THE SECRET OF DYING WELL: THE MANY FACES OF CELL DEATH AND ITS RELEVANCE FOR TREATING DISEASES OF OUR TIME

Prof Anna-Mart Engelbrecht

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THE SECRET OF DYING WELL: THE MANY FACES OF CELL DEATH AND ITS RELEVANCE FOR TREATING DISEASES OF OUR TIME

The dynamic nature of life becomes more and more apparent with every advance in scientific understanding. It is said that the beauty of science lies in the simplicity of its purpose: the search for truth. As Keats so aptly put it in his *Ode on a Grecian urn*: 'Beauty is truth, truth beauty' – that is all ye know on earth, and all ye need to know".

The French physiologist Claude Bernard (1813–1878), regarded as the father of modern physiology, observed that the *milieu interieur* (internal environment) of the body remains remarkably constant despite changing conditions in the external environment. In his book, entitled *The wisdom of the body,* and published in 1932, Walter Cannon was the first to coin the term 'homeostasis' to describe this constant internal environment. He suggested that many mechanisms of physiological regulation have but one purpose – to maintain this internal constancy in the human body. Cells, the fundamental building blocks of the human body, maintain the existence of life as thousands of intricate pathways and masses of information are rapidly processed in these 4–135 μm units to maintain homeostasis.[1] The body consists of trillions of cells – about 100 000 cells are produced every second. To counterbalance this proliferation, a similar number die every second.[2] This homeostasis is tightly controlled by programmed cell death, which is a ubiquitous process essential for cellular turnover. When this system is disrupted, even slightly, various disease states, including heart disease and cancer, can ensue.

PROGRAMMED CELL DEATH

Morphological investigations of cell death quickly revealed that there is more than one way to die. Besides apoptosis and necrosis, autophagy has emerged within the field of cell death, each exhibiting distinct morphological characteristics.

Apoptosis (type I cell death) is characterised by distinct morphological changes in the cell. The physical process begins with the detachment and shrinkage of the apoptotic-competent cell.[3] This is followed by nuclear chromatin condensation (pyknosis) and its degeneration into internucleosomal fragments (karyorrhexis) by endonucleases to produce the classic DNA-laddering pattern. Subsequently, permeabilisation and blebbing of the plasma membrane gives rise to discrete apoptotic bodies. A membrane phospholipid termed phosphatidylserine (PS), typically contained within the inner plasma membrane, becomes exposed to the outer membrane surface during the morphological transformation.[4] The process culminates in the recognition of these apoptotic-competent cells by phagocytes which proceed to engulf the apoptotic bodies in a rapid, yet irreversible manner.[5] The externalised PS lipids aid in the recognition of the apoptotic bodies and their subsequent phagocytosis.[6] Clearance occurs without invoking an inflammatory response.[7]

While apoptosis is characterised by an active participation of the affected cell in its own demise, necrosis, on the other hand, is a passive, catabolic and degenerative process (see Figure 1). Necrosis generally represents a cell's response to gross injury and can be induced, for example by exposure to an overdose of cytotoxic stimuli. The early events of necrosis are mitochondrial swelling followed by rupture of the plasma membrane and release of cytoplasmic constituents, which include proteolytic enzymes.[8,9] Nuclear chromatin shows patchy areas of condensation and the nucleus undergoes slow dissolution (karyolysis). Necrosis triggers an inflammatory reaction in the tissue and often results in scar formation.

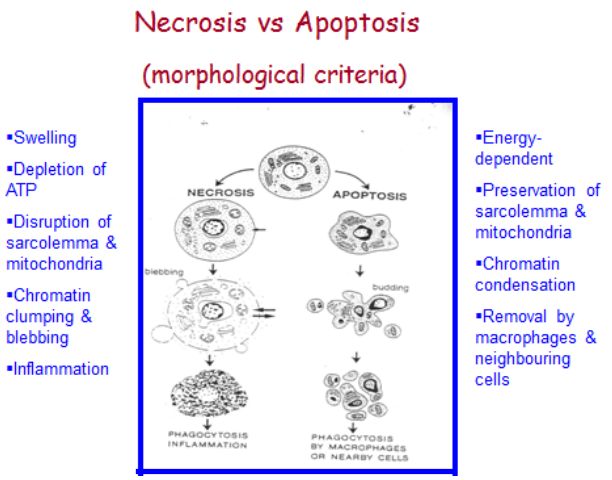

Figure 1: Morphological characteristics of a necrotic and an apoptotic cell

The pattern of cell death may not always have the classical features of either apoptosis or necrosis. Numerous examples of cell death have been described in which the morphological and/or biochemical changes resembled neither typical apoptosis nor necrosis, but often had features of both.[10,11] In some cases, the integrity of the plasma membrane was preserved but DNA degradation was random, without evidence of internucleosomal cleavage. In other situations, DNA degradation was typical of apoptosis but nuclear fragmentation and other features of apoptosis were not apparent.

