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MOLECULAR REGULATION OF NEURONAL APOPTOSIS IN PC12 CELLS

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Submitted to the Department of Biochemistry in partial fulfilment of the requirements for the degree of Master of Science

University of Ottawa
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ABSTRACT

Apoptosis is a normal and essential biological process for the differentiation and maintenance of cellular populations in multicellular organisms. One of the most significant advances in research into apoptosis has been the realization that it is a genetically regulated process. New gene induction is required not only for the onset of apoptosis but also for its progression in an orderly manner. In this study apoptosis was induced in neuronal PC12 cells by 2-5 μM VM26 (teniposide, a known apoptotic inducer) and changes in the expression of genes considered to be involved in apoptosis were examined by RT-PCR. Cell death, DNA fragmentation and morphological changes associated with apoptosis were evident within 8 hours after exposure to VM26 indicating that VM26 is capable of inducing apoptosis.

Within 8 hours of VM26 exposure, clusterin (TRPM-2) and IGFBP2 (Insulin-like growth factor binding protein 2) were both upregulated by 3 and 4 fold respectively. Cathepsin B (RSG-2) and RSG-3 (embigin) exhibited marginal changes in expression, only about 1.2 fold, over the 24 hour time course. Finally, IGFBP5 (Insulin-like growth factor binding protein 5) displayed a substantial decrease in expression, with a 4 fold decrease compared to control. Since IGFBPs appear to play a role in apoptosis, the effects of IGF-I treatment on cells undergoing apoptosis was examined. Treatment with IGF-I led to a dose-dependent protective effect on apoptosis induced by VM26.

In the presence of 200 ng/ml IGF-I, cell viability was 52.1% at 24 hours compared to non-treated cells, at 35.8% viability. In addition, IGF-I was able to modulate cell

viability, in a manner that did not involve induction of proliferation as assessed by cell counts in IGF-I treated and untreated PC12 cells.

Thus the findings in this study offer exciting information in the area of neuronal apoptosis, a phenomenon believed to be involved in a number of neurodegenerative diseases. In addition the results of our studies into the potential anti-apoptotic effects of IGF-I may ultimately lead to IGF-I therapy becoming an important treatment strategy for many of these neurodegenerative conditions. Furthermore, this research highlights the role of IGFBPs in the control of cell survival and indicates that this may be a productive area for further research.

DEDICATION

To my family

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ATP	adenosine 5' -triphosphate
bp	base pairs
BSA	bovine serum albumin
BCL-2	B-cell lymphoma-2
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5'-triphosphate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis N,N,N',N'-tetra-acetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
gm	gram
h	hour (s)
HPRT	hypoxanthine-guanine phosphoribosyl transferase
ICE	interleukin-converting enzyme
IGF-I	insulin-like growth factor-1

IGFBP2	insulin-like growth factor binding protein 2
IGFBP5	insulin-like growth factor binding protein 5
IL-3	Interleukin-3
IL-6	Interleukin-6
kbp	kilobase pairs
kDA	kilodaltons
mRNA	messenger ribonucleic acid
min	minute (s)
NAIP	neuronal apoptosis inhibitory protein
NGF	nerve growth factor
NP40	nonidet P-40
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PC12	pheochromocytoma cell line-12
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFGE	pulse field gel electrophoresis
pH	$-\log_{10}[H^+]$
RNA	ribonucleic acid
rpm	revolutions per minute
RSG2	regression selected gene 2 (cathepsin B)
RSG3	regression selected gene 3 (embigin)

RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
Taq	*Thermophilus Aquaticus*
TGF-α	transforming growth factor
TNF	tumor necrosis factor
tRNA	transfer ribonucleic acid
TRPM-2	testosterone repressed prostate message-2 (clusterin)
U	units of enzyme activity
UV	ultraviolet
V	volts
VM26	teniposide
w/v	weight per volume

INTRODUCTION

I. 1.0 OVERVIEW OF APOPTOSIS

Apoptosis is a normal and essential biological process for the differentiation and maintenance of cellular populations in multicellular organisms. In general, it is characterized by cell surface blebbing, cell shrinkage and chromatin DNA degradation. It can occur in response to both physiological and endogenous stimuli and, in contrast to other forms of cell death, does not elicit an inflammatory response. Apoptosis is an active energy-dependent process usually dependent upon the expression of specific regulatory genes together with endonucleases, proteases and several other genes whose functions are yet to be elucidated.

I. 2.0 APOPTOSIS VS. NECROSIS

The visible characteristics of apoptosis were originally described by Wyllie, Kerr and colleagues who coined the term "apoptosis" to describe this phenomenon and to distinguish this form of cell death from necrosis (Kerr *et al.* 1972;Wyllie, 1980). Necrosis is a type of cell death that occurs as a result of tissue damage and involves the disruption of cell membranes or membrane activity, influx of water from the extracellular environment, swelling of the cell organelles and irregular clumping of the chromatin DNA against the nuclear membrane (Kerr *et al.* 1972). The dying cell progressively swells and bursts releasing its contents into the extracellular milieu, resulting in the activation of an inflammatory response, with macrophage invasion and further damage to the surrounding cells (Wyllie, 1980).

In contrast, apoptosis is an organized, active process associated with characteristic morphological and biochemical changes. These morphological changes include a controlled reduction in cellular volume, condensation of chromatin around the periphery of the nucleus, and eventual dissolution of the entire cell into small membrane bound spherical vesicles known as apoptotic bodies (Kerr *et al.* 1972). These bodies are subsequently endocytosed by neighboring cells or resident macrophages, and digested by the lysosomes therein. This manner of removing the dying cell prevents the leakage of any of the intracellular contents and avoids stimulating the immune system. Biochemically, apoptosis usually requires new RNA and protein synthesis (Kerr *et al.* 1972). To describe genes involved in apoptosis the term *thanatogen* from the Greek word for death has been used. *Thanatogens* fall primarily into two categories: those that initiate apoptosis (primary *thanatogens*) and those that ensure the appropriate process of DNA fragmentation and phagocytosis of dying cells (secondary *thanatogens*).

I. 3.0 INCIDENCE OF APOPTOSIS

Apoptosis is a highly conserved physiological process that acts in concert with mitosis to preserve cellular homeostasis or to facilitate tissue re-modelling during development. It can occur in isolated cells that are genetically programmed to die during development, as in *Caenorhabditis elegans* (Yuan & Horvitz, 1990), or may involve all the cells in a regressing tissue such as the intersegmental muscles that are lost at ecdysis in *Manduca sexta* (Wadewitz & Lockshin, 1988). During development of higher vertebrates the cells in many tissues undergo apoptosis. For example, apoptosis of individual cells is

responsible for the topogenesis of neuronal distribution in the retina of the cat and defects in the process have been implicated in a number of retinopathies (Wong *et al.* 1994). Moreover, during neuronal development, widespread cell death occurs at the time when connectivity is established between neurons and their fields of projection (Cowan *et al.* 1984). It has been suggested that competition for limiting trophic factors helps control the size and specificity of the population of surviving neurons (Cowan *et al.* 1984).

Terminally differentiated cells undergo apoptosis in response to a variety of stimuli. For example, the epithelial cells in the distal tubules and collecting ducts of the kidney undergo apoptosis as a result of urethral obstruction, ischemia and hydronephrosis (Kennedy *et al.* 1994). In thymocytes and lymphocytes apoptosis can be induced using radiation (Sellins & Cohen, 1987) and by administration of glucocorticoids (McConkey *et al.* 1989; Cohen & Duke, 1984). Apoptosis can also be induced in hormone-dependent tissues such as the prostate and mammary gland through ablation of the appropriate trophic hormone. The epithelial cells of the prostate undergo apoptosis after castration (Kerr & Searle, 1973; English *et al.* 1989; Sandford *et al.* 1984; English *et al.* 1985; Tenniswood *et al.* 1990), while regression of the lactating mammary gland is induced by the falling prolactin levels associated with weaning (Walker *et al.* 1989; Strange *et al.* 1992). Hence, apoptosis plays a key role during normal development, in adult tissues, during regression of hyperplasia and in pathological atrophy (Kerr & Harmon, 1991). However, accumulating evidence suggests that it is not a single phenomenon but a series of morphologically and biochemically related processes (Clarke, 1990; Zakeri *et al.* 1995).

I. 4.0 MORPHOLOGY OF APOPTOSIS

Cell death in hormone dependent tissues has been particularly well characterized by electron microscopy which has revealed that in the prostate (Sandford *et al.* 1984) and mammary gland (Guenette *et al.* 1994a) apoptosis occurs predominantly in the secretory epithelium and, as in other cells, can be broken down into a number of distinct steps (Bursch *et al.* 1990a).

