

silver impregnation, Foot's modification of Hortega's silver carbonate, stains did not stain fine enough to enable differentiation of fibroblasts from other cells. Verhoeff's stain demonstrated intercalated discs of cardiocytes on paraffin histological slides for light microscopy. Other stains stained cardiocytes without demonstrating their associated intercalated discs. From the finding, Verhoeff's stain could be an appropriate stain for intercalated disc demonstration on paraffin histological sections for light microscopy. These finding partly concurred with the hypothesis that fibroblasts can be demonstrated in different organs in the body using routine and special stains for light microscopy alone, (Haematoxylin and eosin, van Gieson, Verhoeff's, Masson Trichrome and silver stains).

Dermal fibroblasts were abundantly detectable in the foetus, mostly being active fibroblasts. The amount of fibroblasts decreased with age, based on the relative numbers of spindle shaped euchromatic and heterochromatic nuclei as evident under 100 and 200 times magnifications at light microscopic level. The proportional shifted to quiescent form of fibroblasts, with advancing age. This concurred with the studies carried out by Du Toit et al 2007, where by restoration of the loss of dermal fibroblasts or activation of quiescent or senescent fibroblasts was among the proposed treatment options particularly in facial skin rejuvenation.

The present study showed collagen content in the skin decreased with advancing age both in sun protected and sun exposed skin, though, more pronounced in sun-exposed skin. Such decrease was evident by the relative increase, with advancing age, of

empty space normally occupied with ground substance in living state. This is logical as the synthesizing cells, fibroblasts decrease in both, quantitatively and qualitatively, with advancing age (Doljanski F et al 2004). Collagen synthesis reduction was evident by relative increase in space occupied by ground substances. The finding agreed with study done by**,** who suggested facial rejuvenation technology and anti-aging methods by injecting of the patient's own body cultured dermal fibroblasts (youth cultivated in a test tube) to release collagen. According to Du Toit et al 2007, dysfunctional fibroblasts are associated with the aging process of the skin and wrinkle formation. Lee W et al 1997, suggested that age-related loss of collagen in connective tissues undergoing turnover may be a manifestation of a deregulated increase of collagen phagocytosis in which the net loss of degraded collagen exceeds new synthesis. Brincat et al 1987 suggested sex hormone replacement therapy to prevent skin collagen decrease in postmenopausal women. Shuster et al 1975 showed skin collagen decreased with age and was less in the females at all ages. This findings, however, contradicted the study done by Smith JG et al 1962, who found an increase in total collagen in aging skin per unit weight of skin. Further research using appropriate collagen quantification techniques and methods is recommended.

Examination with light microscopy showed the general accepted view that the superficial papillary layer of the dermis contains a fine meshwork of narrow bundles of collagen fibers, whereas the deeper reticular layer contains thicker bundles of fibers, (Abraham L 2002, Leeson L et al 1988, Pinkus H et al 1981, Lever WF et al 1983). In this study, the relative thickness of collagen bundles in reticular dermis decreased with increasing age, the extreme case occurred in eleventh decade. This is in agreement with the studies performed by Lovell CR et al 1987**,** who went further to

suggest that there may be either more type III or less type I collagen in skin of the oldest subjects. Further investigation will be required to find out which of these possibilities is correct.

It is interesting to note that the reduction of thick collagen bundles in reticular fibers was severe, particularly in sun exposed skin, that the collagen fiber thickness in reticular dermis was almost similar to that of papillary layer of dermis in the oldest subject examined at 11^{th} decade. To the contrary, some moderately thick collagen bundles were still evident in reticular dermis of the sun-protected skin of 11^{th} decade subject examined. This suggests the damaging role of ultraviolet irradiation. The preferential reduction in number and diameter of thick collagen bundles with age could be attributed to decrease in load and tensile strength reported in aging human skin, (Vogel HG et al 1983). Mohammad AB et al 2004, found that dermal fibroblasts from different layers of human skin are heterogeneous in expression of collagenase and type I and III procollagen mRNA. Assuming Mohammad AB et al 2004 were correct, it means, some dermal fibroblasts are preferentially expressing type I procollagen and other preferentially expressing type II procollagen in different layers or micro-locality of the skin. This also will imply that solar damage targets more fibroblasts that preferentially express type I procollagen, resulting in reduction of thick type I collagen synthesis as observed by decrease of thick collagen bundles in reticular layer of dermis in sun-exposed skin with advancing age. This, however, remains to be elucidated.