Autophagy is defined as a cellular process of self-degradation that is evolutionary conserved amongst species.[12] It occurs constitutively at base-line levels within the cell to ensure homeostasis by removing aged, damaged or accumulated proteins and/or organelles. Beyond its quality control function, the autophagic process can also be massively augmented in response to various intracellular and extracellular stress stimuli.[13] These include nutrient and growth factor deprivation (metabolic stress), radiation and chemotherapy (DNA damage), endoplasmic reticulum (ERt) stress, hypoxia and viral invasion. During starvation conditions, cellular metabolism and biosynthesis can be sustained from the recycled pool of cytosolic biomolecules generated during autophagy.[14] Elimination of excessive reactive oxygen species produced by mitochondria under hypoxia, as well as unfolded or aggregated proteins may prevent these forms of 'cellular garbage' from potentially inducing carcinogenesis. While short-lived proteins are degraded by the ubiquitin proteasome pathway, long-lived proteins are mitigated by autophagy.[15]

Depending on the mechanism by which the cytosolic cargo is delivered to lysosomes for degradation, three different types of autophagy exist. Chaperone-mediated autophagy mediates the direct translocation of a single cytosolic protein, possessing a specific sequence motif, to the lysosome for degradation.[16] This form of autophagy lacks the characteristic vesicular formation and rather employs the use of cytosolic chaperones which selectively recognise and translocate proteins. Micro-autophagy and macro-autophagy (referred to generally as autophagy) are the other two forms of autophagy, characterised by the formation of vesicles which allow for the degradation of cytosol, proteins and/or organelles.[17] While the former entails the direct sequestration and degradation of cargo by lysosomal membrane invaginations, the latter necessitates the vacuolisation of cargo within double-membraned structures, termed the autophagosomes, prior to fusion with the lysosome.

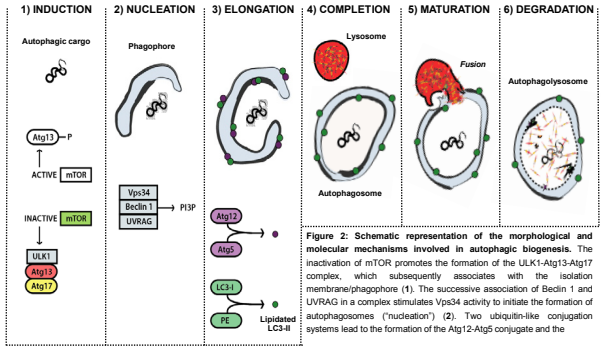

Figure 2: Schematic representation of the morphological and molecular mechanisms involved in autophagic biogenesis. The inactivation of mTOR promotes the formation of the ULK1-Atg13-Atg17 complex, which subsequently associates with the isolation membrane/phagophore (**1**). The successive association of Beclin 1 and UVRAG in a complex stimulates Vps34 activity to initiate the formation of autophagosomes ("nucleation") (**2**). Two ubiquitin-like conjugation systems lead to the formation of the Atg12-Atg5 conjugate and the lipidated phosphatidylethanolamine (PE)-associated LC3-II conjugate, both of which promote elongation (**3**). The LC3-II conjugate becomes incorporated into the membranes of the developing autophagosome. Upon completion (**4**), the maturation phase follows with the fusion of the autophagosome with a lysosome to form an autophagolysosome (**5**). The encapsulated cargo, as well as the inner limiting membrane, becomes degraded by acid lysosomal hydrolyases (**6**).
MSc Thesis: Justin Mills (2013)

The execution process of autophagy (see Figure 2) begins within the cytoplasm with the formation of crescent-like membranous structures termed phagophores, or isolation membranes.[18] This is the initiation stage of the autophagic process. During the elongation stage which follows, these specialised structures expand into double- or multi-membraned autophagosomes, also known as early autophagic vesicles, which sequester cytosol or cellular components for 'bulk' degradation. The cargo may be targeted selectively or non-selectively.[15] The outer autophagosomal membrane first fuses with an endosomal vesicle to form an amphisome.[18] This is followed by fusion with a lysosome to form an autophagolysosome, also known as a late autophagic vesicle. Acid hydrolases within the lysosome digest the inner membrane of the autophagosome along with its encapsulated luminal cargo. These hydrolases include nucleases, proteases, glycosidases and lipases, enabling a broad range of macromolecules to be degraded simultaneously.[12,19]

The many different types of cell death pathways contain a multitude of different biochemical components, many of which are not yet understood. As these pathways are more or less sequential in nature, removing or modifying one component leads to an effect in another. In a living organism, this can have disastrous effects, often in the form of disease or disorder. A discussion of every disease caused by modification of the various cell death pathways would be impractical, but the concept overlying each one is the

same: The normal functioning of the pathway has been disrupted in such a way that the ability of the cell to die normally is impaired. This results in cells that replicate and pass on faulty genetic information to its progeny, increasing the likelihood of the cells becoming cancerous or diseased. The interaction of genetics, the environment and nurture is the foundation for all health and disease, therefore nutrition is an environmental factor of major importance. Using the tools of molecular biology and specifically to understand the factors which induce or inhibit cell death, the aim of our research is to unravel the signalling mechanisms induced by environmental/nutritional factors and to exploit the cell's own machinery to search for new and safe therapeutic modalities.