The first stage, or pre-condensation phase, is often not histologically visible and can last from only a few minutes to several hours after exposure to the apoptotic stimulus. During this phase, many of the genes that are necessary for apoptosis are induced *de novo* or recruited from other functions in the gland, and endonucleases responsible for DNA fragmentation are activated. The length of this phase has been shown to vary from cell to cell within the same tissue, a phenomenon that has been attributed to the micro-heterogeneity in the hormone or growth factor environment of each cell. The second stage of apoptosis and the first visible stage, is referred to as the cytoplasmic condensation phase. It involves the loss of the interactions between the dying cell and its neighbors, degradation of the extracellular matrix (ECM) and an overall decrease in the cytoplasmic volume (Wyllie, 1987). This phase is followed by the nuclear condensation phase, but often the stochastic nature of apoptosis in glandular tissue, makes the clear temporal separation of these two phases difficult to discern. In isolated cells such as granulosa cells, studies suggest that the cytoplasmic phase generally precedes the nuclear condensation phase. The nuclear condensation phase is characterized by fragmentation and margination of chromatin DNA, producing the hyperchromatic, pyknotic nucleus characteristic of apoptotic cells (Wyllie *et*

al. 1981;Wyllie *et al.* 1986; Kyprianou & Isaacs, 1988). The final stage in the process of apoptosis is the fragmentation of the apoptotic cell into several apoptotic membrane-bound *bags* or apoptotic bodies which are subsequently phagocytosed by neighboring epithelial cells or macrophages and degraded by the lysosomal enzymes either activated in the host cell or in the apoptotic body itself (Bursch *et al.* 1990a; Bursch *et al.* 1990b). Remarkably, this latter process occurs without the leakage of the intracellular components into the extracellular space, thereby ensuring that the complement cascade system is not activated, and that there is no inflammatory response (Kerr *et al.* 1972). It has been speculated that the asynchronous nature of apoptosis may help prevent eliciting a major immune response by giving time for surviving cells to engulf and remove the dying ones.

I. 5.0 BIOCHEMISTRY OF APOPTOSIS

One of the most striking characteristics of apoptotic cells is their pyknotic nuclei, resulting from the degradation of chromatin DNA into fragments and its re-distribution to nuclear margins. When extracted and electrophoresed on an agarose gel the chromatin DNA appeared to have been cleaved in an orderly manner, producing the characteristic nucleosomal DNA ladder which has now become recognized as one of the hallmarks of apoptosis in a variety of systems (Arends *et al.* 1990; Eastman & Barry, 1992)..

DNA fragmentation is believed to occur in a stepwise fashion. The first step, which is seen in virtually all cells undergoing apoptosis, involves DNA cleavage at inter-rosette sites generating DNA fragments that are 300 kbp in size. These fragments are subsequently cleaved at interloop sites generating fragments of 50 kbp. The final cleavage event occurs

in some, but not all cells, and produces oligonucleosome fragments (multimers of 200 bp), and is responsible for the hallmark DNA ladder (Walker & Sikorska, 1994). Detailed analysis suggests that this fragmentation of chromatin requires a number of independent enzyme activities each having significantly different cation requirements. It has been proposed that depending on the degree of DNA degradation the requirement for Ca^{2+}or Mg^{2+} may be different. In the first stage of degradation there is a dependence on Mg^{2+}, the second cleavage event requires the additional presence of Ca^{2+}, and the final cleavage requires elevated levels of Ca^{2+} (Walker *et al.* 1995). In keeping with this idea, increasing intracellular Ca^{2+} with ionophores, or decreasing Ca^{2+} with chelators can alter the rate of cell death, and level of DNA fragmentation (Kluck *et al.* 1994; Zhivotovsky *et al.* 1994). In some model systems there also appears to be a requirement for Mg^{2+} for apoptosis and DNA fragmentation (Cain *et al.* 1994). Given this variation in cation requirement it has been proposed that candidate nucleases such as DNase I (Peitsch *et al.* 1993), DNaseII (Barry & Eastman, 1993), and nuc 18/cyclophilinA (Montague *et al.* 1994), may be involved in different aspects of the process. To date, no single nuclease responsible for DNA fragmentation during apoptosis has been discovered, but the use of putative nuclease inhibitors to block apoptosis suggests nucleases do play a role in the process (Batistatou & Greene, 1993).

I. 5.1 DNA FRAGMENTATION

There is growing evidence to suggest that proteases may be required to facilitate DNA fragmentation, either to proteolytically activate the nuclease, or to initiate the

degradation of the nuclear matrix to render the DNA more accessible to nucleases. Recent studies suggest that a family of cysteine proteases may play a key role. Through studies on the nematode, *C. elegans* in which apoptosis is responsible for the elimination of 131 of the 1089 cells during development, a gene referred to as ced-3 was isolated, sequenced and determined to be essential for apoptosis (Yuan & Horvitz, 1990). Comparison to the sequences in the databases has demonstrated that ced-3 is homologous to the mammalian interleukin-1β (IL-1β) -converting enzyme (ICE). As a member of the cysteine protease family, this enzyme cleaves the 31 kDa IL-1β precursor between Asp-116 and Ala-117 residues to generate the mature 17.5 kDa IL-1β cytokine (Nicholson *et al.* 1995). There is considerable evidence to suggest that members of the (ICE)-like cysteine protease family serve as an intracellular proteolytic cascade that leads to the activation of endonuclease(s) responsible for DNA fragmentation during apoptosis. One of the substrates for an ICE/ced-3 like protease is poly(ADP-ribose) polymerase (PARP), an enzyme that plays a role in DNA repair, genome integrity, and inactivation of a putative endonuclease (s) believed to play a role in apoptosis. It has been proposed that in the early stages of apoptosis the sequential proteolytic activation of several ICE-like proteases culminates in the cleavage of PARP in the nucleus (Lazebnik *et al.* 1994). The cleavage of PARP, prevents its recruitmenent to sites of DNA damage, prevents the subsequent genome repair, and allows the activation of the Ca^{2+}/ Mg^{2+} dependent endonuclease which cleaves the DNA. The activation of this pathway has been strongly implicated in the induction of cell death in cells of neuronal and hematopoietic origin (Jacobson & Evan, 1994; Enari *et al.* 1995; Tewari *et al.* 1995).

Comparative studies using PFGE suggest that many cells undergoing apoptosis, may

not completely fragment their DNA (Dusenbury *et al.* 1991;Walker *et al.* 1991) even though the enzymatic activities necessary to do so are present in the isolated nuclei (Pandey *et al.* 1994). This demonstrates that, although all cells appear to have the enzymatic apparatus necessary for DNA fragmentation, subtle differences in chromatin structure, proteolytic activation, intranuclear pH as well as activating ion concentrations (Ca^{2+} and Mg^{2+}), or inhibiting ion concentrations (Zn^{2+} and K^{+}) may significantly alter the extent of DNA fragmentation in individual cell types (Barry & Eastman, 1992; Furuya *et al.* 1994).

I. 6.0 MOLECULAR BIOLOGY OF APOPTOSIS

One of the most significant advances in research into apoptosis has been the realization that apoptosis is a genetically regulated process. New gene induction is required, not only for the onset of apoptosis, but also for its progression in an orderly manner. The active nature of apoptosis has been demonstrated in numerous model systems, through the use of RNA or protein synthesis inhibitors which can delay or block apoptosis (Mesner *et al.* 1992).

I. 6.1 BCL-2

Interestingly, many of the genes with putative roles in apoptosis have also been shown to be aberrantly expressed in a number of cancers. One gene originally identified on this basis was Bcl-2. This gene was isolated from folliclar B-cell lymphomas in which there was the inappropriate survival of lymphocytes (Tsujimoto *et al.* 1984). Further studies into the function of this gene have revealed that both *in vitro* and *in vivo* Bcl-2 was able to block

or delay apoptosis. Overexpression of Bcl-2 in neuronal, hemopoietic, and thymocyte cells has also been shown to confer a resistance to apoptosis (Vaux *et al.* 1988; Hockenberry *et al.* 1990; Nunez *et al.* 1990; Sentman *et al.* 1991; Strasser *et al.* 1991; Garcia *et al.* 1992). Consistent with these findings Bcl-2 is found to be overexpressed in a number of hormone-refractory prostate tumors, suggesting that the aberrant expression of this protein may contribute to the inappropriate survival of tumors, and confer resistance to apoptosis induced by many standard chemotherapeutic agents such as methotrexate, vincristine, and cisplatin (Tung & Fritz, 1985; Colombel *et al.* 1993; Ohmori *et al.* 1993). Bcl-2 protein has been localized in several cell compartments, such as inner and outer mitochondrial membrane, nuclear envelope, endoplasmic reticulum, cell membrane and intracytoplasmic vesicles (Hockenberry *et al.* 1990; Jacobson *et al.* 1993). This broad distribution suggests it may be involved in the regulation of transmembrane transport, signalling, regulation of intracellular Ca^{2+} and activation of endonucleases which play a key role in apoptosis. Bcl-2 expression in the neuronal system may also protect neurons from death resulting from oxidative stress, and variations in Bcl-2 might reflect variations in the level of cell death in different cells in the brain (Migheli *et al.* 1994).

