Lalini Reddy. *et al*. / *International Journal of Biological & Pharmaceutical Research.* 2012; 3(4): 550-559.

e- ISSN 0976 - 3651
Print ISSN 2229 - 7480

International Journal of Biological & Pharmaceutical Research
Journal homepage: www.ijbpr.com

IJBPR

MOLECULAR IMMUNOGENETICS OF APOPTOSIS: EXPERIMENTAL DILEMMAS

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ABSTRACT

There have been several research articles published on the biological and biochemical nature of apoptosis. These have included studies on the molecular genetics of apoptosis. Apart from the genes that are involved in the apoptotic cascade, there are several other genes that are either activated or inhibited when cell lines are exposed to apoptotic stimuli. This article addresses the simplicity and complexity of the genetic nature of apoptosis in a variety of cell lines.

Key Words: Apoptosis, Programmed Cell Death, Caspases, Bax, Bcl-2 gene family.

INTRODUCTION

Apoptosis, or programmed cell death, is a process which old and infected cells undergo when they do not receive an adequate supply of extracellular growth factors (Conlon and Raff, 1999) or are affected by pathological insults (Bratton and Cohen, 2001; Wahl and Carr, 2001) and chemical agents (Gorman *et al*., 1997; Madesh and Hajnoszky, 2001). There have been several studies reported about apoptosis involving the use of freshly isolated polymorphonuclear neutrophils (PMN) from the circulation, and the isolation and expression of apoptotic genes from PMN using reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR in those studies was used to measure the expression of homologous genes like *bcl-2* and *bcl-X1*. These genes are responsible for promoting apoptosis once they are activated by apoptotic stimuli. However, concurrently, RT-PCR is also used to study the expression of the heterologous inhibiting genes like *bcl-2*, *bax-α* and *bcl-X* (Weinmann *et al*., 1999). It is important to note that the analysis of heterologous and homologous genes is not an accurate measure of apoptosis because they say nothing about the outcome of this complicated process. Therefore in the PMN study, *caspase-3* activation was also measured using RT-PCR (Weinmann *et al*., 1999). The measuring of *caspase-3* expression is commonly studied to confirm that the apoptotic stimuli that is exploited and administered did indeed induce cell death, and, furthermore it, provides some evidence about the proteolytic cleavage of the procaspase-3 gene and whether it is cleaved into caspase 3 or not during apoptosis (as adapted from knowledge about the apoptotic pathways) (Conlon and Raff, 1999).

RT-PCR in the PMN study used site-specific primers so as to achieve gene products of particular segments of genes that are amplified (amplicons). In addition to the PMN-treated cells, the cell line (HL-60) (juvenile cancer cell of pro-myeloid origin) and BL-41 (B-cell line) served as the positive controls. These were the only cell lines that showed positive for *bcl-2* activation after RT-PCR (Weinmann *et al*., 1999). *bcl-2* gene activation was absent in PMN, even when they were treated with apoptotic stimuli after 2, 6, 22 or 34 h (read article by Lagasse and Weissman, 1994; Weinmann *et al*., 1999).

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bcl-X is an homologue of *bcl-2* and it was found to be present in all 3 of the mentioned cell lines. Furthermore, *bax-α* pro-apoptotic gene, was also expressed in all 3 cell lines and this indicated the possibility that 100% heterodimerisation existed between *bcl-2* and *bcl-X1* in the PMN cell line (Weinmann, 1999). It is important to remember that although heterodimerisation is one possibility in the situation above, other genes such as *bak*, which are also found in PMN, play an inhibitory role in the apoptotic process and that is what points programmed cell death in the direction of apoptotic gene regulation. Studies have also found expression of the *bak* gene in HL-60 cells and BL-41 treated cell lines (Kroemer, 1997; Oltvai and Korsmeyer, 1994).

The machinery/genes involved in the control of apoptosis belong to the *bcl-2* gene family, which constitute the *bcl-X1, bax-α* and *bak* genes. However, although gene expression studies are popular, very few papers have reported genetics as a research discipline that could reveal important scientific information about apoptosis. Much thought has been given to the fact that genetics is much more labour-intensive compared to research performed merely at the basic science level, yet researchers boast that protein level work is much more conclusive. Even though the latter may be the case, since proteins are the highest level of nucleic structure innovative research should start at DNA and/or RNA levels. This point was emphasised in the PMN study, in which western blotting did not detect the expression of Bax proteins in PMN, however, when RT-PCR was performed, the *bcl-2* and *bax-α* genes were present (Weinmann, 1999). This highlights the reliability of performing RT-PCR in apoptotic studies as the most reliable method and which we have found to be true at the Durban University of Technology, Department of Biotechnology and Food Technology molecular laboratory.