THE SEARCH FOR NEW AND SAFE THERAPEUTIC STRATEGIES

OMEGA-3 FATTY ACIDS: NATURE'S OWN THERAPY?

The notion that fatty acid intake may influence the incidence of cancer and heart disease has been studied intensively *in vivo* and *in vitro* for more than 40 years and seems to be widely accepted. Ranging from epidemiological studies to those conducted using cell culture models, most studies provide evidence that omega-3 (n-3) fatty acids, especially the long-chain polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found abundantly in fish oil, are able to inhibit the development of cancer or slow down tumour growth.[20,21] However, the cellular mechanisms responsible for these effects have not yet been clarified, although various hypotheses exist, including modifications to eicosanoid metabolism and activation of different cellular signalling pathways.[22,23]

Epidemiological evidence for a link between diet, cancer incidence and heart disease

Epidemiological studies have demonstrated a lower incidence of certain malignancies amongst certain populations. This phenomenon was most notably described in the Inuit population of Greenland[24–27] and a related phenomenon was also seen in Mediterranean countries, where olive oil (composed mainly of oleic acid) is an important part of the diet.[28–31] The typical Greenland Eskimo diet is high in lipids, contains predominantly large quantities of seal and fish and is rich in omega-3 fatty acids.[32,33] When the plasma lipids of native Greenland Eskimos were analysed and compared to that of Danes, the native Eskimos had higher plasma lipid concentrations of alpha-linolenic acid (ALA), EPA and DHA of the n-3 series.[34] Yet as time passed the cancer profile of these populations changed markedly to resemble that of the American Caucasian population,[26,27] which might be due to the adaptation of a Western lifestyle. Nonetheless, those early studies of the dietary habits of the Greenland inhabitants and the once rarity of cancer amongst them led to the first suggestions of the possible anticarcinogenic effects of PUFAs.

During this time, much attention was also given to the proposal that such a plasma lipid profile was favourable towards a decreased incidence of atherosclerosis and ischaemic heart disease.[34,35]

The n-6/n-3 imbalance

Since that time, it has become known that n-3 fatty acids are protective against a host of diseases such as cardiovascular disease (CVD), inflammation, neurodegenerative diseases and especially cancer.[36] However, the typical Western diet consumed today is deficient in these important PUFAs, resulting in an increased risk for modern diseases such as those mentioned previously. In addition to the apparent n-3 PUFA deficiency, the Western diet has elevated n-6 PUFA content, especially Linoleic Acid (LA) and Arachidonic Acid (AA).[36,37] It is believed that the foods available to our ancestors (before agricultural practices and animal domestication were taken on) were rich in n-3 PUFAs and contained them in a ratio with n-6 PUFAs of approximately 1:1. Such a fatty acid profile in food led the human body to establish a genetic pattern without genes that would enable it to synthesise fatty acids or convert them to another form. The n-6:n-3 PUFA ratio has increased over time, as the Western diet today contains a ratio of 15–20:1. Unfortunately, the human body cannot adjust its genome to suit such a lipid profile in such a short time, making modern man susceptible to devastating modern disorders. It is thus necessary to supplement our diets to enrich tissues with n-3 fatty acids and correct for the n-6/n-3 imbalance.

The role of fatty acids as constituents of membrane phospholipids in normal and tumour cells

The fatty acids contained in membrane phospholipids are essential in determining certain physical properties of a membrane, such as fluidity and flexibility. They also regulate cellular functions, including the movement of ions and metabolic products across the membrane, receptor binding and eicosanoid production.[38] Mammalian cells *in vitro* readily take up lipids such as fatty acids from their culture medium.[39] Supplementation of media with different fatty acids is thus a simple method to manipulate the fatty acid composition of membrane phospholipids in cultured cells. When membrane phospholipid composition is modified, so are various cellular processes and responses. These include carrier-mediated transport, activities of membrane-bound enzymes, binding properties of membrane receptors, cytotoxicity, growth, modulation of cellular signalling events, and eicosanoid synthesis, amongst others.[36,38,39]

The role of omega-3 fatty acids in the regulation of signalling pathways to induce cell death in cancer cells

Cancer is often described as a disorder of the balance between cell proliferation and cell death.[40] During the progression of cancer, the tumour cells acquire a variety of phenotypic properties that allow them to proliferate both swiftly and limitlessly, invade the surrounding tissues, survive without their normal microenvironment, and metastasise.[41]

These features are usually acquired progressively over a protracted period of time as a result of increased genomic instability that leads to down-regulation of tumour suppressor genes and up-regulation of oncogenes.[42] One such oncogene encodes the serine/threonine kinase Akt, also known as protein kinase B (PKB) (a member of the PI3-K pathway) and has emerged as a crucial regulator of widely divergent cellular processes, including apoptosis, cell proliferation, differentiation and metabolism.[43–46] Disruption of normal PI3-K signalling has been documented as a frequent occurrence in several human cancers and appears to play an important role in their progression.[37,47–48] The PI3-K signalling pathway should therefore be considered as a potential target for chemotherapy.