Bcl-2 activity is believed to be regulated by a 21 kDA protein displaying extensive amino acid homology to Bcl-2, known as Bax. By heterodimerizing with Bcl-2, Bax is able to counter the death repressing activity of Bcl-2, suggesting that the ratio of Bax to Bcl-2 in a cell an important regulator of apoptosis (Oltvai *et al.* 1993). Two forms of another gene, Bcl-x, also appear to regulate apoptosis by heterodimerizing with Bcl-2. The longer protein, Bcl-x_L helps Bcl-2 confer a resistance to apoptosis, while the shorter version, Bcl-x_s acts as

a dominant inhibitor of Bcl-2 function (Boise *et al.* 1993). A new homolog of Bcl-2 called Bak has been shown recently to promote apoptosis by binding to the anti-apoptotic members (Bcl-2 and Bcl-x_L) of the Bcl-2 family (Chittenden *et al.* 1995). Bcl-2 and its family members thus appear to play a central role in apoptosis, however further studies on its function, other members of the family and dimerization partners are necessary to understand its exact role.

I. 6.2 c-MYC

Another gene shown to play a role both in cancer and in apoptosis is the proto-oncogene, c-Myc. c-Myc is a cell cycle regulator, whose levels are elevated in the prostate following castration (Colombel *et al.* 1992). The expression of c-Myc in cells of fibroblast and myeloid lineages, has been associated with their increased susceptibility to undergo apoptosis (Askew *et al.* 1991; Bissonnette *et al.* 1992; Evan *et al.* 1992; Fanidi *et al.* 1992). The involvement of a proliferation-related gene like c-Myc suggests the existence of a high turnover state in the tissues, in which cell proliferation and cell death are likely to coexist, their relative quantities being determined by the microenvironment of the cell (Evan *et al.* 1992). The striking coincidence of proliferation and death within the same areas in many tissues gives credibility to this view (Shi *et al.* 1992). The c-Myc gene appears to act in concert with growth factors such as IGF-I, PDGF to regulate the level of apoptosis in a given cell. These growth factors appear to act downstream of c-Myc to prevent c-Myc induced apoptosis. It is also of note that Myc dimerizes with a partner protein, Max which then

interacts with another protein, MAD. This complex interaction is similar to that seen with Bcl-2 and its dimerization partners, indicating that interactions of this sort may play a key role in regulating protein function. (Amati *et al.* 1993).

I. 6.3 p53

Studies have also revealed a potential link between expression of the tumor suppressor gene p53 and apoptosis. This gene is commonly mutated in over half of all human tumors, implying that the gene likely plays a fundamentally important step in the pathogenesis of cancer (Hollstein *et al.* 1991;Vogelstein & Kinzler, 1992). A functional p53 gene appears to be essential for the induction of cell death in lymphocytes exposed to various cytotoxic agents as well as a number of other cell lineages including: thymic T cells (Clarke *et al.* 1993; Lowe *et al.* 1993), myeloid precursor cells (Lotem & Sachs, 1993), and intestinal epithelial cells (Clarke *et al.* 1994; Merritt *et al.* 1994). In its capacity as a cell cycle regulator, p53 appears to allow cell cycle arrest to facilitate DNA repair prior to DNA replication. In the event that DNA damage is too severe to be repaired, p53 appears to direct the cell towards apoptosis, or increase the susceptibility of the damaged cell to apoptosis (Miyashita *et al.* 1994; Selvakumaran *et al.* 1994). It is likely that more than one gene is involved in the regulation of a complex, highly ordered process such as apoptosis. In M1 leukemic cells for example, overexpression of Bcl-2 can prevent cell death suggesting that Bcl-2 may act downstream of both p53 and c-Myc to override their effects, and promote the inappropriate survival of leukemic cells (Lotem & Sachs, 1993).

I. 6.4 NAIP

Recent studies into genes associated with the control of apoptosis in the neuronal system, has revealed two genes that have the opposite effect of p53 and may actually help prevent inappropriate or extensive neuronal cell death. These two genes, NAIP (Neuronal Apoptosis Inhibitory Protein) and SMN (survival motor neuron gene) have been shown to directly decrease the extent of cell death in the neuronal system. Deletions in both these genes have been associated with the development of the autosomal recessive disease, Spinal Muscular Atrophy, a progressive neurodegenerative disease that is characterized by degeneration of the anterior horn cells of the spinal cord (Liston *et al.* 1996). Expanding knowledge in the area of genes both promoting and inhibiting apoptosis, may pave the way for the development of new therapies for a variety of conditions such as SMA and other neurodegenerative diseases.

I. 6.5 CLUSTERIN (TRPM-2)

The rat ventral prostate undergoing apoptosis has provided a good model for the study of novel genes involved in apoptosis. A number of different strategies have been used to identify such genes and the first gene to be cloned and characterized in this context was TRPM-2 (testosterone repressed prostate message 2), now referred to by convention as clusterin (Léger *et al.* 1987). This widely expressed, multi-functional glycoprotein is the most abundant protein induced in the prostate following castration and clearly plays an integral

role in the apoptotic death of secretory epithelial cells in the prostate and mammary gland. (Montpetit *et al.* 1986;Betuzzi *et al.* 1989; Strange *et al.* 1992;Wong *et al.* 1993). It has subsequently been shown that clusterin is associated with both developmental and induced cell death. However, its expression is not exclusively confined to dying cells (Griswold *et al.* 1986; Sylvester *et al.* 1991; Zakeri *et al.* 1992; Bursch *et al.* 1995). Clusterin has been implicated in reproductive cell function, lipid transport, complement regulation and endocrine secretion (Little & Mirkes, 1995). Its induction in disease states such as glomerulonephritis, polycystic kidney disease, renal tubular injury, atherosclerosis, myocardial infarction and neurodegenerative conditions including Alzheimer's disease and epilepsy has also been noted (Wong *et al.* 1994; Rosenberg & Silkensen, 1995). In retinitis pigmentosa, morphologic and biochemical data from photoreceptor cells undergoing apoptosis suggests that clusterin is induced in the retinal pigment epithelium and may play a role in tissue regression and membrane remodelling (Smith *et al.* 1995).

While the exact role of this gene in apoptosis has not been firmly established, the protein appears to be involved in controlling cholesterol efflux from the membranes of the dying cells, and facilitating the transfer of cholesterol to ApoA-I/HDL, and in protecting the membrane from complement fixation (Wilson *et al.* 1994). It thus serves to facilitate membrane remodelling and the removal of redundant membranes while protecting the membranes from recognition by the immune system. This is a critical process that must occur as the apoptotic cell shrinks, prior to fragmentation, into apoptotic bodies and is the key feature distinguishing apoptosis from necrosis. It has also been shown that over-expression of clusterin in prostate cancer cells protects them from apoptosis induced by

Tumor Necrosis Factor (TNFα) (Sensibar *et al.* 1995), suggesting that the absolute level of clusterin expression, or the timing of the expression, may be critical in determining the sensitivity of the cells to apoptotic stimuli.

I. 6.6 RSG 2 (CATHEPSIN B)

Further studies on gene induction in the rat ventral prostate has identified a sequence originally called RSG-2, but now referred to as cathepsin B, that increases significantly following castration and codes for a cysteine protease (Guenette *et al.* 1994b). Although the physiological role of cathepsin B is not yet understood, it is present in the lysosomes of many cell types (Kominami *et al.* 1985; Recklies & Mort, 1985) and has been implicated in the degradation of specific components of the extracellular and basement membrane. Of particular interest, is the potential role of cathepsin B in neurodegenerative conditions where increased enzyme activity has been reported in both multiple sclerosis, and in neuritic plaques associated with Alzheimer's disease (Cataldo & Nixon, 1990; Bever & Garver, 1995). In these plaques the enzyme is associated both with lysosomes and with extracellular lipofuschin granules indicating that the enzyme is likely secreted into the extracellular matrix. Cathepsin B degraded components of the basement membrane which is a prerequisite for the cytoplasmic condensation of a dying cell (Sloane & Honn, 1984). Temporally, the proteolytic activation of cathepsin B correlates with the expression and activation of a number of other extracellular proteases which form a proteolytic cascade that initially degrades any protease inhibitors, abolishing their effects, and subsequently degrading many components of the basement membrane. The induction of these ECM-proteases and other

lysosomal enzymes has led to the suggestion that the degradation of the ECM during apoptosis requires the co-ordinate activation of several lysosomal enzymes. The enzymatic activity disrupts the interactions between normal epithelial cells and the extracellular matrix leading to apoptosis, a phenomenon termed "anoikis" (Frisch & Francis, 1994).