Personally, this indicated that the detection of Bax proteins could have been extremely low in the PMN control cell line because its gene, together with the *bcl-X1*, *bax-α* and *bcl*-2 genes were expressed in the HL-60. In mature PMNs *bcl-2* mRNA was completely absent (Weinmann, 1999). This highlights the fact that gene and protein expression techniques cannot really be compared in a meaningful way no matter how much one attempts to optimise and/or standardise a particular technique, because gene/protein results vary among cell lines that are used during research. The fact that the *bcl-2* gene was expressed in HL-60 cell line and not in the mature PMN cell line is also an indication, from an apoptotic pathway standpoint, that *bcl-2* is downregulated and that the PMN cell line can thus be interpreted as 'apoptosis-induced' cells (Weinmann, 1999). At this point of the discussion it is important to point out the importance of using mature cell lines in apoptotic studies. The absence of *bcl-2* expression in mature PMN, indicates that there is a possibility of there being some differences in the evolution of the apoptotic machinery between mature and juvenile cell lines of the same or different origins.

The use of PMN from transgenic mice (mice that interbreed and have mixed genes) is an excellent example when studying *bcl-2* gene expression. However, as a key point, it is necessary to note that, *bcl-2* and *bcl-X1* are the death antagonising splice variants of the *bcl-X* gene and the anti-apoptotic counterparts of the *bax-α* gene. The *bax-α* gene is the death-promoting splice variant of the *bax* gene (Sedlak *et al*., 1994). Weinmann (1999) found that while in PMN *bcl-2* is expressed, in transgenic mice no such expression exists. Weinmann attributed this to there being a genetic drift in PMN genes among mice that interbreed compared to those that don't interbreed. Weinmann concluded that the difference in genetic drift has an effect on the overall outcome of *bcl-2* gene expression and the outcome of apoptosis (Weinmann, 1999).

In addition to Weinmann's conclusion, Sedlak *et al* (1994) suggested that the upregulation and downregulation of *bax-α* and *bcl-X* genes in the formation of homodimers and/or heterodimers, also affect the results obtained for *bcl-2* genes in transgenic mice, but he reinterated that this was only demonstrated in other cell systems like K562 (liver cancer), HEp-2 (larynx cancer), A549 (lung cancer), and HeLa (cervical cancer) cell lines.

Caspase 3 is involved in the regulation of *bcl-X1* expression since in the study involving PMN, *bcl-X1* function was enhanced when it was downregulated and this abolished the role of *bcl-X1* (Weinmann, 1999). *Bcl-X1* has been found to be regulated in PMN cell lines by *caspase 3*. Cheng *et al* (1998) considered Bcl-X1 protein turnover as being more significant compared to its transcriptional regulation because turnover allows for cellular Bcl-X1 protein levels to be kept high, thereby inhibiting apoptosis while simultaneously increasing the detection of *caspase 3* genes.

Since *bcl-2* is the first activated gene in apoptosis, its regulation determines the amount of substrate that is available for *caspase 3* expression in cell lines that are induced to undergo apoptosis (e.g. by TNF-α mitogens) (Cheng *et al*., 1998). In PMN cells, the downregulation in Bcl-X1 protein levels induce apoptosis by there being a shift in ration between Bax-α and Bcl-X1 protein expression, brought about by the direct regulation of apoptosis by *caspase 3* (Yang and Korsmeyer, 1996).

There are additional factors that can promote programmed cell death, one being ageing of cell lines as was the case with PMN. Ageing of cell lines have been found to have a profound effect on apoptosis by decreasing the expression of Bcl-X1 proteins and thus enhancing the overall susceptibility of PMN to undergo programmed cell death. In addition, there is a controversy as to whether it is the increase in the Bax-α / Bcl-X1 ration or merely because of the ageing process, which may induce spontaneous apoptosis by enhancing the cell death process (Weinmann

et al., 1999). Therefore, in order to fully study apoptosis in any cell line, many members of the *bcl-2* gene family need to be tested so as to understand apoptotic gene expression, and to make more clearly justifiable conclusions about the process of apoptosis.

Apart from the three different pathways of apoptosis (see Hengartner, 2000), apoptosis involves 3 different phases (Zou *et al*., 1997). The first phase involves intracellular signalling in which case, a ligand binds onto a receptor that is embedded in the cell membrane and triggers a cascade of events that lead to apoptosis. The second phase involves the regulation of those events by the *bcl-2* gene family, and the third phase is apoptosis (an irreversible process that is confirmed by the cleavage and activation of caspases) (Zou *et al*., 1997).

Caspase-3, which is formed from the splicing of its precursor, pro-caspase-3 proteins, requires the activation of apoptosis protease activity factor (*Apaf-1* and *Apaf-3*) and the release of cytochrome c (*Apaf-2*) in order for its own expression to be detected. However, there has also been evidence that *caspase-3* activation can also occur independently of cytochrome c (Reed, 1997; Zou *et al*., 1997). In the study involving PMN, the inhibition of *caspase-3* activity confirmed the fact that apoptosis is not possible in that cell line (Weinmann, 1999). This highlighted the biological relevance of this gene in the process of apoptosis.