In order for a therapeutic agent to be truly effective, it should be toxic to cancer cells without harming normal cells. It has been demonstrated both in animal and cell culture models that PUFAs can significantly reduce tumour size *in vivo* and suppress cell viability and induce apoptosis of cancer cells *in vitro*.[49-52] However, the molecular mechanisms involved in the cytotoxic effect of PUFAs on cancer cells and the intracellular mechanisms responsible remain to be elucidated. Therefore, in order to assess the signalling mechanisms involved in the cytotoxic effect of PUFAs, we treated CaCo-2 (colon cancer) and NCM460 (normal colon epithelial) cells with a saturated (palmitic acid, PMA), a monounsaturated (oleic acid, OA) and two polyunsaturated fatty acids (arachidonic acid, ARA, omega-6; docosahexaenoic acid, DHA, omega-3) and determined their effects on cell viability, apoptosis and protein phosphorylation events in the PI3-K pathway. This study was undertaken to evaluate the chemopreventive/antiproliferative potential of saturated, monounsaturated, and polyunsaturated (omega-6 and omega-3) fatty acids on a colon cancer cell line and to investigate their mechanism of action.

The fatty acid composition of membrane phospholipids can influence the function of certain membrane proteins, such as receptors, transporters and enzymes. Such changes would also have downstream signalling effects and could ultimately change the profile of gene expression. The fatty acids were differentially incorporated in the cell membranes of the cancer and the normal epithelial cells. In normal NCM460 cells, DHA induced a three-fold increase in the phospholipid content and was also further β-oxidised to EPA. Our results demonstrated a much better incorporation of added fatty acids into NCM460 cells' membrane phospholipids compared to CaCo-2 cells. This also might play a role in the dual effect of these fatty acids in normal versus cancer cells. Furthermore, when the total saturated, monounsaturated and polyunsaturated fatty acids in NCM460 and CaCo-2 cells were compared, a two-fold increase in the omega-3 fatty acid content was observed in normal cells compared to cancer cells. The reduced omega-3 fatty acid content in control CaCo-2 cells versus control NCM460 cells might thus explain the beneficial effect of DHA (an omega-3 fatty acid) when treating cancer cells.

The enrichment of tumour cell membranes with fatty acids has important therapeutic potential and has also been shown to enhance the cytotoxicity of therapeutic agents. Therefore, the next aim of this study was to determine the cytotoxic potential of various fatty acids on CaCo-2 cells and normal colon epithelial cells (NCM460 cells). None of the fatty acids used in the current study were toxic to normal colon epithelial cells, but had differential effects on the cancer cells. DHA was the only fatty acid that significantly reduced the viability of the CaCo-2 cells. Although many mechanisms have been proposed to explain the tumour suppressive effects of these fatty acids, the molecular signalling mechanisms are still poorly elucidated. Therefore, the third aim of this study was to determine the effects of DHA on the PI3-K signalling pathway (see Figure 3). PI3-K is an essential part of the signalling network that blocks programmed cell death (apoptosis) and enables cells to survive when they are in a favourable environment. These pathways are activated so that a continuous survival signal from growth factors is required to block apoptosis.[53] Upon growth factor activation of receptor tyrosine kinases, PI3-K is recruited to the receptor in the plasma membrane and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) on the 3-OH group, generating phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PI3-kinases are composed of a catalytic subunit (p110) and a regulatory subunit (p85). DHA and AA significantly reduced the regulatory subunit (p85), while no differences were observed in the catalytic subunit of PI3-K with any of the fatty acids. The decreased PI3-K level induced by DHA also correlates with increased apoptosis in our cell model. PI3-K is considered one of the intracellular pathways responsible for the transmission of anti-apoptotic signals for controlling cell survival and over-expression of PI3-K in cells has been shown to cause a significant increase in survival of cells exposed to ionising radiation.[54] We have demonstrated that DHA treatment inactivates PKB to decrease pro-apoptotic factors, which include BAD and Forkhead kinase (FKHR) phosphorylation. This, in turn, leads to increased apoptosis as indicated by increased caspase-3 and poly-ADP-ribose polymerase (PARP) cleavage. Our results confirmed the antiproliferative and antitumourigenic efficacy of DHA in colon cancer cells, together with its safety regarding normal colon epithelial cells. Although this study proposes one possible pathway for DHA's effect on cell viability, the PI3-kinase signalling pathway and apoptosis, many other possible modes of action undoubtedly exist.

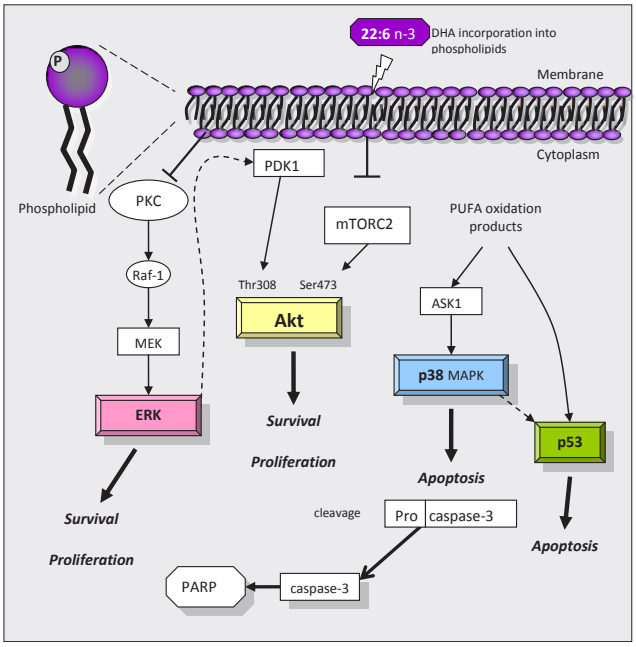

Figure 3: DHA inhibits pro-survival pathways and induces apoptosis in colon cancer cells (MSc thesis: J-L du Toit-Kohn, 2006)