The striking finding was the great discrepancy in the organization of collagen fiber architecture between sun-protected and exposed skin. Sun protected skin, upper third medial aspect of thigh, maintained and had its fibers organized in layers throughout all age groups. To the contrary, sun exposed skin showed disorganized collagen fibers as early as 3rd decade, the situation worsened with advancing age. This refuted the hypothesis that there is questionable difference in collagen morphology of the skin regardless of; age, sex, site and race of an individual. The findings however agreed with a study done by Du Toit et al 2005. Further studies are needed to know whether this disorganization in collagen bundles has any benefits for survival in sun-exposed skin. In addition, Du Toit et al 2005, also found aging of skin to start at about twenty five years and became visible at 28 years of age. It however showed earlier commencement when compared to that done by El-Domyati M et al 2002, who found disorganization of collagen architecture to begin in the 4th decade.

The hypothesis that elastic fiber content of skin shows degradations in sun-exposed versus sun-protected skin of an individual was not proved in the present study. The relative elastic fibers detection by Verhoeff's stain under 100 and 200 times magnifications at light microscopic level, diminished with advancing age in sun protected skin and vice versa in sun-exposed skin. The increased detection of elastic fibers in sun-exposed skin alone is inadequate to substantiate that they represent degraded fibers. Biochemical study techniques are needed to prove that. This finding, however, agrees to that done by El-Domyati M et al 2002, who showed gradual increase in staining intensity of elastin in sun-exposed skin with age and decrease in staining intensity of elastic fiber with advancing age in sun-protected skin. The accumulated elastin were disorganised and appeared to occupy spaces left by

collagen. This finding was also noted by Bernstein EF et al 1996, who suggested up-regulation of elastin promoter activity in dermal fibroblasts was responsible for the excess elastic tissue but not the reason for the aberrant morphology of the elastotic material. The abnormal elastic fibers accumulation in sun-exposed skin has also been document by various researchers, including Seite S et al 2006, who found that lysozyme prevented elastin degradation by normal degradation mechanisms of human leukocyte elastase (HLE) after binding to the damaged parts of the elastin network and by direct lysozyme-HLE interaction, which reduces HLE proteolysis.

More over, thinning of the epidermis and flattening of the dermal-epidermal junction with increasing age was noted in sun exposed skin. The most probable reason is due to disappearance of dermal papillae and epidermal buds seen in advanced age skin (Contet-Audonneau LJ et al 1999). This finding contradicts that reported by Domyati et al 2002, who found increase in epidermal thickening in sun-exposed skin.

The fibroblast is a ubiquitous cell in the human body, (Abraham L 2002, Leeson L et al 1988). They are found in all organs of the body for as they elaborate and maintain the integrity of the connective tissue ECM . In lung, fibroblasts are found in the alveolar septum. Usually are few in numbers, only proliferate and transformed to myofibroblasts whenever there is irreversible injury to the alveolar cells. In myofibroblastic state, fibroblasts replace lung parenchyma by fibrolous tissue (Abraham L 2002, Gu L et al 2004). In the present study, it was witnessed replacement of some alveolar spaces (lung parenchyma) by fibrous tissue. Fibrous tissue replacement of lung parenchyma seriously interfere with lung function as the replacing fibrous tissue is not adapted for the gaseous exchange. Fibroblast-

myofibroblast differentiation is induced by TGF-beta1 in a Smad proteins-dependent manner (Gu L et al 2004). Fibroblasts secrete connective tissue matrix septa in lung parenchyma, (Pakurar AS et al 2004). Compliancy of the lung is enhanced by longitudinal elastic fibers in the walls of terminal and respiratory bronchioles and alveolar. Elastic fibers were not stained in area where fibrous tissues replaced lung parenchyma in the present study. The loss of elasticity and breakdown of elastic fibers gives rise to emphysema, characterised by chronic airway obstruction, (Abraham L 2002).

Some fibroblasts were observed in the heart. They were found in the cardiocytes interstitial connective tissue septa. Among its function, is to secrete connective tissue matrix which support the organs by filling all empty spaces, and fix blood and lymphatic vessels and nerves in place, (Abraham L 2002). They have a key role in wound healing by secreting the necessary matrix and chemical mediators.