TNF-α, a cytokine (chemical messenger), has been proven to accelerate the process of apoptosis in (PMN) cell lines because it enhances Bax-α / Bcl-X1 ration. This has been said to be the result of direct proteolytic cleavage, and positive feedback mechanism, of *caspase 3*. Weinmann (1999) has thus concluded that the Bax-α/Bcl-X1-mediated control of apoptosis via *caspase 3*, is biologically important for achieving programmed cell death of old and infected cells.

However, apart from old and infected cell lines, there are numerous natural products and tested plant extracts that exhibit cytotoxic, anti-proliferative, and even proapoptotic activity toward cancer cell lines. These include compounds that suppress various kinds of tumours (Aggarwal *et al*., 2003; Duvoix *et al*, 2005; Sharma *et al*, 2005). In general, when medicinal compounds for cancer exhibit the characteristic of anticancer suppressors, they are considered useful therapeutic agents by people who suffer from specific type of cancers. If such natural and plant-extracted compounds are useful to the public, pharmaceutical companies are compelled to produce and manufacture them in the form of modalities (such as in the form of tablets, vaccines, capsules and various medicinal formulations etc.) to pharmacies and to hospitals and/or doctors for sale to patients.

Compounds trigger apoptosis via the three mechanisms, but it is extremely important to conceptualise the way in which these mechanisms operate when cancer cell lines are treated with apoptotic stimuli. This is imperative because understanding the pleiotropic activity (occurs when one gene influences multiple phenotypic traits) of administered compounds, determines the ultimate fate of apoptosis i.e. whether it occurs or not (hence the use of the MTT cytotoxicity assay and RT-PCR). For example, the pleiotropic activity of curcumin has been studied quite extensively to facilitate our understanding about the mediation of the regulation of multiple signalling pathways, so that oncologists and geneticists in search of cancer cures, know if the genes that govern these signalling pathways are operating efficiently (Sharma *et al*., 2005; Skommer *et al*., 2007). This makes genetic profiling of transcriptional changes during apoptosis pivotal. The pleiotropic activity of many plant extracts and fractions like *Bulbine natalensis* and *Bulbine frutescens* has never been documented in the literature, but by comparing the results of *caspase 3* and *bax* gene expression with the cytotoxicity results of *Bulbine* crude fractions (aqueous and organic solvent extractions) for the different plant organs (*B. natalensis*: root, corm and leaves; and *B. frutescens*: root and leaves), some idea about the relationships between the toxicity of crude fractions and their effect on the expression of the selected apoptotic markers (*caspase 3* and *bax*) can be envisaged. However, this is only one plant example of many. The two apoptotic markers can thus give insight into the mechanisms of apoptosis that are induced by crude plant fractions made using different solvents.

Depending on the type of cell line used during apoptotic studies, the level at which apoptotic gene expression is altered varies. This genetic alteration manipulates the apoptotic signalling proteins they encodes for. In addition, the tumour and metastasis supressors, cell adhesion and migrating factors, transcriptional/splicing factors, protein biosynthesis regulators and many other proteins undergo conformational changes (Skommer *et al*., 2007). In the HF49 cells line, curcumin has been found to have an altering effect on the expression of genes that encode for apoptotic signalling pathways, and since these pathways affect cancer cell status in terms of viability and/or cell death, it has been concluded that curcumin has the ability to exerts widespread effects in this cell type (genetic diversity) (Skommer *et al*., 2007). This provides information about the true chemotherapeutic value of curcumin. A simple example, in which the programmed cell death machinery can be demonstrated, is in unaffected cells when B lymphocytes contribute toward the dissemination of tumour cells, and result in lymphocytic leukaemia, B-cell malignancies, and acute lymphoblastic / leukaemia cells (see article published by Skommer *et al*., 2007). B-lymphocytes, whether they are infected or not, like other cells, have receptors and proteins on their surface that influence the locomotion B lymphocytes and tumorigenesis. These receptors include SDF-1 (ligand stromal cell derived factor-1) and the C × CR4 (C × C chemokine receptor 4). Skommer *et al* (2007) found that

when B-lymphocytes were treated with curcumin at pulse intervals of 8, 24, and 36 h, the expression of their locomotory receptors, were downregulated with increasing time. This was found using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). This downregulation indicated to those authors the possibility of apoptosis. A good example of the former situation is where curcumin does not act as an optimal antigen/ligand to the C × CR4 receptor on the B-lymphocytes. Other studies involving curcumin have found similar results indicating that receptor-ligand cell death is not only specific to B-lymphocytes (Skommer *et al*., 2007), but to other cell lines types as well.