I. 7.0 APOPTOSIS AND SURVIVAL SIGNALS

Unique cell surface molecules and extracellular matrix components, specific gene expression or repression, and the presence or absence of extracellular signals, all appear to work in concert to modulate apoptosis in different cell types. The external signals that lead to apoptosis are as varied as those that lead to differentiation and proliferation and can include both the withdrawal and the presence of these extracellular signals (Williams & Smith, 1993). Indeed, dependence on essential survival factors for suppression of apoptosis appears to be very widespread (Raff, 1992). In haemopoietic stem cells, for example, the primary function of colony stimulating factors appears to be suppression of apoptosis (Williams *et al.* 1990) and this effect allows an intrinsically determined pathway of differentiation to be followed. In contrast the stimulation of some cell surface molecules such as the TNF-α receptor and the APO-1 antigen (Fas antigen) can often induce cell death by apoptosis, although this is not always the outcome (Nagata & Golstein, 1995).

I. 7.1 GROWTH FACTORS AND IGFBPS

There is considerable evidence to suggest that soluble growth factors such as insulin-

like growth factor (IGF-I, IGF-II), Nerve Growth Factor (NGF), Epidermal Growth Factor (EGF), Transforming Growth Factor α (TGFα) and Fibroblast Growth Factor (FGF) may play a crucial role in preventing apoptosis. Recently, members of the insulin like growth factor (IGF) family have emerged as key physiological regulators of apoptosis (Arteaga, 1992; Pietrzkowski *et al.* 1992; Reeve *et al.* 1993). IGF-I for example, has been found to protect a number of cell types from cell death. These include, neurons (Deluca *et al.* 1996; Zawada *et al.* 1996), vascular smooth muscle cells (Bennett *et al.* 1995), myocardium (Buerke *et al.* 1995), preovulatory follicles (Eisenhauer *et al.* 1995; Chun *et al.* 1996), mammary tissue and breast cancer cells (Geier *et al.* 1994;Geier *et al.* 1995;Neuenschwander *et al.* 1996), terato-carcinoma (Granerus *et al.* 1995), prostate (Guenette & Tenniswood, 1994), blood cell and lymphocyte precursors (Muta & Krantz, 1993; Kelley *et al.* 1996; Minshall *et al.* 1996), mouse embryo fibroblasts (Sell *et al.* 1995; Li *et al.* 1996) and colorectal carcinoma cells (Wu *et al.* 1995).

Through interactions with its cognate receptor, IGF-I has been shown to prevent apoptosis induced by the drug VM26 in BALB/c3T3 cells (Sell *et al.* 1995) and apoptosis occurring in IL-3 dependent haematopoietic cells following withdrawal of IL-3 (Rodriguez-Tarduchy *et al.* 1992). Thus IGF-I appears to play a central role in tissue homeostasis, by repressing the induction of apoptosis, and allowing differentiation or mitosis to occur. The permissive effect of IGF-I appears to act downstream of the cell cycle regulator c-Myc gene, blocking the entry of the cell into the apoptotic pathway. Hence, both elevation of c-Myc and down-regulation of IGF-I appear to be necessary for the induction of apoptosis (Evan *et al.* 1992; Amati *et al.* 1993; Evan & Littlewood, 1993; Harrington *et al.* 1994).

Some of the most dramatic and best characterized examples of apoptosis occurring in response to growth factor removal has been in the central nervous system (Martin *et al.* 1988). The neuronal system has provided a good model for studying apoptosis and through these studies and others, it appears that, to exert their effects, growth factors like IGFs must not only interact with their cognate receptors but also with specific components of the ECM and specific binding proteins known as insulin-like growth factor binding proteins (IGFBPs). A number of IGFBPs have been identified in a variety of different tissues; however, much less work has been done on the potential role of IGFBPs in cell survival compared to the work done on IGFs. Guenette and Tenniswood (1994) demonstrated that IGFBP5 is dramatically induced early in the prostate following induction of apoptosis, and appears to associate with specific components of the ECM (fibronectin and tenascin) which are only expressed by the stroma after castration. This has lead to speculation that IGFBPs may attenuate the IGF signal, triggering apoptosis and leading to the induction of a cascade of other genes involved in the process, including cathepsin B and clusterin. However, more recently, Lee *et al.* (1996) have shown that IGFBP5 is associated with cell survival and another binding protein, IGFBP2 may be associated with cell death following ischemia-reperfusion injury of the perinatal rat brain. These observations are supported by those of Nordqvist *et al.* (1996) who showed that IGFBP2 levels increased in regions of the brain damaged by contusion injury. Furthermore, retinoic acid up regulates the expression of IGFBP5 in osteoblasts further supporting a role for this binding protein in cell survival (Zhou *et al.* 1996).

The role of IGFBPs in apoptosis, is not well understood; however, it is apparent that

they have widespread roles, not only in the prostate but also in other tissues. In the brain for example, the role of IGFs, and IGFBPs is being studied in the context of neurodegenerative conditions. Alzheimer's disease is characterized by deficits in memory, cognitive function and neuropathologically by the loss of neurons and the presence of neuritic plaques and neurofibrillary tangles (McKhann *et al.* 1984). Due to their putative function as neurotransmitters and neuroregulators in the CNS, neurotrophic factors have been suggested to play a role in Alzheimer's disease. In addition to the role of NGF, speculation is revolving around the role of IGFs and specific IGFBPs which are found to be elevated in the cerebrospinal fluid of some patients with Alzheimer's disease (Sandberg *et al.* 1988). It is suggested that elevations of IGF and specific IGFBPs may be an attempt by the neuronal system to compensate for neurodegeneration. *In vitro* studies have found that depending on the cell system and specific IGFBP used, they may stimulate or inhibit the biological effect of IGFs (Conover & Powell, 1991; McCusker *et al.* 1996). The role of specific IGFBPs appears to be dependent at least in part on the interactions of these proteins with specific components of the ECM underlying these specialized cell types. Thus while soluble growth factors such as IGF-I evidently have widespread role in different systems, further study will be necessary to improve our knowledge of their actions.

I. 8.0 APOPTOSIS AND THE EXTRACELLULAR MATRIX (ECM)

The ECM clearly plays an important role in cell adhesion, cell migration, and cell signalling. In addition, cell-specific growth factors and enzymes are immobilized in the ECM, at the cell surface, to facilitate their physiological functions and in some cases prevent

their proteolytic degradation. Recently, the ECM has also been shown to play a role in regulating protein secretion and gene expression in certain tissues by mechanisms involving both membrane and nuclear events, including the binding of some components of the ECM to transcription factors (Jackson *et al.* 1991).

The ECM varies from cell type to cell type, but in general contains a number of collagenous and non-collagenous glycoproteins and proteoglycan components including fibronectin, collagen, laminin, vitronectin and heparan sulfate proteoglycan (Walicke, 1989; Kofoed *et al.* 1990; Paulsson, 1992; Damsky & Werb, 1996). These components play a key role in promoting neuronal differentiation, and survival and may operate through several candidate neuronal receptors. Interestingly, it has been shown that certain ECM components, namely heparan sulfate proteoglycans, are found specifically colocalized to amyloid plaques in the brains of Alzheimer's patients, and it has been suggested that heparan sulfate proteoglycan, bearing neurotrophic activity, are synthesized and deposited in the developing plaque by astrocytes and neurons to stimulate the growth of other neurites into the plaque (Jackson *et al.* 1991).

In the developing neuronal system, a growing body of evidence suggests that both glia-derived and ECM-derived molecules as well as other local cell-cell interactions between neurons and non-neuronal cells, may play a role in preventing apoptosis of developing neurons. Thus it is becoming increasingly apparent that any comprehensive and valid conceptualization of the events that control neuronal apoptosis must take into account the role of targets and afferents as well as neuronal interactions with non-neuronal cells and the ECM (Oppenheim, 1991).

I. 8.1 FAS/APO-1

Recently, a cell surface receptor called Fas/APO-1 belonging to the NGF/TNF family of receptors was isolated. Fas/APO-1 is believed to play a role in the development of the immune system and the maintenance of tolerance to self antigens, thereby preventing autoimmune diseases such as lupus from occurring. Fas/APO-1 is a 48 kDa membrane-spanning glycoprotein receptor, which, upon binding with an anti-Fas/APO-1 monoclonal antibody or Fas-ligand signals the lymphocyte to die by apoptosis (Elkon, 1994). The Fas/APO-1 system not only provides another model in which to study apoptosis, but also opens up the possibility of activating apoptosis using antibodies directed against cell surface molecules as a therapeutic modality (Cohen, 1993).

I. 8.2 RSG-3

Another group of molecules shown to play a role in cell surface interactions, as cell adhesion molecules and cell surface receptors, belong to the Vκ immunoglobulin gene superfamily. (Huang *et al.* 1990). Typical examples are cell surface immunoglobulins, T-cell receptors (Thy), neuronal cell adhesion molecule (N-CAM), myelin-associated glycoprotein (MAG), major histocompatibility antigen class I and II, and interleukin-6 (IL-6) receptors (Williams & Barclay, 1988; Williams & Gagnon, 1982). One of these family members referred to as RSG-3 (Regression Selected Gene 3) has been shown to be upregulated in rat ventral prostate following castration (Sridhar, 1993). RSG-3 is the rat homolog of the murine 70 kDa glycoprotein, embigin, which is displayed on the cell surface and shown to enhance cell-substratum adhesion (Huang *et al.* 1990). It has been speculated that as an

epitope it may play a role in the recognition and subsequent phagocytosis of apoptotic bodies or alternatively, may serve as a cellular receptor for cognate epitopes on neighboring cells. The function of this gene and other members of this family is yet to be fully understood. However, its expression in systems undergoing apoptosis may provide insight into their role in cell-cell or cell-surface adhesion, their function during cell remodelling and disruption of membranes that occurs during apoptosis (Sridhar, 1993).