Fibroblasts in culture rapidly develop the ability to attach to the wall of the container and move across its surface. Such adherence and mobility are attributed to the development of a system of microfilaments called stress fibers and was also reported by Buckley IK et al 1967. These may measure up to 2µm in diameter and may branch or radiate from focal points (Goldman RD 1975). Microfilament constituting stress fibers are mainly composed of actin, including α-smooth muscle actin, as shown by immunofluorescence and imunoelectron microscopy with specific antibodies (Goldman RD et al 1975, Willingham MC et al 1981, Skalli O et al 1986). In immunostaining for light microscopic study, these detailed facts could not be

depicted, however, culture fibroblasts showed interesting cytoplasmic extensions which could be suggestive of stress fibers. Previous studies have shown that stress fibers also contain actin-associated proteins such as myosin, tropomyosin, alpha-actinin, and filamin (Skalli O et al 1988). In addition to stress fibers, cultured fibroblasts develop gap junctions (Bellows CG et al 1981), analogous to that observed between myofibroblasts in vivo (Gabbiani G et al 1978). In primary passage fibroblastic populations, the presence of α-smooth muscle actin has been reported (Skalli O et al 1986, Vandekerckhove J et al 1978, Leavitt J et al 1985). The expression of α-smooth muscle actin in cultured fibroblasts represent the feature of fibroblastic cultures themselves which may be related to functions exerted by fibroblasts under particular environmental conditions in vivo.

Cytoskeletal proteins such as desmin and smooth muscle myosin heavy chains are also variably expressed by cultured fibroblasts derived from different organs or pathologic tissues. The expression, however, is generally very low, and in several populations, as the case for this study, these cytoskeletal proteins are not expressed (Dsmouliere A et al 1992).

The forces generated by cultured fibroblasts are traction, rather than contraction forces, as shown by experiments in which fibroblasts distorted a sheet of silicon on which they were grown (Harris AK et al 1981). Several observations suggest that stress fibers are probably the force-generating elements in wound contraction, since they contract upon addition of adenosine triphosphate on glycerinated fibroblasts (Hoffmann-Beerling H 1954, Isenberg G et al 1976, Burridge K 1981), and

microinjection experiments revealed that they are functionally analogous to skeletal musle fibers (Kreis FE et al 1980, Burridge K 1981).

In floating collagen of fibrin matrices, fibroblasts developed stellate morphology with long processes and cytoskeletal meshwork. In contrast, fibroblast cells in anchored collagen matrices become bipolar, and orient along lines of tension. These cells then develop prominent stress fibers and cell-to-stroma attachment sites (fibronexus), and resemble myofibroblasts, This indicate that fibroblasts are able to contract a tissue matrix in vitro without differentiating into myofibroblasts, and that the appearance of myofibroblasts correlates with the appearance of stress within the matrix. Fibroblasts in floating versus anchored collagen matrices also show differences in cell proliferation and DNA synthesis. After contraction of floating collagen matrices there is a marked decline in cellular DNA synthesis; the cells become arrested, and cell regression begins. In contrast, fibroblastic cells in anchored matrices continue to proliferate and to synthesize DNA. Furthermore, and taking into account that the tension forces developed in a restrained collagen lattice by myofibroblasts obtained from human skin granulation tissue explants are more intense than those exerted by human dermis fibroblasts . These were also demonstrated by Germiain L et al 1994.

CHAPTER 7: CONCLUSION

- Light microscopy
- Fibroblast tissue culture study

In conclusion, fibroblasts are ubiquitous cells that are distributed widely in the human body. In the present study it was possible to demonstrate fibroblasts in the skin, heart and lung by Haematoxylin and eosin and Van Gieson staining techniques at light microscopic level. Masson Trichrome, Verhoeff's and silver stain could not clearly stain fibroblasts. Masson Trichrome was superior in demonstrating the collagen in shin, heart and lung. Fibroblasts are key cells for the integrity of the extracellular matrix and its enormous role in human body. According to Abraham L, 2002, some of ECM roles includes to serve as; (1) the reservoir of hormones controlling the growth and differentiation, (2) the media through which nutrients and metabolic wastes are exchanged between cells and their blood supply, (3) filling media to the spaces left after injury to tissue whose cells do not divide (cardiac muscles), which forms a scar as shown in the present study where fibrous tissues replaced lung parenchyma , and (4) fibrin, collagen, and fibronectin in the ECM signal cells to divide and migrate like fibroblasts and migrating keratinocytes which use the fibronectin cross-linked with fibrin as an attachment site to crawl across during wound healing. The present study demonstrated variation of dermal collagen morphology with age, and site of the body in the reticular layer of dermis. Generally the thickness of reticular collagen bundles increased with foetal from foetal to mid adulthood, and then start to decrease dermal collagen were of medium thicknesses where as adult collagen fiber were more thicker. The importance of further studies on fibroblasts can therefore not be over emphasized.

In this fibroblast culture study, more consistent fibroblast proliferation was observed in foetal calf enriched mediums.