The *C × CR4* gene is found in FL-cells and in normal germinal center B-cells (Duvoix *et al*., 2005). It has been discovered that when both types of cells are treated with monoclonal anti-C × CR4 antibodies, they conjugate with C × CR4 receptors which are present on the surface of the B-lymphocytes. However, it has been published that C × CR4 surface gene expression has been evident in the FL-cells, with their antibody neutralisation being the cause of apoptosis and inhibition of the proliferation of several NHL-derived cells (including the primary FL cells). This is of clinical importance in NHL models (Bertolini *et al*., 2002), but Vlahakis *et al* (2002) has doubts as to whether C × CR4 is actually involved in the induction of both the prosurvival or proapoptotic signalling pathway, because it is a G-protein coupled chemokine receptor.

Although the focus has been on many different kinds of genes and scenarios, it is important to remember that the expression and activation/inhibition of even one gene in any of the apoptotic mechanisms, determine the fate of the other genes as well. However, just like the study in follicular lymphoma B-cells, it is not possible to perform research and appraise results of all the apoptotic stimuli-responsive genes, since it may be beyond our comprehension. Therefore two genes, *bax* and *caspase-3*, have been found to be sufficient 'selected' based on their importance in programmed cell death machinery, in general – without taking homologous and heterologous selected gene expression into account. The B lymphoma study provided confidence that it is possible to use just two genes to give information on the expression of other genes in the apoptotic pathway, and to determine whether it induces apoptosis or not, and whether receptor-ligand binding occurs successfully or not may also be assumed, but off course one needs to determine if the compound being tested is hydrophilic or hydrophobic in nature first.

In addition to the genes involved in apoptotic mechanisms, there are also other genes that are responsive to apoptotic stimuli, and may shadow the actual process of apoptosis. These compound-responsive genes are *PER* (period homolog), *SR family* members (serine-arginine rich), *PPIF* (gene encoding cyclophilin D), *PTPN6*, *PTPN7 (*gene encoding protein thyrosine phosphatase), *DuSP5* (gene encoding dual-specificity phosphatase 5), *CDC14B* (a functional ortholog of budding yeast), *NQO1*, *TR1* ('apoptotic decision-making genes'), *CD20* (gene that encodes the CD20 ligand receptor) and *CCL2* (chemokine C-C motif ligand 2). To keep things universal, the discussion on the above mentioned genes would be kept as general as possible so that it is applicable to various types of apoptotic stimuli since there are many plant extracts and fractions that are novel and of medicinal importance.

As we know, all the cells of our body (including infected cells that are not part of the body), except the red blood corpuscles, are nucleated. Situated within the cytosol of our cells are the mitochondria, nucleus and the cytoskeleton. The mitochondria is involved in energy production, the nucleus regulates the activities of the mitochondria and other cellular processes that involve transcription and translation, and the cytoskeleton forms the backbone of the cell, something like the structural framework. When a cell is subjected to apoptotic conditions, the mitochondria, nucleus and the cytoskeleton are responsible biological indicators that determine the ultimate fate of such a cell, but are driven by the expression of various apoptotic specialist genes like *caspase 3*, *bax*, *caspase 9* and various others (read Hengartner, 2000 to gain more insight into the biochemistry of apoptosis).

When a cell is treated with apoptotic stimuli *in vitro*, the *PER* gene becomes overexpressed (Gery *et al*., 2005; Hua *et al*., 2006). This inhibits the growth of cancer cell lines and causes them to remain small in size. The fact that cell size is an indicator of cancer progression and/or pathogenesis (the ability of a 'sickness' to invade the body) means that a smaller cell size is an indicator of either cell cycle arrest and/or apoptosis. It has been found that mice which are deficient in *mPER2* (mRNA PER2 gene) genes are more prone to cancer (Fu *et al*., 2002). This means that *PER* gene expression provides a protective mechanism against apoptosis. However, when apoptosis occurs, the *SR family* member genes become regulated to a greater degree and this is particularly important for the regulation of alternative splicing of *caspase 9* into *procaspase 3* and *caspase 3* (Massiello and Chalfant, 2006), the indicators of apoptotic cell death. When apoptosis occurs optimally by regulation of *caspase 9* splicing, the stability of *caspase 9* mRNA and genetic shuffling (due to the process of translation) within the nucleus is conserved; and structure of the genome is maintained (Li and Manley, 2005). However, an important point to note is that although the process of alternative splicing within the nucleus of a cell has an influence on the cell cycle, by degrading DNA, the *SR family* members contribute to the stability of the nucleus by inhibiting the caspase-activated DNase, an enzyme that degrades DNA (Li *et al*., 2005). This ensures that the execution of apoptosis/cell death occurs in an organised fashion.