OMEGA-3 FATTY ACIDS AND HEART DISEASE

Coronary heart disease (CHD) remains one of the leading causes of death in all Western industrialised countries. Although epidemiology and human intervention trials consistently show an inverse relationship between dietary omega-3 fatty acid consumption and mortality from heart disease[55–57] the mechanism of action of these fatty acids remains to be elucidated. Long-chain PUFAs play an important role in various biological processes in cardiac muscle cells. PUFAs can either act as second messengers or as reversible modulators to amplify, attenuate or deviate a signal at a precise intracellular location.[58]

Previous studies have shown that a variety of extracellular stimuli, including ischaemia-reperfusion, profoundly affect the activation of the mitogen-activated protein kinases (MAPKs), which includes extracellular signal-regulated protein kinase (ERK), p38 and c-Jun NH_2-terminal protein kinase (JNK).[59-62] All three MAPKs have been shown to play pivotal roles in transmission of signals from cell surface receptors to the nucleus and are involved in cell growth, differentiation and apoptosis.[63-65] In response to MAPK activation, a family of dual-specificity phosphatases becomes transcriptionally induced, leading to dephosphorylation and inactivation of specific MAPKs within 30 to 60 minutes.[66] Currently ≈9 dual-specificity phosphatase family members have been described, each of which has a slightly different substrate specificity, tissue distribution, subcellular localisation or inducible expression profile.[66] MKP-1 (mitogen-activated protein kinase phosphatase-1) is an important member of this family, which regulates inactivation of p38, JNK and ERK.[67-69] However, no evidence existed for an interaction between EPA and the activation/inhibition of the MAPKs and the pro-survival kinase PKB/Akt in neonatal rat cardiomyocytes during simulated ischaemia (SI) and reperfusion. Therefore, in order to assess the mechanisms of protection of long-chain PUFAs in injured/apoptotic heart cells, we treated neonatal cardiomyocytes with EPA prior to and after simulated ischaemia and determined their effects on cell viability, apoptosis and the activation patterns of the MAPKs and PKB/Akt. To further evaluate the significance of findings obtained in a cell model of simulated ischaemia, the effect of the PUFAs on functional recovery of isolated globally ischaemic perfused rat hearts was also studied.

Our results have shown that EPA protects cardiomyocytes against simulated ischaemia-reperfusion injury. It is suggested that their beneficial actions are due to: (i) the increased phosphorylation of ERK which is PKC-dependent, (ii) the induction of a tyrosine phosphatase, leading to the reduced phosphorylation of the pro-apoptotic p38, and (iii) the activation of an as yet unknown pathway distal to PI3-K. Both increased ERK phosphorylation and the inhibition of p38 phosphorylation may contribute to the increased cell viability and decrease in apoptosis induced by EPA. Finally, the increased cell viability and reduced apoptosis induced by EPA studied in this cell model were also reflected by the significant improvement in functional recovery of the globally ischaemic heart during reperfusion. Thus, EPA might offer an alternative, non-pharmacological strategy to protect the heart against ischaemia-reperfusion injury.

AUTOPHAGY AND CHEMOTHERAPY: EXPLOITING THE CELL'S OWN MACHINERY AS ADJUVANT THERAPY

Anthracyclines, such as doxorubicin (DXR), are considered as some of the most effective chemotherapeutic agents for the treatment of breast cancer.[70] Unfortunately, anthracyclines are also cytotoxic and affect a wide variety of systems. Progressive myocardial damage and skeletal muscle atrophy are possible results of their cumulative effect.[71] As the cardio- and muscle toxicity of these drugs is cumulative and dose-limiting, methods for inducing cell death at earlier time points in the treatment regime are urgently needed. However, this is not the only challenge that researchers and clinicians are faced with. Despite the fact that DXR induces damaging effects on the heart, cancer cells are becoming inherently resistant to DXR-induced cell death.

Chemo-resistance

The resistance of cancer cells to chemotherapy (chemo-resistance) is a multifaceted challenge faced by oncologists and their cancer patients. Initially, patients are susceptible to chemotherapy (chemo-sensitive) and cancer free directly after their course of treatment. However, 50% to 70% of patients relapse within one year as a result of poor surgical outcomes in which whole tumours are not completely resected, subsequently leading to the formation of another tumour.