Remarks: Because of the characteristics of “cell senescence”, that is typical of cultured fibroblasts, a cell proliferation rate of 75% is considered good (Boss et al 2000, Cristafolo et al 1998 and Matsua et al 2004). Some studies have shown that it is possible to culture fibroblasts in serum free media. This was possible in this study but the results were clearly inferior to those observed in serum enriched media. The clinical implications of this study are important, because if fibroblast cell therapy is to be considered for human use, serum free media will be needed. Serum rich media carry the possibility of transmission of zoonotic diseases to man (Mad Cow Diesease) and therefore it would be preferable to use serum free culture media (Boss et al 2000).

CHAPTER 8:

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APPENDIX 1: T-TEST: TWO-SAMPLE ASSUMING EQUAL VARIANCES

	facial fibroblast	*Thigh fibroblasts*
Mean	**2.171428571**	**2.794117647**
Variance	0.793277311	0.471479501
Observations	35	34
Pooled Variance	0.634779882	
Hypothesized Mean Difference	0	
df	67	
t Stat	-3.245701713	
P(T<=t) one-tail	0.000915218	
t Critical one-tail	1.667915512	
P(T<=t) two-tail	**0.001830437**	
t Critical two-tail	1.996008905	

Reject the null hypothesis because p value <0.05

B=sun exposed A= sun protected

Appendix 1: t-Test: Two-Sample Assuming Equal Variances

	facial collagen	*Thigh collagen*
Mean	**2.171428571**	**2.794117647**
Variance	0.793277311	0.471479501
Observations	35	34
Pooled Variance	0.634779882	
Hypothesized Mean Difference	0	
df	67	
t Stat	-3.245701713	
P(T<=t) one-tail	0.000915218	
t Critical one-tail	1.667915512	
P(T<=t) two-tail	**0.001830437**	
t Critical two-tail	1.996008905	

Reject the null hypothesis because p value <0.05

B=sun exposed C= sun protected

APPENDIX 2: STAINING PROCEDURES.

Haematoxylin and Eosin (H &E): Histological Technique

After Harris 1990, Mallory 1938

- De-wax sections, Xylene for 5 minutes.
- Rinse in serial changes of alcohols, 100 – 70%.
- Rinse in tap water.
- Stain with Harris Haematoxylin -10 minutes.
- Wash and blue, in running tap water- 1minute.
- Differentiate in acid alcohol (1% hydrochloric acid in 70% alcohol) -10 seconds.
- Wash and blue, in running tap water -5 minutes.
- Stain with Eosin -4 minutes.
- Wash in tap water.
- Dehydrate, clear and mount.
- Examine on the light microscope at low and high magnifications (x 100, x 40).

Masson Trichrome Staining

1. Slide were deparaffinised and sections rehydrated :

   3 x 3’ xylene (blot excess xylene before going into ethanol),

   3 x 3’ 100% ethanol,

   1 x 3’ 95% ethanol,

1 x 3’ 80% ethanol,

1 x 5’ De-ionised H2O,

2. Mordant in Bouin’s solution at room temperature overnight in a hood. Be careful, Bouin’s solution is hazardous and the picric acid, when in less than 10% water, is very explosive. Used Bouin’s solution should be placed in an appropriate waste container.

   **Bouin’s solution intensifies the final coloration of the tissue.

3. Slides were washed in running tap water to remove yellow colour from sections.

   Then, rinsed briefly in distilled water.

4. Stained in Working Weigert’s iron haematoxylin solution for 5 minutes. Haematoxylin solution was freshly made by adding equal volumes of Solution A (1% haematoxylin in 95% ETOH) and Solution B (1.2% ferric chloride and 1% acetic acid in distilled water). The working solution was satisfactory for approximately 10 days.

   **Haematoxylin stains nuclei blue-black.

5. Washed in running tap water for 5 minutes. Rinsed in deionised water.

6. Stained in Biebrich scarlet-acid fuchsin for 5 minutes,

   Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded.

   **Biebrich scarlet-acid fuchsin stains cytoplasm and muscle red.

7. Rinse in deionised/ distilled water.

8. Place the slides in phosphomolybdic/phosphotungstic acid solution for 5-10 minutes. Freshly prepare working phosphotungstic/phosphomolybdic acid solution by mixing 1 volume of phosphotungstic acid solution and 1 volume of phosphomolybdic acid solution with 2 volumes of distilled water.

   Discard after one use. Formation of precipitate in phosphomolybdic acid solution does not affect performance.