Even though apoptotic signal transduction events within a cell is controlled by the process of alternative

splicing of *caspase 9*, signal transduction in a cell treated with apoptotic stimuli is regulated by three important genes that form part of the apoptosis receptor-ligand mechanism. These genes are: *TPN6*, *PTPNT*, and *DuSP5*. They are responsible for cell growth, differentiation and oncogenic transformation and *DuSP5* silencing (an indication that *PTPN6* is present but it is not shown to be expressed/transcribed/translated) (Mizuno *et al*., 2002). These genes have been found to contribute to the aggressiveness and virulence of various types of cancers during the induction of receptor-ligand apoptosis in B-cells. In contrast, the expression of the *DuSP5* gene indicates the suppression of the growth of various types of cancers (Ueda *et al*., 2003).

The energy (ATP) provided to drive transcription/translation and the signal transduction process for apoptosis to occur, is provided by the mitochondrion, which has a unique structure. Apart from its large surface area due to the folding of the inner mitochondrial membrane, the mitochondrion also has pores that are embedded in the membrane which constitutes the component *PPIF*. *PPIF* is important in Ca^{2+} and oxidative cancer or normal cell damage (Baines *et al*., 2005). However, it has been reported that *PPIF* has no protective role in apoptotic cell death involving the mitochondrial pathway. A chemical inhibitor, cyclophilin D has actually been found to stimulate necrosis rather than apoptosis during the modulation of the cell cycle (Wlodkowic *et al*., 2006), but its effect is counteracted by the expression of the *SR family* member genes.

The structural backbone of our cells is the cytoskeleton which is made up of filaments (micro- and macrofilaments) and tubules (micro- and macrotubules). Associated with the nucleoli (the region of the nucleus in which protein synthesis/translation occurs), the centrosome (the region that holds the spindle fibres of chromatids during cell division so that each cell contains an equal number of chromosomes) and intra-cellular filaments, are the *CDC14 β gene* (Cho *et al*, 2005). The function of the tubules of the cytoskeleton is dictated by a tubulin diacetylate enzyme/gene called SIRT2. The *SIRT2* gene depends on ubiquitin to initiate cell death by destabilising the microtubules of the cytoskeleton, however, more research is ongoing to clarify the role of the *CDC14 β* in apoptosis. It has been discovered, though, that the expression of *CDC14 β* phosphatase in lung adenocarcinoma cells treated with apoptotic chemicals, is inhibited and this has been evidenced by slow cell division which inhibits the progression of tumours (Chen *et al*., 2007).

The mitochondria, nucleus and the cytoskeleton are embedded within the cytosol of the cell. However, the cytosol also contains genes/proteins that have some relation to the apoptotic process. As already mentioned, oxidative stress can also cause apoptosis. The *NQO1* gene ((NADCP) H: quinone oxidoreductase 1) encodes for a flavoprotein that is found within the cytosol of the cell. However, these genes only exist within the cytosol when when cells are exposed to chemical carcinogens, antioxidants, oxidants and radiation i.e. when the cell undergoes cell cycle arrest (Lu, 2005). During apoptosis, *NQO1* expression declines the function of *p53*; the initiator gene of apoptosis. *NQO1*, and *TR1*, both provide a defense mechanism toward cellular disintegration because even though they encode for different enzymes (*TR1* encodes for thioredoxic reductase 1), they are both involved in regulating intracellular processes such as DNA synthesis, transcription and cell growth (Chauhan *et al*., 2003).

The differentiation of B-lymphocytes into precursor B-lymphocytes cells requires *CD 20* antigens that are present in the cytosol of cells. It has been demonstrated that *CD20* gene expression is an excellent indicator that *CD20* is effective against the treatment of lymphomas. It has also been shown that apoptotic stimuli, like curcumin and rituximab, reduces the expression of *CD20* genes in FL cells which has prompted scientists to investigate the efficacy of curcumin and rituximab as a combination drugs for the treatment of cancers (Hiddemann *et al.*, 2005; Maloney, 2003).

Thus far only the *TPN6*, *PTPNT* and *DuSP5* genes/ligands have been described as important signal transduction genes involved in apoptosis. However, just like those genes, chemokines can only carryout their function if they are bound to a ligand. The binding of a chemokine to a ligand receptor on the surface of a cell (normal or infected), initiates a cascade of signal transduction events that result in cell death. *CCL2*, for example, encodes for the chemokine ligand 2 receptor, which becomes upregulated when cells are treated with apoptotic compounds like curcumin. This upregulation has been shown to cause macrophages to become infiltrated at the site of infection or tumorigensis, resulting in enhanced phagocytic death of the infected cell lines e.g. when cervical cancer cells are treated with cisplatin (Nakamura *et al*., 2004). Therefore the expression of signal transduction genes during apoptosis can obscure gene expression results when detecting apoptotic markers in cancer cell lines exposed apoptotic stimuli. This makes the study of signal transduction genes pivitol so as to eliminate their effect on the expression of apoptotic markers.