The micro-environment (stroma) consists of endothelial cells, carcinoma-associated fibroblasts, adipocytes, mesenchymal cells, mesenchymal stem cells and cells from the immune and inflammatory systems. As these stromal cells communicate with each other,[72] accumulating evidence shows that the cells residing in this niche could potentially induce chemo-resistance in tumour cells by influencing cancer cell sensitivity to apoptosis; local release of soluble factors such as interleukin-6 (IL-6) that promote survival; the generation of specific niches within the tumour micro-environment which consist of subpopulations of tumour cells that may gain a survival advantage following initial drug exposure; and the conversion of the cancer cells into cancer-initiating cells or cancer stem cells. Although the cross-talk between the stromal cells and tumour cells are crucial in developing tumours, cells located in the intra-tumour, avascular regions face other challenges posed by this niche. This specific region is characterised by low nutrients and oxygen (hypoxia), acidic extracellular pH and populations of quiescent cells which subsequently impact on the success of chemotherapy in solid tumours.[73] The rapid rate of tumour growth requires increased levels of energy production through cellular metabolism. However, inadequate vascularisation progressively leads to a hypoxic tumour environment. The hypoxic environment requires that energy demands are satisfied through glycolytic pathways rather than the more efficient oxidative phosphorylation route known as the Warburg Effect.[74,75] Additionally, the high interstitial pressure prevents clearance of metabolic end-products such as lactic acid. Enhanced hypoxia further leads to the up-regulation of growth factors such as platelet-derived growth factor B (PDGF-B), transforming growth factor ß (TGF-ß), insulin-like growth factor 2 (IGF-2) and epidermal growth factor (EGF).[76] Angiogenesis is regarded as the key contributor of metabolites and oxygen found in large tumours, thus allowing tumour progression.[73] An additional prevailing mechanism thought to be involved in chemo-resistance is autophagy.

Autophagy

As mentioned previously, autophagy is characterised by the development of autophagosomes that engulf organelles and other cellular components such as proteins before fusing with the lysosome, which result in mass proteolysis. This has been shown to occur mainly in response to nutrient starvation, hypoxia, ATP depletion or signals prompting cellular remodelling and is mainly controlled via the mTOR signalling cascade. The current hypothesis in the literature reveals that autophagy might more accurately be described as a cell survival mechanism that acts alongside cell death but does not necessarily lead to it. Although cell death through autophagy has been suggested as a mechanism of tumour cell survival,[77-78] it still remains a controversial matter whether autophagy leads to tumour formation or suppression. In addition, a study by Zhang and co-workers[79] revealed that hypoxia induces mitochondrial autophagy to prevent an increase in the level of reactive oxygen species and subsequent cell death. Thus, when viewed dynamically, it is becoming apparent that autophagy acts to delay cell death and may only lead to it in a last desperate effort while attempting to keep cells alive. However, this can only occur once autophagy has progressed and persisted beyond the so-called 'point of no return' (PONR), resulting in apoptosis or possibly even necrosis.[80] There is, however, a great lack of knowledge regarding the progression of autophagy with time in oxygen and nutrient deficient tumour micro-environments. This could be due to autophagy being recognised predominantly as a death mechanism in the cancer environment as well as due to the lack of investigation on autophagy in the different tumour micro-environments. This lack of knowledge raises areas of concern and questions which require clear answers: What is the specific role of autophagy in tumours and what is the contribution of autophagy to cellular survival during therapy? Whether cell death through autophagy or autophagy itself actually occurs in breast cancer is still largely undetermined, therefore further investigation in this field is required. Another concept that still remains to be explored is the possibility that malignant cells in the advanced stages of carcinogenesis undergo autophagy as a pro-survival mechanism.

Nutrient deprivation and autophagy

Since ancient times therapeutic fasting has been practiced as a method of healing in various health conditions. It appears that the body's innate response against disease and infection is to limit its nutritional intake. Since such a phenomenon exists, the concept of controlled nutrient starvation is increasingly becoming an appealing concept worth exploring by physiologists and oncologists.

One of the earliest studies on therapeutic starvation was conducted in 1988 by Raffaghello and co-workers;[81] normal and cancerous yeast cells were starved and then exposed to chemotherapy. They found that normal cells survived the toxic dosage but the cancer cells did not. This inspired an animal trial in which tumour bearing mice were starved for 48 hours after which a high dose of chemotherapy was administered. This study proved to be successful as 43% of the non-starved mice died, but only one of the starved mice. Once chemotherapy was terminated, a normal diet was resumed and the starved mice regained all the lost weight. With the great success of the animal trial, Raffaghello and co-workers[82] proceeded with a human trial in 2010, in which ten patients starved for 48 to 140 hours prior to and 5 to 56 hours after chemotherapy reported reduced side-effects of the toxic drug.

It is known that cancer cells alter metabolism to suit their specific growth requirements and express chronic proliferation and thus require an increased need for energy and carbon/nitrogen sources for biomass production. As a major contributor to energy substrates during stressful conditions, autophagy supports cancer cells to meet their enhanced metabolic demands. The role that autophagy plays in cancer metabolism remains to be elucidated completely. Not much is known about how autophagy selectively accesses major energy sources such as amino acids, lipids and carbohydrates, and to what extent these sources contribute

to survival of cancer cells during stressful conditions such as hypoxia and nutrient deprivation. Therefore, one of the aims of our study was to determine whether the manipulation of autophagy can sensitise cancer cells to chemotherapy and protect normal cells against its detrimental side-effects.