   **This allows for uptake of the aniline blue stain.

9. Stain sections in aniline Blue solution for 5 minutes.

   **Aniline blue stains collagen blue.

10. Rinse slides briefly in distilled water.

11. Place slides in 1% acetic acid solution for 3-5 minutes. Discard the solution.

    **With rinsing in acetic acid after staining, the shades of colour become more delicate and transparent.

    **If blue staining of connective tissues faded, the section has probably been over differentiated in acetic acid solution.

12. Dehydrate to xylene,

    2 x 3’ 95% ethanol,

    2 x 3’ 100% ethanol (blot excess ethanol before going into xylene),

    3 x 5’ xylene.

13. Leave slides in xylene overnight to get good clearing of the ethanol.

14. Cover slip slides using Permount or Polymount (xylene based).

- Place a drop of Permount on the slide using the glass rod, taking care to leave no bubbles. (Don't stir the Permount with the rod too much and make sure that Xylene still covers the slide).
- Angle the cover slip and let fall gently onto the slide. Allow the Permount to spread beneath the cover slip, covering the tissue.
- Dry overnight in the hood or at 37°C.

RESULTS:

Nuclei stain black,

Cytoplasm, muscle, erythrocytes stain red,

Collagen stain blue.

NOTES

1. Light green may be substituted for Aniline blue.
2. 5% phosphotungstic acid for 5 minutes, must be substituted when using light green.
3. When staining liver biopsies, the collagen enhancement is better light blue, than dark blue.

Van Gieson Staining

METHOD

1 Bring sections to distilled water.

2 Stain nuclei with Celestin Blue.....3 mins

3 Rinse in distilled water.

4 Stain in haematoxylin……...3 mins

5 Wash well in tap water..... ……..3 mins

6 Flood with Curtis stain .....….....3 mins

7 Blot.

8 Dehydrate rapidly in alcohols, clear and mount.

RESULTS

Nuclei .....Blue

Collagen .....Bright red

Cytoplasm, muscle, fibrin and red blood cells ….....Yellow

Foot's Modification Of Hortega's Silver Carbonate Method For Reticulum.

Staining Procedure

1. Deparaffinise sections through series of xylene and dehydrate,
2. Wash thoroughly in tap water,
3. Oxidize in 0.25% solution of potassium permanganate for 5 minutes,
4. Rinse in tap water,
5. Bleach in 5% solution of oxalic acid for 10 minutes,
6. Wash well in tap water, followed by distilled water,
7. Place in warm silver ammonium carbonate solution in the incubator or hot water bath at 37°C to 43°C. for 10 to 30 minutes. Tissues will show a yellow

to brown coloration when impregnated. Remove from water bath and allow cooling briefly.

8. Rinse in distilled water,
9. Reduce in 20% solution of neutral formalin for 5 minutes. Tissue will become amber in color.
10. Wash well in tap water,
11. Tone in 0.2% gold chloride solution for 5minutes. Section will be grayed in this solution.
12. Wash well in tap water.
13. Fix in a 5% aqueous solution of sodium thiosulphate for 2 minutes. The thiosulphate will remove all unreduced silver.
14. Wash well in tap water.
15. Counterstain if desired with alum hematoxylin and van Gieson's stain for nuclei and collagen. The counterstain is usually unnecessary in well-stained slide, for the coarser connective tissue will be brownish-pink and well differentiated from the fine black reticulum
16. Dehydrate in two changes of absolute alcohol
17. Clear in two or three changes of xylene and mount.

RESULTS.

Coarse connective tissue fibers- brown-pink,

Reticulum-black to dark violet,

Nuclei- black.

Verhoeff's Method

Method

1. Dewax sections and bring to water.
2. Cover with staining solution 15 to 30 minutes.
3. Rinse in water.
4. Differentiate in 2% aqueous ferric chloride until elastic tissue fibers appear black on a grey background
5. Rince in water.
6. Rinse in 95% alcohol to remove any staining due to iodine alone.
7. Counterstain as desirable (van Gieson is conventional, although eosin may be used).
8. Blot to remove excess stain.
9. Dehydrate rapidly through alcohols.
10. Clear in xylene and mount in DPX.

RESULTS

Elastic tissue fibers- black,

Other tissues according to counterstain.

APPENDIX 3: AUTOMATED IMMUNOCYTOCHEMICAL PROCESSING SCHEDULE.