In smooth muscle cells (SMCs), cytokines have been found to have proapoptotic potential for example the cytokines, interleukin-1, tumour necrosis factor (TNF-α) and interferon (IFN-γ) which are produced by inflammatory cells (Mallat and Tedgui, 2000). These cytokines are known, in general, to recruit macrophages to the site of infection and induce apoptosis by receptor-ligand interactions (Fas-Fas-L) (Boyle *et al*., 2001). In Smooth Muscle Cells (SMCs), however, the process of apoptosis is mediated by DAP kinase proteins (Deiss *et al*., 1995; Cohen *et al., 1997;* Cohen *et al*., 1999; Raveh *et al*., 2000) which has been found to have an effect on the

expression of Bax proapoptotic proteins, due to their interaction and association with one another (i.e. Bax-DAP interactions).When the expression of Bax in SMC and antherosclerotic plaques increase, the plaques become instable, causing them to rupture. This results in thrombus formation at the site of injury (Mallat and Tedgui, 2001). Oxidative stress (due to reactive oxygen species), as encountered previously, oxidises LDL (low density lipid containing the apoptotic compound, ceramide) and oxysterols resulting in apoptosis in SMC (Kockx and Herman, 2000; Mallat and Tedgui, 2000). When the compounds mentioned trigger apoptosis in SMCs, DAP kinase mediates programmed cell death by first increasing the level of Bax and p 53 (activation of *p53*) which in turn causes *bcl-2* gene expression to decrease (Bennett *et al*., 1995).

DAP kinase is a pro-apoptotic calmodulin-regulated serine/threonine kinase which is known to carry interesting molecules like c-terminal death domain (Deiss *et al*., 1995). DAP regulates itself by autophosphorylation as well as by possessing an intrinsic kinase activity that is stimulated by Ca^{2+}/ calmodulin (Cohen *et al*., 1997).

It has been found in gene profiling studies (using RT-PCR) that when the level of DAP kinase in carotid endarterectomy specimens and mammary arteries were upregulated (14.9 fold), caspase and Bax activity were 3.3× and 3.6× less upregulated relative to DAP kinase (Martinet *et al*., 2002). In antherosclerotic plagues, Martinet *et al* (2002) found DAP to be overexpressed at the protein level. Futhermore, the RT-PCR failed to show differential expression of the TNF receptor (Martinet *et al*., 2002) which implied to those researchers that this cytokine was unable to effectively induce apoptosis, possibly due to mutation/s of the TNF-α receptor caused by the apoptotic stimuli perhaps.

It has been published that in regions of high lipid content in SMC, DAP kinase has a high affinity. Since the lipidic regions contain Bax proteins/genes, DAP kinase forms an association with those proteins; which initiates and mediates apoptosis (Martinet *et al*., 2002). Martinet *et al* (2002) found that when SMCs are treated with aggregated LDL (agLDL), the level of lipids in the cytoplasm increased after 3 days of treatment, and that this caused DAP kinase to be overexpressed transforming SMCs into foam cells (Martinet *et al*., 2002). It has been proven that the formation of foam cells, cause *bax* and *TNF-receptor 2* expression to increase; resulting in *DAP kinase* and *bax* gene transcription (Martinet *et al*., 2002). However, in order for programmed cell death to occur, caspase-3 or poly (ADP-ribose) polymerase-1 has to be cleaved and this was evidenced by the SMCs study in which uncleaved caspase 3 results in SMCs having normal cellular morphology with foam cells being adherent (see and read the study by Martinet *et al*., 2002).

Another example of apoptotic stimuli is the ceramide that is found in agLDL (Schissel *et al*., 1996). In antherosclerosis lesions, the apoptotic effect of ceramide depends on the action of DAP kinase (Pelled *et al*., 2002). Studies have shown that when SMCs are treated with ceramide in pulse-doses, the expression of DAP kinase protein and its RNA precursors increase only 6 h after treatment. However, a significant remark was made in that even though one would expect ceramide to cause cell death, it doesn't always. Martinet *et al* (2002) found that SMCs were ambiguous by staining positive under propidium iodine after 24 h of treatment, yet being annexin V and cleaved caspase-3 negative (Martinet *et al*., 2002). Inbal *et al* (2002) and Martinet *et al* (2002) suggested that could be due to the possibility of autophagy (type II programmed cell death) given the fact that the overexpression of DAP kinase in human plague cause cell death independent of caspases, or because DAP kinase requires additional apoptotic factors like chemical reagents, enzymes etc. to achieve apoptosis (type I cell death).