I. 9.0 APOPTOSIS IN THE NEURONAL MODEL SYSTEM

To date a significant amount of research into the molecular components of cell death has been performed on regressing prostate tissue, one of the most characterized apoptotic model systems. Following removal of the trophic hormones required for tissue maintenance, the rat ventral prostate undergoes rapid apoptosis, and has served as a good model for studying apoptosis. Although differences between various systems undergoing apoptosis exist, some fundamental similarities appear to exist, allowing parallels to be drawn between the well established prostate model and the relatively poorly established neuronal model.

It is now widely accepted that apoptosis is an integral part of the neuronal system, and its potential importance is only now being realized. Homeostasis in every system is dependent upon a delicate balance of cell proliferation and cell death which, if disturbed, can result in disease. In the neuronal system, insufficient apoptosis may thus contribute to the development of various tumors. On the other hand, premature or extensive apoptosis may be the key to a number of neurodegenerative diseases such as spinal muscular atrophy, Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral

sclerosis, retinitis pigmentosa and various forms of cerebellar degeneration (Thompson, 1995).

During normal neuronal development in mammals up to 30% of cells appear to die by apoptosis which helps to ensure the precise matching of pre-synaptic and post-synaptic pools of cells. The determination of neuronal survival appears to occur via competition for a limited supply of target-derived neurotrophic support. In culture, studies suggest neuronal cells rapidly undergo apoptosis in the absence of trophic factors; going from an apparently healthy state to one marked by degeneration, death and phagocytosis in a very short time period lending support to the competition theory (Oppenheim, 1991). The active nature of apoptosis in this system has been demonstrated through the use of RNA and protein synthesis inhibitors which can block or delay the process (Mesner *et al.* 1992), as do nuclease inhibitors (Batistatou *et al.* 1993). This feature is similar to other model systems of apoptosis. Apoptosis can also be seen following the treatment of neurons with a variety of drugs. Both trophic factor withdrawal and drug treatment have become popular methods for studying apoptosis in cultured neurons. An advantage of studying apoptosis in cultured neurons as opposed to apoptosis in tissues, is the availability of large homogeneous cell populations which facilitate molecular and biochemical analysis (Mesner *et al.* 1992). In addition it is possible to carefully control the microenvironment of the cells which can improve the reproducibility of the results.

I. 10.0 APOPTOSIS AND NEURODEGENERATIVE DISEASE

A cardinal feature of human neurodegenerative disorders is the extensive cell death

of a specific group of neurons. Presently, the molecular mechanisms which underlie neuronal cell death are poorly understood, but it has been suggested that both endogenous and/or exogenous neurotoxins may play a critical role in inducing apoptosis in some, if not all neurodegenerative diseases (Walkinshaw & Waters, 1994). For example, in Alzheimer's disease the principal protein component of the amyloid plaques that build up in the brain, known as Aβ peptide, has been shown to induce apoptosis in neurons. This leads to the profound degeneration of many types of neurons and may account for the numerous neurological deficits that patients afflicted with the disease encounter (Laferla *et al.* 1995). Similarly, in Parkinson's disease as yet unidentified environmental or metabolic toxins may be responsible for inducing neuronal apoptosis (Walkinshaw & Waters, 1994). Recent *in vitro* studies suggest that this apoptosis may be inhibited by the administration of nuclease inhibitors, and in fact this may be the mode of action of the drug Desipramine which is presently used to treat Parkinson's disease. The active nature of apoptosis, also might make some neurodegenerative conditions, amenable to therapeutic intervention using specific inhibitors of RNA or protein synthesis (Walkinshaw & Waters, 1994).

Competition for neurotrophic factors plays a key role in the neuronal system where neurons receiving enough trophic factor survive, while those that don't, perish. Motor neuronal disorders, such as the loss of spinal cord motor neurons in amyotrophic lateral sclerosis or the degeneration of spinal cord motor neuron axons in certain peripheral neuropathies, present a unique opportunity for therapeutic intervention with neurotrophic factors such as IGF-I. The administration of a growth factor such as IGF-I, may exert a neuroprotective effect on cells by suppressing a cell death program which could be activated

in some forms of neuropathology (Lewis *et al.* 1993). It is unclear exactly how these neurotrophic factors effect survival, but it has been postulated that they may act through the Bcl-2 proto-oncogene.

The identification and analysis of genes involved in apoptosis may not only provide basic insight into the development of the neuronal system, but also contributes to our understanding of pathological disorders and reveal potential targets for treatment strategies. It is also possible that the mechanisms underlying cell death related to the disregulated expression of genes or the lack of trophic factors, may play a crucial role in the idiopathic degeneration of neurons occurring later in life, associated with aging (Milligan *et al.* 1994). Undoubtedly further studies are required, which will help advance knowledge in this exciting area of apoptosis--an unquestionably widespread phenomenon.

I. 11.0 STATEMENT OF PURPOSE

In this thesis the role of survival factors and genes that may be involved in the interactions between cells and the ECM in the death of PC12 neuronal cells was examined. The DNA damaging agent VM26 (teniposide) was used as a powerful inducer of apoptosis, as indicated by assessing three markers of apoptosis. In addition, changes in the steady state expression of the genes clusterin, cathepsin B, IGFBP2, IGFBP5 and RSG-3 were evaluated during various stages of the apoptotic process, using an RT-PCR approach. Changes in the expression kinetics of these genes have been shown in other model systems of apoptosis, and hence these genes were chosen for study in the neuronal system, where apoptosis is undoubtedly playing a very significant role in both normal and pathological conditions.

MATERIALS AND METHODS

PART 1: INDUCTION OF APOPTOSIS IN PC12 CELLS BY VM26

M. 1.0 CELL LINE

The rat adrenal pheochromocytoma cell line (PC12), ATCC CRL 1721 used in this study is a single clonal line established by Greene and Tischler (1976) from a transplantable rat adrenal pheochromocytoma. PC12 cells respond reversibly to nerve growth factor (NGF) by induction of the neuronal phenotype. The clonal cells synthesize and store the catecholamine neurotransmitters, dopamine and norepinephrine, but not epinephrine. PC12 cells are useful model systems for neurobiological and neurochemical studies (Greene & Rein, 1977).

PC12 cells were grown in 80 cm^2 flasks in 20 ml of RPMI 1640 (Gibco BRL, Bethesda, MD.) culture medium supplemented with 5% fetal bovine serum (FBS), 10% horse serum and 20 μg/ml of gentamycin sulfate (Sigma Chemical Co., St. Louis, MO.). Cells were grown at 37°C in incubators with a humidified atmosphere of 95% air, 5% CO_2. Cultures were treated with 5 μM teniposide (VM26, 15 mM stock solution in ethanol; Bristol-Meyers Squibb, Candiac, PQ) and/or IGF-I (0.25 μg/μl stock, UBI, Lake Placid, NY) for varying lengths of time as indicated. Control cultures were treated with the equivalent volume of vehicle.

M. 1.1 CELL MORPHOLOGY DURING APOPTOSIS

PC12 is a cell line that grows partly attached to a substratum and partly in suspension.

For morphological studies cells were initially grown in flasks and then transferred to glass coverslips in a 6 well plate two days prior to the treatment with VM26, to allow them to attach. At the indicated time points after treatment, coverslips bearing the cells were rinsed briefly in 10X PBS, fixed for 5 min in 3% paraformaldehyde (J.B. EM Services Inc., Dorval, Quebec) in PBS, immersed for 1 min in 1 μg/ml of Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) in PBS for 1 min at room temperature then mounted on slides in 116-120 μl Vectashield (Vector Laboratories Inc., Burlingame, CA) mounting medium. Cells were viewed through an Olympus BX50 Microscope equipped with phase and epifluorescence optics and photographed using Ilford XP2-400 film.

M. 1.2 CELL COUNTING AND VIABILITY

Drug-treated and control PC12 cells were flushed from the sides of the flask by pipetting and pooled in a volume of 40 ml at each time point. 50 μl of cells were removed from the pooled fraction and mixed 1:1 (v/v) with a 0.4% trypan blue solution (Sigma Chemical Co., St. Louis, MO). Cells were then counted using a hemocytometer and cell viability was assessed based on trypan blue exclusion.