PM serial N°: M210857

Processing Module: BOND 1

Tray: 3

Dispense volume: 150 µL

Run events: Batch 216 Start time: 10/30/2007 3:35 PM

Batch progress: Finished

Time	Event N°	Description	Parameter	Value	Parameter ID
10/30/2007					
3:34:40 PM	1001	Slide Staining Assembly locked			
3:35:41 PM	1027	Batch accepted			
3:35:41 PM	1028	Batch start pressed	Slide ID	01SQ	2007
Slide ID	01SN	2007			
Slide ID	01SL	2007			
Slide ID	01SJ	2007			
Slide ID	01SH	2007			
Slide ID	01SF	2007			
Slide ID	01SB	2007			
Slide ID	01S3	2007			
Slide ID	01SD	2007			
Slide ID	01S1	2007			
By user:	BondPowerUser	2045			
Requested batch start time:	10/31/2007 03:30	2093			
10/31/2007					
3:46:34 AM	1032	Reagent dispensed	Reagent name:*No Reagent		2006

150 µL dispense position 2506

All slides 2030

4:18:44 AM 1032 Reagent dispensed Reagent name:*Bond Dewax Solution 2006

150 µL dispense position 2506

All slides 2030

4:20:02 AM 1032 Reagent dispensed Reagent name:*Bond Dewax Solution 2006

150 µL dispense position 2506

All slides 2030

4:20:47 AM 1032 Reagent dispensed Reagent name:*Bond Dewax Solution 2006

150 µL dispense position 2506

All slides 2030

4:22:01 AM 1032 Reagent dispensed Reagent name:*Alcohol 2006

150 µL dispense position 2506

All slides 2030

4:22:47 AM 1032 Reagent dispensed Reagent name:*Alcohol 2006

150 µL dispense position 2506

All slides 2030

4:23:37 AM 1032 Reagent dispensed Reagent name:*Alcohol 2006

Intermediate position 150 µL 2503

All slides 2030

4:25:07 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

4:25:51 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

4:26:40 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

Intermediate position 150 µL 2503

All slides 2030

4:33:34 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

Initial fill 2502

All slides 2030

4:44:00 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

4:46:42 AM 1032 Reagent dispensed Reagent name:SMA 2006

Reagent UPI: 00567618 2025

150 µL dispense position 2506

All slides 2030

5:02:58 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:03:36 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:04:21 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:14:26 AM 1032 Reagent dispensed Reagent name:*Peroxide Block 2006

Detection system UPI: 01038728 2026

150 µL dispense position 2506

All slides 2030

5:20:01 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:22:38 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

Open fill 150 µL 2501

All slides 2030

5:24:05 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:26:46 AM 1032 Reagent dispensed Reagent name:*Post Primary 2006

Detection system UPI: 01038728 2026

150 µL dispense position 2506

All slides 2030

5:35:20 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:37:56 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:40:42 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:44:38 AM 1032 Reagent dispensed Reagent name:*Polymer 2006

Detection system UPI: 01038728 2026

150 µL dispense position 2506

All slides 2030

5:53:12 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:55:49 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

5:59:17 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:02:39 AM 1032 Reagent dispensed Reagent name:*Mixed DAB Refine 2006

150 µL dispense position 2506

All slides 2030

6:04:22 AM 1032 Reagent dispensed Reagent name:*Mixed DAB Refine 2006

150 µL dispense position 2506

All slides 2030

6:15:41 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:16:18 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:17:02 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:18:56 AM 1032 Reagent dispensed Reagent name:*Bond DAB Enhancer 2006

Reagent UPI: 01023831 2025

150 µL dispense position 2506

All slides 2030

6:24:31 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:26:53 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:30:55 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:35:20 AM 1032 Reagent dispensed Reagent name:*Hematoxylin 2006

Detection system UPI: 01038728 2026

150 µL dispense position 2506

All slides 2030

6:40:55 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:46:12 AM 1032 Reagent dispensed Reagent name:*Bond Wash Solution 2006

150 µL dispense position 2506

All slides 2030

6:47:31 AM 1032 Reagent dispensed Reagent name:*Deionized Water 2006

150 µL dispense position 2506

All slides 2030

6:47:32 AM 1003 Batch completed successfully

6:47:51 AM 1002 Slide Staining Assembly unlocked

11/6/2007 11:35 AM 1/1

APPENDIX 4:

Immunocytochemistry:

Recommendations for Use for Primary Antibody

Monoclonal Mouse Anti-Human Smooth muscle actin

Clone 1A4

Code No./ Code/ Code-Nr. M0851

Indication:

For in vitro diagnositic use.

Monoclonal Mouse Anti-Human Smooth Muscle Actin, Clone 1A4, was used for immunocytochemistry. The antibody labels smooth muscle cells, myofibroblasts and myoepithelial cells, and is a useful tool for the identification of leiomyomas, leiomyosarcomas and pleomorphic adenomas. Differential identification was aided by the results from a panel of antibodies. Interpretation must be made within the context of patient's clinical history and other diagnostic tests by a qualified pathologist.