Therefore it is evident that apoptosis is a very complicated and sophisticated process from a genetic, biochemical, pathological, immunological, molecular and physiological perspective. In SMCs, apoptosis is a major event that utilises many proteins, but like other cell lines it depends on the cleavage of caspase proteins which are regulated by posttranscriptional mechanisms, protein-protein interaction, post-translation modifications, e.g. phosphorylation, proteolytic cleavage, glycosylation and subcellular localisation regulation (Martinet *et al*., 2002). However, like all cells, SMCs also require a balance between proapoptotic and antiapoptotic proteins for them to be viable (Martinet *et al*., 2002).

Some cell lines (like SMCs) undergo apoptosis by differential expression of Fas-signalling molecules and by the transcription factor nuclear factor-KB, which controls the expression of the inhibitor of apoptosis protein-1 (Apaf-1) (Erl *et al*., 1999). In human cancer cell lines, for example, the expression of DAP kinase mRNA and proteins are lost, and thus contributes toward tumorigenesis (Inbal *et al*., 1997). In the case of SMCs, it may be that additional factors in the vessel walls of human plaques in relation to lipid uptake, cause activation of the *DAP kinase* gene and that this could be the reason as why *DAP kinase* gene expression was 3× higher compared to *Bax* gene expression (Martinet *et al*., 2002).

The molecular events/genetics involved in the induction of apoptosis is important to develop treatment strategies for neurological, muscular, blood and other types of cancers. The *p53* gene regulates apoptosis, as already mentioned. However, the overexpression of *p53* also triggers apoptosis in many cell types including, cultured cortical, hippocampal, and sympathetic neurons (Slack *et al.*, 1996; Xiang *et al*., 1996; Jordan *et al*., 1997).

When the DNA in infected or old cells becomes damaged, the *p53* gene mediates cell cycle arrest and this helps the damaged cells to be killed by preventing their

growth and proliferation. For example, damaged nerve cells are ridded and regulated by the association of *p53* with *TrKA* and *p75* neutrophil receptors (Aloyz *et al*., 1998). A discussion on the latter two genes is irrelevant to the direct process of apoptosis, and would therefore not be discussed in further detail. In mature neurons, *p53* is an important regulatory molecule when nerves are damaged (Hughes *et al*., 1999). For example, in mice that possess a null mutation for the *p53* gene, brain damage induced by isclaemia or kianic acid appears to be much less (Crumrine *et al*., 1994; Morrison *et al*., 1996) compared to the cultured neurons from mice that are *p53*-deficient (Xiang *et al*., 1996, 1998). However, mice that are *p53*-deficient are also susceptible to brain damage caused by glutamate (Xiang *et al*., 1996, 1998). In this example, kianic acid and glutamate are examples of apoptotic stimuli, and it indicates that the status of the cell lines possess certain apoptotic genes, is important to consider prior to conducting scientific studies because not all cell lines that undergo apoptosis possess the same apoptotic markers e.g. K562 cell lines are *p53* deficient, but are susceptible to the process of apoptosis via all three mechanisms of apoptosis (read Hengartner, 2000 for a detailed explanation of the mechanisms of apoptosis)

When the *p53* gene mediates apoptosis, it initiates the first gene of the apoptotic pathway, *bax*. The function of the bax gene differs with the type of cell being studied (Ding and Fisher, 1998). Studies have shown that *bax* gene expression levels do not increase when cells are treated with camptothecin or radiation in cortical and hippocampal neurons (Johnson *et al*., 1998; Xiang *et al*., 1998). However in those cell types, Bax proteins are translocated from the cytoplasm to the mitochondrial membrane, where Bax proteins forms pores in the membrane of the mitochondrion and results in depolarisation and the release of cytochrome c (Wolter *et al*., 1997; Goping *et al*., 1998; Zhang *et al*., 1998). Cytochrome c enhances the interaction of *Apaf 1* with *caspase 9* causing the activation of the caspase cascade (Li *et al*., 1997; Zou *et al*., 1997). As it is already known, Bax promotes cell death when it is in its homodimeric form. In addition, Bax is inactive in its heterodimeric form with its anti-apoptotic family members. This indicates that *bax* can be activated when its anti-apoptotic members are inactive (Oltvai and Korsmeyer, 1994).

In different cell lines, *p53* regulates apoptosis at different degrees. Since the *bax* gene has consensus sequences in its promotor region that are analogous to the *p53* gene, it is involved in apoptosis. This is the reason as to why *bax* is regulated by *p53* gene expression; transcriptionally, in neuronal cells as well as in other cell systems. The *bax* gene has been believed to modulate the *caspase 3* gene expression in its proenzyme apoptotic signalling cascade, at least until it is cleaved into a cysteine proteinase (Miyashita *et al*., 1994; Miyashita and Reed, 1995).