M. 1.3 PULSE FIELD GEL ELECTROPHORESIS (PFGE)

M. 1.31 SAMPLE PREPARATION

PC12 cells (0.5-1.5 x 10^6 cells, equivalent to 10-20 μg DNA) were flushed from the sides of the flask, pelleted and washed once in 1 ml of nuclear homogenization buffer (15 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine,

60 mM KCl, 15 mM NaCl) and resuspended in 100-250 µl of the same nuclear buffer, ensuring a uniform suspension. The suspension was added to an equal volume of 1.5% low melting point agarose solution melted and maintained at 37° C, containing 10 µl of proteinase K, 20 mg/ml stock, (Gibco BRL, Bethesda, MD). The slurry was mixed quickly and added to a 1 cc syringe which was placed upright in the refrigerator for 5 min to solidify the slurry. The plug was then removed and placed into a 15 ml tube containing 3 ml TEEN digestion buffer (10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 25 mM EDTA, 10 mM NaCl), 150 µl 10% (w/v) laurylsarcosine and 20 µl proteinase K solution (20 mg/ml), and incubated overnight with rotation at 37° C. Following incubation, the plug wash was removed and placed in a tube with 2X volume of absolute ethanol, and 1/10th volume of 2.5 M sodium acetate, pH 5.2, at -20° C overnight. The plug was rinsed for 30 min in fresh, ice-cold TE buffer (10 mM Tris-HCl pH 8.5, 1 mM EDTA) with rotation. After washing, the plug was retracted into the syringe and stored at 4°C until time of loading on the gel.

M. 1.32 SAMPLE ANALYSIS BY PFGE

PFGE was carried out using an Autobase Electrophoresis System (Q-life,Kingston, ON) using a programmed ROM card designed to separate fragments in the 0.2-800 kbp range (Romcard No. 3, 24 h runtime). The electrophoresis buffer, 1X TBE (10X TBE stock is 0.892 M Tris, pH 8-8.5, 0.89 M boric acid, 25 mM EDTA) was recirculated and maintained at a steady temperature of 20-24° C (3-4 buffer changes/hour, cooled to 14° C). Electrophoresis was carried out through a 0.8% agarose gel. The plugs were prepared as described above, sliced from the syringe (3 mm width containing DNA from (0.5-1.5 x 10^6

cells) and loaded into dry wells and sealed with 1% low melting point agarose precooled to 37° C.

Using the pre-programmed conditions the gel was run and then stained with 200 µl/L of 10 mg/ml stock ethidium bromide for 30 minutes. Destaining was done in warm water and visualization was achieved by placing the gel on a transilluminator. The gel was either photographed on Polaroid MP4 film or the image was captured using a Panasonic CCD Camera and Data Acquisition System.

PFGE DNA size markers were run on each gel as described in the Figure legends. They included a 1kb DNA ladder (size range 1.0-12.0 kbp; Gibco BRL, Bethesda, MD.), HindIII digest of lambda DNA (size range 2.0-23.1 kbp; Gibco BRL, Bethesda, MD.), yeast chromosomes (size range from 225-1900 kbp; New England Biolabs, Beverly, MA.) and low range DNA size markers (size range from 0.13-194 kbp; New England Biolabs, Beverly MA.).

PART 2: GENE EXPRESSION IN APOPTOTIC PC12 CELLS

M. 2.0 RNA ISOLATION FROM PC12 CELLS

To ensure the isolated RNA was of high quality, all solutions used in the preparation of RNA, except those containing Tris-HCl, were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) overnight, then autoclaved (Fedorcsak & Ehrenberg, 1966). Tris-HCl solutions were prepared in DEPC-treated and autoclaved distilled water. All glassware and pipette tips were autoclaved prior to use, gloves were worn throughout the procedure and the RNA was maintained on ice at all times.

Total RNA was isolated from PC12 cell cultures using a modification of the detergent lysis method (Favoloro *et al.* 1980). Cells were grown to confluency in tissue culture flasks and were harvested at the indicated time points. After repeated pipetting to flush cells adherent to the flask, the cells were pelleted by spinning for 5 min at 1000 rpm at 4°C. Media was removed and the cell pellet was washed once in 1X PBS and further pelleted by centrifugation at 13,500 rpm. The cell pellet was resuspended in TSM (10 mM Tris-HCl, pH 7.4, 140mM NaCl, 1.5mM $MgCl_2$) + 0.5% (v/v) NP-40, vortexed lightly and left on ice for 2-3 min. The suspension was then spun for 10 sec in a microfuge to pellet the cells. The supernatant was removed to a clean tube and mixed with TSE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) + 0 .2% (w/v) SDS. Two extractions with 50% phenol chloroform: isoamyl alcohol (24:1 v/v), were then carried out. The RNA was precipitated from the aqueous phase by 1/20 volume of 3 M NaCl, and 2 volumes of 100% ethanol at -20° C overnight. The pellet was recovered, dried, redissolved in sterile double distilled water and stored at -70°C.

M. 2.1 RNA INTEGRITY AND QUANTITATION

The integrity of the RNA was determined by electrophoresis of 5 µg total RNA on a 1% agarose gel in 1X TAE buffer (40 mM Tris-acetate, pH 8.5 and 2 mM EDTA) containing 1 µg/ml of ethidium bromide. The gel was then photographed on a transilluminator using a Polaroid DS-34 camera and positive-negative film number 55. The RNA was quantitated by measuring the absorbance at 260 nm, based on a 40 µg/ml solution of RNA giving an absorbance at 260 nm of 1.0.

M. 2.2 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

M. 2.21 REVERSE TRANSCRIPTASE REACTION

500 ng of RNA was diluted to 12 µl, incubated at 70° C for 5 min and then quick-chilled on ice. To each RNA sample 4 µl of 5X RT buffer (250 mM Tris-HCl pH 8.3, 275 mM KCl, 15 mM $MgCl_2$), 2 µl 100 mM DTT, 1 µl (10pmoles) of Downstream primer, dNTPs (2 mM of each dNTP), and 1 µl (5U) of Superscript (BRL, Burlington, ON) were added. The reaction was incubated at 42° C for 1h, and then at 50° C for 30 min. Yeast tRNA (GIBCO BRL, Bethesda, MD) was used as a negative control. Water in the place of RNA was also used as a negative control for the RT-PCR reaction. RNA isolated from the rat ventral prostate 4 days after castration and from rat kidney was used as a positive control.

M. 2.22 POLYMERASE CHAIN REACTION (PCR)

5 µl of the reverse transcription product were then added to tubes containing 5 µl 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 5 µl of upstream and downstream primer (10 pmoles each), 6.0 µl 10 mM dNTPs, 7.0 µl 50 mM $MgCl_2$. The 50 µl reaction mixture was placed in a 0.2 ml thin-walled PCR tube, and placed in the Stratagene Robocycler 96 Temperature Cycler. A hot start was performed and 1 µl Taq Polymerase (Gibco BRL, Bethesda, MD) was added to the reaction mixture following incubation at 95°C for 5 min and 72°C for 1 min. The reaction mixture was subjected to 30 cycles of 95°C (denaturation) for 1 min, 55°C (annealing) for 1 min and 72°C (extension) for 1 min. Following the 30 cycles, the PCR reaction tube was incubated for 1 cycle at 72°C for 15 min and cooled to 15°C.

M. 2.23 RESOLVING RT-PCR PRODUCTS

35 µl of the PCR product was combined with 5 µl loading buffer (50% glycerol, 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 1 mM EDTA, pH 8.0) and the samples were electrophoresed in a 1.0% agarose gel prepared in 1X TAE at 70V for 3h. Ethidium bromide stained samples were visualized by UV transillumination at 306 nm and photographed using a Polaroid DS-34 camera and positive-negative film number 55.

M. 2.30 SOUTHERN ANALYSIS OF RT-PCR PRODUCTS

M. 2.31 SOUTHERN BLOTTING

RT-PCR products fractionated on agarose gels were processed by soaking the gel first in a denaturing solution of 1.5 M NaCl, 0.5 M NaOH for 30 min, and then in a neutralizing solution of 0.5 M Tris-HCl pH 7.5, 1.5M NaCl for 30 min. The gel was then transferred directly to a Pall Biodyne nylon membrane (GIBCO BRL, Bethesda, MD) by capillary blotting overnight using 20X SSC (0.3M sodium citrate pH 7.0, 3 M NaCl) as transfer buffer according to the protocols provided by the supplier. Membranes were cross-linked with UV using a Stratalinker 2400 (Stratagene, La Jolla CA.) at 254 nm at 1200 µjoules for 30 sec prior to prehybridization.

M. 2.32 OLIGONUCLEOTIDE END-LABELING

Oligonucleotides were synthesized on the ABI 392 synthesizer and were end-labeled according to Sambrook et al (1989). 100 ng of oligonucleotide was mixed with 1X polynucloetide kinase buffer (50mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol,

0.1mM spermidine-HCl, 0.1 mM EDTA), 50 μCi γ^{32}P ATP (NEN Dupont, Mississauga, ON), and 20U of T4 polynucleotide kinase (NEN Dupont, Mississauga, ON), in a final volume of 50 μl and incubated for 30 min at 37° C. The reaction was stopped by the addition of 1 μl of 500 mM EDTA and unincorporated nucleotides were removed by passing the reaction over a Sephadex G50 spin column. The specific activity of the final probe ranged from 10^8-10^9 cpm/μg of DNA.