The manipulation of autophagy increases the efficacy of the chemotherapeutic agent doxorubicin in cancer cells

Understanding how cancer cells are able to avoid and tolerate the effects of short-term starvation of nutrient supply could prove vital if future anticancer strategies are to be successfully developed based on this premise. Indeed, current research has already begun to investigate these avenues, and the recent employment of controlled starvation of cancer patients has exposed a potentially feasible and reproducible therapeutic approach. In a series of studies, we investigated the ability of a commonly used cancer cell line (MDAMB231), compared to a non-tumourigenic control line (MCF12A), to tolerate a short-term bout of nutrient restriction. Using this model, additional experiments were designed to provide mechanistic insight into these initial findings. Using this knowledge, studies were conducted in an attempt to exploit the novel anticancer potential of using nutrient starvation in conjunction with chemotherapy *in vitro*. A new and innovative cancer model was then developed to expand these findings into the *in vivo* setting. Initial experiments established that a fast growing, metastatic cancer cell line (MDAMB231) was more sensitive to amino acid starvation than a non-tumourigenic line (MCF12A). It was shown that short-term deprivation of amino acids resulted in increased cell death and a proliferation arrest in the cancer cells. Most cells are known to possess proficient intracellular mechanisms that are able to maintain amino acid levels during times of starvation. The up-regulation of autophagy through the autophagosomal-lysosomal pathway has been implicated as a mechanism used by cancer cells to maintain cellular ATP levels during nutrient-poor conditions. Normally autophagy functions as a cytoplasmic quality control mechanism to remove protein aggregates and damaged organelles. Recently published findings have revealed that human cancers with mutations in H-ras or K-ras may require autophagy for tumour survival and sustained growth.[83] Although these mutations are not common in breast cancer, genetic modifications in regions coding for proteins elsewhere in the RAS pathway results in high RAS activation in a large percentage of breast malignancies. As the MDAMB231 breast cancer cell line is a known K-ras mutant, it was speculated that autophagy is crucial for this tolerance to acute nutrient starvation.[83]

Experimental evidence demonstrated that autophagy inhibition resulted in decreased cell survival and reduced proliferation levels during acute nutrient starvation in the MDAMB231 cell line. After a few hours without exposure to nutrients, protection was lost and intracellular cell death programmes initiated. Surprisingly, the slower growing, non-cancer cell line was more tolerant to these periods of short-term starvation. Autophagy frequently has been implicated as a potential survival mechanism in malignant tumours, based on the premise that the degradation products released following autophagy-mediated breakdown of cytoplasmic materials can be utilised for protein synthesis or as substrates for ATP production. Unfortunately, there is little direct evidence to support this hypothesis in cancer cells. Based on evidence from our previous experiments, the next study attempted to provide a rare insight into the autophagy-mediated changes in amino acid levels that occur during a short-term starvation event. We further supply some mechanistic support to explain the finding that autophagy protects MDAMB231 (cancer) and MCF12A (normal) cells during amino acid starvation. Unlike most existing studies into this phenomenon, the model design used here allowed for the delineation of the impact of amino acid starvation alone (a known trigger for increased autophagy) while all other nutrients remained constant. It was successfully demonstrated, by inhibiting autophagy pharmacologically (bafilomycin A1) or biologically (ATG5 siRNA), that autophagy is a vital process for increasing tolerance to nutrient deprivation. Interestingly, it was shown that both cell lines utilised in these studies exhibited a short-lived autophagy-mediated surge in amino acid levels. While amino acid levels quickly decreased in the MDAMB231 cancer cells afterwards, presumably due to the high metabolic and biosynthesis needs of these cells, they remained elevated in the slower growing MCF12A normal cells. As autophagy inhibition blunted this protective response, it was inferred that generation of amino acids by autophagy is a vital mechanism during adaptive tolerance to short-term amino acid starvation. The discovery of an analogous elevation in free fatty acid levels during similar conditions (which could be blunted by autophagy inhibition) strengthened the hypothesis that increased autophagy results in the increased intracellular availability of basic protein and organelle constituents for reuse elsewhere in the cell. In an attempt to understand how these basic cellular building blocks were utilised by the cell, changes in ATP levels were examined during starvation in the presence or absence of autophagy inhibition. Surprisingly, autophagy-related processes were implicated in the maintenance of ATP levels within these cell lines during nutrient starvation. MDAMB231 cells were revealed to be particularly reliant on the ATP homeostasis conferred by increased autophagy during the first hours of amino acid deprivation. Together, these three studies demonstrated how a cancer cell line that depends on autophagy for survival is able to avoid cell death during short-term starvation by generating basic cellular building blocks and utilising them for cellular processes such as ATP maintenance.