Caspases are present in a number of different forms within old and infected cells. To determine whether caspases (*caspase 1, 2* and *3*) activated drug *p53*-mediated cell death, researchers infected cellular granular neurons (CGNs) with Adp53 (a recombinant adenovirus that has an expression cassette for the human *p53* gene). They found that *caspase-3* was activated in a time-dependent manner relative to the control neuron cells (neurons not infected with Adp53; but instead with AdLacZ, a recombinant adenovirus that has an expression cassette for the *β-galactosidase* reporter gene) in that there was a 3 fold increase in *caspase 3*-like activity after 2 days of treatment, and a 13 - 14 fold increase after 3 days. Western blot analysis confirmed these results by showing a decrease in the level of proenzyme caspase 3 protein during *p53*-mediated cell death. The same results were obtained for *caspase 1*, but western blot analysis indicated no cleavage of the procaspase 2 protein at 48 and 72 hrs. This proved that *caspase 1* and *caspase 2* have no role in neuronal apoptotic cell death, but that apoptosis is induced by *p53* (Cregan *et al*., 1999).

Since *p53* has been reported as a mediator of apoptosis and *bax*, the first activated gene of apoptosis, the need of *bax* gene expression in *p53*-mediated apoptosis is important to understand. Studies have shown that when mice, that are deficient and proficient (wild-type) in the *bax* genes are treated with Adp53 (experiment) or AdLacZ (control), the wild-type neurons exhibit a decrease in cell survival after 2 days of infection with Adp53, and that this increases with a loss of 80% in cell viability by the 4^{th} day. Contradictingly, the *bax*-deficient neurons exhibit a marginal decrease in cell death while the control treated neurons remained viable for about 5 days after infection. The fact that *bax*-deficient neurons did not survive after treatment, indicates that the *bax*-gene does not participate in apoptotic cell death in all cell types and that this is a unique feature seen in neurons (Cregan *et al*., 1999), but not limited to neurons.

To clear any misunderstanding about cell death in *bax*-deficient neurons, scientists screened such neurons for the presence of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) proteins. They found that in *bax*-proficient neurons, infection with Adp53 caused significant increase in the frequency of TUNEL-positive cells in comparison to the control-infected cells. In contrast, the *bax*-deficient neurons only showed an increase of 10% in TUNEL-positive cells. This implies that *p53*-mediated cell death requires Bax to initiate cell death in a variety of cell types. This reinforces the statement that *bax* is transcriptionally regulated by *p53*. However, it has been found that when *p53* is overexpressed, the levels of

Cortical, hippocampal, and sympathetic neurons (slack *et al.*, 1996) to consider that *p53*-mediated cell death does not necessarily involve Bax expression (Cregan *et al*., 1999).

The 13-fold increase in the activity of *caspase 3* in Bax-proficient neurons after 3 days, and absence of *caspase 3* activity in Bax-deficient neurons, indicate that Bax is required to activate *caspase-3* during *p53*-induced cell death (Cregan *et al*., 1999). Previous studies have shown that Bax plays a pivotal role in caspase activation when cerebellar granule neurons are deprived of potassium (Miller *et al*., 1997). This implies that Bax overexpression has the potential to induce *caspases* in neuron cells (Vekrellis *et* al., 1997; Martinou *et* al., 1998) and therefore mutations in the *bax* gene can possibly result in the production of cells against cell death. This emphasises that Bax has a protective function in addition to *caspase 3* activation.

It has been found *caspase 3*-deficient neurons are resistant to *p53*-induced cell death, that are infected with Adp32 in transgenic mice which carry a null mutation for the Bax or caspase 3 (CCP32). Researchers have found that in CCP32-deficient neurons, the neuronal cells appeared intact under the light microscope. This means that although *caspase 3* is involved in cell death, there is a possibility the effects of *bax* and *p53* are involved in additional cell death pathways (Cregan *et al*., 1999). Since *bax* and *caspase 3* are necessary indicators of apoptotic cell death, and since *caspase 3*-deficient neurons die and do not exhibit pykotic morphological changes (cleavage of DNA into interchromosomal fragments following the breakdown of the nucleus) suggest that it is possible for cell death to occur via an independent programme that does not show the typically expected results of *caspase 3* gene activation (Enari *et al.*, 1998; Sakahira *et al*., 1998).

CONCLUSION

In conclusion, there are many confounding variables that affect apoptotic studies. The results obtained during analysing apoptotic results from scientific studies depend on the genetic lineage of the cell line used and the compound (plant extract, natural product, chemical stimuli, radiation e.t.c.) administered to the cell line. Furthermore, the activation of apoptotic markers during the apoptotic cascade of events (see Hengartner, 2000) depend and are also independent of other genes such as *bax, p53* and those that are involved in transduction events of cell proliferation and cell death.

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