M. 2.33 OLIGONUCLEOTIDE HYBRIDIZATION

The nylon membranes were prehybridized in 6X SSPE from a 20X SSPE stock (50 mM NaH_2PO_4 pH 7.4, 3 M NaCl, 50 mM EDTA), 5X Denhardt's solution from a 100X Denhardt's stock (0.2% polyvinyl pyrrolidine, 0.2% BSA, 0.2% Ficoll), 50 % Formamide and 120 μg/ml of heat denatured sonicated calf thymus DNA at 42° C for 4 h. Hybridization was performed in the same buffer with 100 ng of denatured radiolabeled probe (10^8-10^9 cpm/μg) at 42° C for 24 h. Membranes were subsequently washed at room temperature, initially with four washes in 6X SSC, 0.1% SDS for 5min, and once in 0.1X SSC for 30 min at 50° C. The membranes were wrapped in plastic wrap and autoradiographed using Cronex X-ray film in Kodak X-Omatic cassettes with intensifying screens overnight at room temperature.

M. 2.40 PRIMER DESIGN

All primers used in the RT-PCR experiments and oligonucleotides were synthesized on the ABI 392 oligonucleotide synthesizer. Two PCR primers were designed for each gene studied, one was designated the upstream primer while the other was designated the downstream primer. The primer sequences used for each gene of interest and the expected size in kbp of the product of RT-PCR are given in the table below.

TABLE 1: Primers used for RT-PCR and Southern Hybridization

GENE	PRIMER	SEQUENCE
HPRT	UPSTREAM	5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3'
	DOWNSTREAM	5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3'
	PRODUCT SIZE	220 bp
Clusterin	UPSTREAM	5'-GAC TCC AAA GAG GCC ACA CCA T -3'
	DOWNSTREAM	5'-GAT TCC CTC CTT GAG ACT CCT AC-3'
	PRODUCT SIZE	1410 bp
Cathepsin B	UPSTREAM	5'-CTT CCT ACA GTG TGT CTG ACA GCGA-3'
	DOWNSTREAM	5'-GAG AGG CTA GGA TGT AGG CTG TGAT-3'
	PRODUCT SIZE	570 bp
IGFBP2	UPSTREAM	5'-ACA GCA GGT TGC AGA CAG TGA GGAT-3'
	DOWNSTREAM	5'-CTG CTC GTT GTA GAA GAG ATG GCA C-3'
	INTERNAL	5'-GCC AGT TCC TTC ATG CCT GAC TTA G-3'
	PRODUCT SIZE	504 bp
IGFBP5	UPSTREAM	5'-TTC TCC ACA CAC TCT CGC TCT CCT G-3'
	DOWNSTREAM	5'-GGC CTT GGT CAG ATT CCT GTC TCA T-3'
	INTERNAL	5'-ATG GTG ATC AGC GTG GTC CTC CTG C-3'
	PRODUCT SIZE	625 bp
RSG3	UPSTREAM	5'-CCT TGG AGA AGA GCT CAG AGG AA-3'
	DOWNSTREAM	5'-AAG CAG GAT GAT GGC CAC TAA GA-3'
	PRODUCT SIZE	408 bp

* Southern Hybridization was only performed on IGFBP2 and IGFBP5.

M. 2.50 POSITIVE CONTROLS FOR RT-PCR

M. 2.51 ANIMAL CARE

All procedures utilizing animals were approved by the University of Ottawa animal care committee and conformed to the standards required by the Canadian Council on Animal Care.

M. 2.52 RAT VENTRAL PROSTATE

Male Sprague-Dawley rats, (250-300 g) were maintained in a controlled environment (14 h light, 10 h dark), and received Purina Rat Chow and water ad libitum. The animals were castrated via the scrotal route under light halothane anaesthesia. Animals were sacrificed by cervical dislocation at day 4 post castration, and the prostate glands were excised and processed immediately as outlined below.

M. 2.53 RNA ISOLATION FROM RAT TISSUE

Total RNA was isolated from freshly homogenized rat tissues using the LiCl/urea procedure (Auffray & Rougeon, 1979) with minor modifications (Tenniswood & Simpson, 1982). Briefly, the tissue was weighed and homogenized on ice in a fixed initial volume of extraction buffer (20 ml/g of tissue: 3 M LiCl, 6 M urea, 50 mM NaOAc, pH 5.5) and sonicated (3 x 15 sec bursts, with 1 minute cooling intervals between each burst). SDS was added to 0.1% and the sample was incubated at 4°C overnight. The sample was centrifuged at 16,000 rpm for 30 min and the pellet was recovered, drained, and resuspended in wash

buffer (4 M LiCl, 8 M urea, 50 mM NaOAc, pH 5.5, 0.1% SDS). The sample was centrifuged as described above and the pellet was recovered and dissolved in half the initial volume of 50 mM NaOAc, pH 5.5 and extracted with an equal volume of phenol (saturated with 50 mM NaOAc, pH 5.5) and an equal volume of chloroform. The organic and aqueous phases were separated by centrifugation (4,200 rpm, 5 min, room temperature) and the aqueous phase was retained. The organic phase was re-extracted with 10 ml of 50 mM NaOAc and the aqueous phases were pooled. The combined aqueous phases were re-extracted in an equal volume of phenol/chloroform (saturated with 50 mM NaOAc, pH 5.5) and once with chloroform. The RNA was precipitated from the aqueous phase by adding 1/10th volume 3M NaOAc, pH 5.5 and 2.5 volumes of cold absolute ethanol. The RNA was precipitated at -20°C overnight and pelleted at 10,000 rpm for 1 h at 0°C. The pellet was recovered, dried, redissolved in sterile double distilled water and stored at -70°C.

PART 3: IGF-I PROTECTION STUDIES

M. 3.0 IGF-I PROTECTION ASSAY

PC12 cells were grown to 50-70% confluence in RPMI 1640 medium supplemented with 5% FBS, 10% horse serum, 20 μg/ml gentamycin sulfate as indicated earlier . For experiments with IGF-I added to the serum, the indicated concentration of IGF-I (UBI, Lake Placid, NY) from the stock (0.25 μg/μl) was added to the flask at the 0 h time point, coincident with the time of treatment with VM26. Cell viability was assessed by trypan blue exclusion, and cell counts were determined by counting in a haemocytometer, as described earlier.

M. 3.1 DOSE RESPONSE CURVE FOR IGF-I

To assess the effect of changing the concentration of IGF-I on PC12 cells induced to die with 5 μM VM26, IGF-I was added at concentrations of 50, 100, and 200 ng/ml at 0 hr. At 0 and 24 h of incubation, cell counts and viability was assessed with the aid of a haemocytometer and trypan blue exclusion.

M. 3.2 EXTENT OF IGF-I PROTECTION

To assess the effect of changing the concentration of VM26 on PC12 cells grown in the presence of 100 ng/ml IGF-I, PC12 cell cultures were treated with varying concentrations of VM26. Cells were treated at the 0 h time point with 1 μM, 2 μM, or 4 μM VM26. At 0 and 24 h incubation, cell counts were assessed with the aid of a haemocytometer and trypan blue exclusion.

M. 3.3 IGF-I EFFECTS ON PC12 PROLIFERATION

To assess the effect of treating cells with 100 ng/ml IGF-I, cells were counted using a haemocytometer at the 0 h time point, at 24 h following treatment with IGF-I, and at 24 h without treatment with IGF-I.

RESULTS

PART 1: INDUCTION OF APOPTOSIS BY VM26

R. 1.0 INTRODUCTION

Apoptosis is a distinct form of cell death which is recognizable by membrane blebbing, chromatin condensation and DNA fragmentation into oligonucleosomal fragments giving the characteristic ladder pattern on an agarose gel (Kerr & Harmon, 1991). Cells typically undergo apoptosis in response to an exogenous stimulus or the disappearance of a necessary trophic factor. In this study VM26 a chemotherapeutic drug, which interacts with topoisomerase II, was used to induce apoptosis. The morphological and biochemical criteria mentioned above were used to characterize apoptosis as it occurred in the PC12 cell cultures.