Introduction:

Cytoplasmic actins, which belong to the microfilament system of cytoskeleton proteins , are the most conserved eukarytic proteins being expressed in mammals and birds. The actin protein consists of six isoforms, varying in their amino acid sequence, but all having the same molecular mass of 42kDa. The isoforms show more than 90% overall sequence homology, but only 50-60% in their 18 N-terminal region appears to be a major antigenic region. There are different: α, β- and ɣ-actins, respectively. The β- and ɣ-actins may be present in muscle cells as well as most other cell types in the body, including non-muscle cells.

Reagent provided:

Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 0.005 mol/L. Tris/HCl, pH 7.2, and containing 15mmol/L NaN3.

Clone: 1A4. The 1A4 clone is identical to the anti-asm-1. Isotype: IgG2a, kappa.

Mouse IgG concentration: see label on vial.

Immunogen:

N-terminal synthetic decapeptide of α-smooth muscle actin coupled to keyhole limpet haemocyanin (KLH).

Specificity:

In Western blotting and SDS-PAGE immunoblotting of α-smooth muscle isoform of actin, the antibody labels a band corresponding to α-smooth muscle actin.

As demonstrated by Western blotting and /or Immunocytochemistry, the antibody cross-reacts with the α-smooth muscle actin equivalent protein in chicken, cow and rat.

Precautions:

1. For professional users.
2. This product contains Sodium azide (NaN3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.

3. As with any product derived from biological sources, proper handling procedures should be used.
4. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
5. Unused solution should be disposed of according to local, State and Federal regulations.

Storage:

Store at 2-8°C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the user must verify the conditions. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact DakoCytomation Technical Services.

Specimen preparation:

Paraffin sections: The antibody can be used for labelling paraffin-embedded tissue sections fixed in formalin. Pre-treatment of tissues with heat-induced epitope retrieval is recommended. Optimal results are obtained with 10mmol/L. Tris buffer, 1 mmol/L EDTA, pH 9.0. Less optimal results are obtained with DakoCytomation Target Retrieval Solution, High pH, code No. S 3308, or 10mmol/L citrate buffer, pH 6.0. However, DakoCytomation Target Retrieval Solution, code No. S1700 was found inefficient. Pre-treatment of tissues with proteinase K was found destructive of

epitope. The tissue sections should not dry out during the treatment or during the following immunocytochemical staining procedure.

Frozen sections and cell preparations: The antibody can be used for labelling acetone-fixed, frozen sections.

Staining procedure:

Dilution: Monoclonal Mouse Anti-Human Smooth Muscle Actin, code No. M0851, may be used at a dilution range of 1:50-1:100 when applied on formalin-fixed, paraffin-embedded sections of normal human colon using 20 minutes heat-induced epitope retrieval in 10mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0, and 30 minutes incubation at room temperature with the primary antibody. Optimal conditions may vary depending on specimen and preparation method, and should be determind by each individual laboratory. The recommended negative control is DakoCytomation Mouse IgG2a, code No. X0943, diluted to the same mouse IgG concentration as the primary antibody. Unless the stability of the dilute antibody and negative control has been established in the actual staining procedure, it is recommended to dilute these reagents immediately before use, or dilute in Dakocytomation Antibody Diluent, code No. S0809. Positive and negative controls should be run simultaneously with patient specimen.

Visualization: LSAB™ +/HRP kit, code No. K0679, and EnVision™ +/HRP kits, code Nos. K4004 and K4006, are recommended. For frozen sections and cell preparations, the DakoCytomation APAAP kit, code No. K0670, is a good alternative if endogenous peroxidase staining is a concern. Follow the procedure enclosed with the selected visualisation kit.

Automation: The antibody is well suited for immunocytochemical staining using automated platforms, such as the DakoCytomation Autostainer.

Performance characteristics:

Cells labelled by the antibody display a cytoplasmic staining pattern.

Normal tissues: The antibody labels smooth muscle cells in blood vessels and, additionally, salivary ducts and myoepithelial cells around acini in salivary glands. Smooth muscle cells in 35/36 normal uterine myometria were also positively labelled. Further, a temporal labelling of perisinusoidal liver cells has been observed. In frozen tissues, the antibody labels myofibroblasts and myoepithelial cells around acini and ducts of the breast, whereas epithelia (adeno, squamous), lymphocytes, cardiac and skeletal muscle cells, endothelial cells, fat cells, Schwann cells and fibroblast are negative.