Many clinical trials are beginning to assess the effectiveness of compounds known to regulate autophagy in patients receiving anticancer therapy. As mentioned earlier, short-term starvation has shown promise in alleviating some of the symptoms associated with chemotherapy. Our previous results demonstrated that nutrient deprivation elicited specific and dynamic alterations to autophagy in

MDAMB231 (cancer) cells. Using this data as a platform, we established that increased autophagy, associated with amino acid starvation in these cells, correlated with decreased cell survival during DXR treatment. Interestingly, a sustained elevation of autophagy in MCF12A cells (normal breast epithelial cells) during similar treatment was associated with protection from cell death during DXR treatment. As our data showed that a non-cancer cell line was protected if starved of amino acids during DXR treatment, while a cancer cell line with high basal autophagy activity had increased cell death, the next aim of this study was to verify this in an *in vivo* model.

Typically much of the solid tumour is made up of a variety of cell subpopulations that are required for continued growth and invasion. As these heterogeneous neoplasms are comprised of cancer and stromal elements, it is therefore essential that *in vitro* studies are translated *in vivo*. The next study was successful in establishing a novel mammary tumour model in a GFP-LC3 transgenic mouse. A reproducible method to study autophagy in the non-cancer subpopulation of tumours was established for the first time. The final study was undertaken in order to establish if tumours are protected or display decreased survival if basal autophagy flux is up-regulated (through amino acid starvation or rapamycin treatment) in mice during DXR treatment. Our results demonstrated that rapamycin treatment of mice receiving a high cumulative dose of DXR treatment increases animal survival. Furthermore, the combination treatment was just as effective as DXR alone to decrease tumour size in these animals.

The manipulation of autophagy attenuates doxorubicin-induced cardiotoxicity

As mentioned previously, the therapeutic potential of the chemotherapeutic agent DXR is restricted by its serious side-effects (cardiotoxicity) which can lead to congestive heart failure. Although numerous studies have attempted various methods to reduce anthracycline-induced cardiotoxicity, very few have been able to reproduce their results in a clinical setting. We have demonstrated that rapamycin, a potent inhibitor of the mTOR signalling pathway which induces autophagy, possesses cardioprotective effects against anthracycline-induced cardiotoxicity.

We have demonstrated that DXR is a potent inducer of cell death, the ubiquitin-proteasome pathway (UPP), mitochondrial dysfunction and endoplasmic reticulum stress which are all attenuated by rapamycin (inducer of autophagy) treatment. Additionally, the co-treatment of rapamycin and DXR decreased cell death (apoptosis), increased cardiomyocyte size and prevented the decrease in body weight induced by DXR treatment in the *in vivo* model. Furthermore, as rapamycin is currently being used in the clinical setting to suppress tumour growth, its characteristics thus make this drug an ideal adjuvant therapy to either treat or prevent cardiotoxicity in order to potentially inhibit or delay heart failure.

We have demonstrated that autophagy protected cancer cells from the stressful microenvironment of hypoxia and starvation. Furthermore, cancer cells use autophagy to generate ATP during nutrient starvation. The amino acids generated through autophagy are utilised in the Krebs cycle to maintain ATP levels under nutrient starvation conditions. A further up-regulation of the already high basal autophagy levels sensitises cancer cells to DXR-induced cell death. In normal cells, including cardiomyocytes, DXR-induced cytotoxicity is associated with reactive oxygen species (ROS) production, which leads to compromised mitochondrial function and morphology and eventually to cell death. Combination therapy of DXR and rapamycin improves cell viability in normal cells and attenuates apoptosis. Furthermore, it decreases ROS production and preserves mitochondrial function. It also prevents translocation of DXR into the nucleus, where it induces its cytotoxic actions. The model established by us, and the related findings, have presented a novel and unique platform for further research into this remarkable phenomenon, and particularly the role of autophagy in it.

Manuscripts generated from this work:

- Engelbrecht AM, Engelbrecht P, Genade S, Niesler C, Page C, Smuts M, Lochner A. 2005. Long-chain polyunsaturated fatty acids protect the heart against ischaemia/reperfusion-induced injury via a MAPK dependent pathway. Molecular and Cellular Cardiology 39:940–54. [IF 4.965]

- Engelbrecht AM, Du Toit J-L, Louw L. 2008. Differential induction of apoptosis and inhibition of the PI3-Kinase pathway by saturated, monounsaturated and polyunsaturated fatty acids in a colon cancer cell model. *Apoptosis* 13(11):1368–77. [IF 4.066]

- Toit-Kohn JL, Louw L, Engelbrecht AM. 2009. Docosahexaenoic acid induces apoptosis in colorectal carcinoma cells by modulating the PI3 kinase and p38 MAPK pathways. J Nutr Biochem Feb;20(2):106–14. [IF 4.288]

- Sishi BJN, Loos B, Van Rooyen J, Engelbrecht AM. 2013. Autophagy upregulation promotes survival and attenuates doxorubicin-induced cardiotoxicity. Biochemical Pharmacology 85:124–34. [IF 4.405]

- Sishi BJN, Loos B, Van Rooyen J, Engelbrecht AM. 2013. Doxorubicin induces protein ubiquitination and inhibits proteasome activity during cardiotoxicity. *Toxicology* 309:23–9. [IF 3.681]

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