Abnormal tissues: The antibody labelled 24/26 leiomyomas, 6/7 atypical leiomyomas and 21/25 leiomyosarcomas of the uterus, as well as 13/13 extrauterine nongastrointestinal spindled leiomyosarcomas. Moreover, the antibody labelled a variable amount of cells in 8/8 pseudosarcomatous myofibroblastic tumours of the urinary bladder in children. In pleomorphic adenomas, the antibody labelled tumour epithelial cells (myoepithelial cells) in 19/20 cases. In frozen tissues, the antibody, in addition to the labelling of 5/5 leiomyomas and 6/7 leiomyosarcomas, also labelled 4/22 malignant fibrous histocytomas and ½ rhabdomyosarcomas. 6/6 malignant schwannomas were negative, as also 13/13 other soft tissue tumours, including 1 fibrosarcomas, 6 liposarcomas, 1 angio-sarcoma, 1 capillary haemangioma, 1 Triton tumour, and 3 synovial sarcomas.

Procedures:

A. Reagents required but not supplied

1. Standard solvents used in immunohistochemistry.
2. 50 mM tris-buffered saline (TBS) pH.6.
3. Antigen retrieval solution(s)
4. Enzyme retrieval solution(s)
5. Antibody diluent.
6. Primary antibody.
7. Mounting medium.

B. Equipment required but not supplied.

1. Equipment required for antigen retrieval, if recommended for the primary antibody.
2. General immunohistochemistry laboratory equipment.

C. Methodology

Prior to undertaking this methodology, users must be trained in immunohistochemical techniques.

The combination of primary antibody, its dilution, together with the detection system should be validated by the user on a series of known positive and negative controls.

Unless indicated, all steps are performed at room temperature (25□).

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DAB Working Solution:

Add 50µl of DAB Chromogen to 1ml of Novolink™ DAB Substrate Buffer (polymer). Use within six hours of preparation.

Quality control:

Differences in tissue processing and technical procedures in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the following procedures. Controls were fresh surgical specimens formalin-fixed, processed and paraffin wax-embedded as soon as possible in the same manner as any normal non cadaver sample(s).

Positive Control:

Used to indicate correctly prepared tissues and proper staining techniques. One positive tissue control should be included for each set of test conditions/primary antibody in each staining run. A tissue with weak positive staining is more suitable than with strong positive staining for optimal quality control and to detect minor levels of reagent degradation. For recommended positive control tissue see primary antibody instructions for Use. If the positive tissue control fails to demonstrate positive staining, results with the test specimens should be considered invalid.

Negative Tissue Control

Should be examined after the positive tissue control to verify the specificity of the labelling of the target antigen by the primary antibody. For recommended negative control tissues see primary antibody instructions for use. Alternatively, the variety

of different cell types present in most tissue sections frequently offers negative control sites, this should be verified by the user. Non-specific staining, if present, usually has a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Use intact cells for the interpretation of staining results. Necrotic or degenerated cells often stain non-specifically. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by endogenous enzymes such as pseudoperoxidase (erythrocytes), endogenous peroxidase (cytochrome C), or endogenous biotin (eg. Liver, breast, brain, kidney). To differentiate endogenous enzyme activity or non-specific binding of enzymes from specific immunoreactivity, additional patient tissues may be stained exclusively with substrate-chromogen. Streptavidin-HRP or labelled polymer, and substrate-chromogen, respectively. If specific staining occurs in negative tissue control, results with the patient specimens should be considered invalid.

Negative Reagent Control

Use a non-specific negative reagent control in place of the primary antibody with a section of each patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site.

Patient Tissue:

Examine patient specimens last. Positive-staining intensity should be assessed within the context of any non-specific background staining of the negative reagent control. As with any immunohistochemical test a negative result means that the

antigen was not detected, not the antigen was absent in the cells/tissue assayed, If necessary, use a panel of antibodies to identify false-negative reactions.

Limitations:

Immunohistochemistry is multistep diagnostic process that consists of specialised training in section of the appropriate reagents; tissue selection, fixation, and processing; preparation of IHC slide; and interpretation of the staining results.

Tissue staining is dependent on the handling and processing of the tissue prior to staining.

Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues of fluids may produce artfacts, antibody trapping, or false negative results. Inconsistent results may be due to the variations in fixation and embedding methods, or to inherent irregularities within the tissue.

Excessive or incomplete counterstaining may compromise proper interpretation of results.

The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

NovoLink™ Polymer Detection Systems and their components are for use on paraffin-embedded sections with specific fixation requirements. Unexpected antigen expression may occur, especially in neoplasms. The clinical interpretation

of any stained tissue section must include morphological analysis and the evaluation of appropriate controls.