R. 1.1 TRYPAN BLUE EXCLUSION

PC12 cells were exposed to 5 μM VM26 over a period of 24 h and loss of cell viability at the indicated time points was assessed based on trypan blue positive staining. Figure 1A illustrates that PC12 cells became increasingly susceptible to the apoptotic effects of VM26 over time. In the first two hours following drug treatment there was only a minimal increase in the number of cells that were trypan blue positive, by 4 h approximately 6.4% of the cells, a two fold increase over control, had undergone cell death. Over the subsequent 4 h there was a dramatic increase in the percent of cells staining with trypan blue and approximately 30% of the cells, or a nine fold increase over control, was noted at 8 h. The level of cell death continued to increase at a steady rate over the next 16 h and reached

Figure 1. Induction of apoptosis by VM26 in PC12 cells

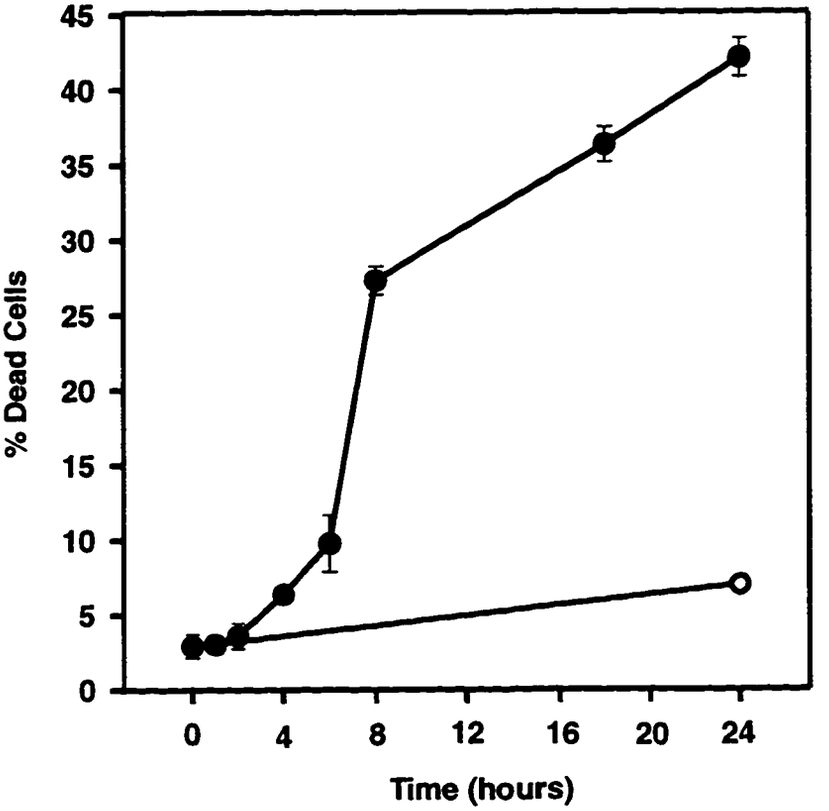
Panel A. Kinetics of apoptosis.
PC12 cells were grown to 70% confluency in RPMI 1640 medium in the presence of 5% FBS, 10% horse serum and 20 μg/ml gentamycin as described in the Methods section. The cells were treated with 5 μM VM26 and harvested after specified periods of time ranging from 0 h to 24 h. Cell viability was assessed by Trypan blue exclusion and cell counts were determined using a haemocytometer. The data shown is the mean ± SEM for three experiments.

Panel B: Analysis of DNA fragmentation

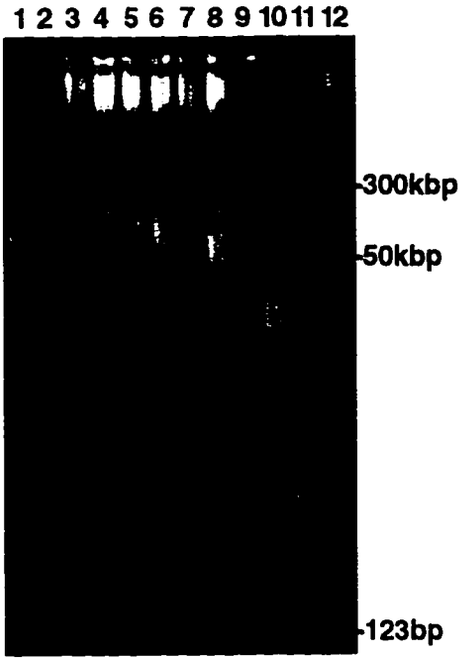
PC12 cells were grown to 70% confluency in RPMI 1640 medium in the presence of 5% FBS, 10% horse serum and 20 μg/ml gentamycin as described in the Methods section. Cells were treated with 5 μM VM26 and harvested at specified periods of time ranging from 0 h to 24 h. Total cellular DNA was processed as described in the Methods section. Low melting agarose plugs containing DNA was prepared and the agarose slices were loaded into dry wells of a 0.8% agarose gel in TBE buffer (89.2mM Tris-HCl, pH 8.3-8.5, 89 mM boric acid and 2.5 mM EDTA). The DNA (5-10 μg per lane) was separated by pulsed field gel electrophoresis using a Q-Life Autobase PFGE system with software-assisted ROM card #3. The gel was run for 24 h and DNA fragments ranging in size from 800 kbp to approximately 0.1 kbp were resolved. Representative DNA sizes are shown alongside the gel in kbp.

Lane 1- lambda DNA ladder
Lane 2- DNA from control untreated cells
Lane 3- DNA from cells harvested at 1h
Lane 4- DNA from cells harvested at 2 h
Lane 5- DNA from cells harvested at 4 h
Lane 6- DNA from cells harvested at 6 h
Lane 7- DNA from cells harvested at 8 h
Lane 8- DNA from cells harvested at 18 h
Lane 9- DNA from cells harvested at 24 h
Lane 10-yeast chromosomal markers
Lane 11-low range DNA size markers
Lane 12-lambda DNA ladder

A


45
40
35
30
25
20
15
10
5
0
% Dead Cells
0
4
8
12
16
20
24
Time (hours)

B


1 2 3 4 5 6 7 8 9 10 11 12
300kbp
50kbp
123bp

a maximum of 42% (a fifteen fold increase) after 24 h of treatment with VM26. In contrast control cells treated with vehicle alone demonstrated minimal increase in trypan blue staining.

R. 1.2 DNA FRAGMENTATION

To establish that this increase in cell death was attributable to apoptosis, DNA fragmentation, a well established feature of apoptosis was studied. DNA fragmentation is catalysed by endogenous endonucleases and proceeds though an ordered series of stages beginning with the production of DNA fragments of 300 kbp, which are then degraded to fragments of 50 kbp. These 50 kbp fragments are further degraded, in some but not all cells, to smaller fragments (10-40 kbp) which then release the small oligonucleosome fragments that are recognized as the characteristic DNA ladder (Walker *et al.* 1993). Studies have shown that DNA from cells undergoing apoptosis can be rapidly deproteinized using SDS lysis and then can be loaded directly into a gel for effective analysis by PFGE (Walker *et al.* 1993). This method was used to examine DNA isolated at different time points from PC12 cells treated with VM26 (Fig. 1B).

PFGE demonstrated that incubation of PC12 cells with 5 μM VM26 led to a progressive accumulation of DNA fragments of 50 kbp and below (lanes 3-9). Prior to 6 h of drug exposure, DNA fragments of >300 kbp, believed to represent chromatin loop domains (Filipski *et al.* 1990; Walker *et al.* 1991) predominated (lanes 2-5) indicating few cells had yet entered apoptosis. However, by 6 h many of the cells had entered apoptosis, noted by the appearance of fragments of less than 50 kbp (lane 6) and the characteristic

laddering pattern faintly visible at 18 h (lane 8). The timing of the appearance of fragments of less than 50 kbp at 6 h correlated well with the sharp increase in the percentage of dead cells noted by positive trypan blue staining (Fig 1A) which occured between 6 and 8 h after drug treatment.

R. 1.3 MORPHOLOGICAL CHANGES

In addition to the biochemical criteria of DNA cleavage, apoptosis is characterized morphologically using immunofluorescence microscopy, by a sequence of distinct cytoplasmic and nuclear changes. Typically the nucleus shrinks, chromatin becomes condensed and marginated and the final step is nuclear fragmentation into apoptotic bodies (Bursch *et al.* 1990a; Arends *et al.* 1990).

Samples for immunofluorescence were examined by both phase contrast microscopy and fluorescence using the DNA-specific fluorochrome, Hoescht 33258 (Fig. 2). Morphological changes consistent with apoptosis were apparent in the nuclei of the PC12 cells treated with VM26. In panels A and A' showing phase contrast and Hoechst staining of the untreated PC12 cells, respectively, the nuclei appeared intact with the heterochromatin uniformly distributed along the nuclear envelope. Panels B and B' show cells after 4 h of treatment with VM26, when the nuclei had started to become phase dark (panel B) with the Hoechst staining showing areas of brighter fluorescence (panel B'). After 8 h of treatment with VM26 some condensation of the nuclear chromatin was evident with partial detachment from the nuclear membrane (panel C). Panel C' shows the typical pattern of chromatin condensation-showing a reticular and fibrillated substructure. In panel D, depicting cells

Figure 2. Apoptotic Morphology of PC12 cells treated with VM26

Cells were grown on poly-L-lysine coated glass coverslips and placed in RPMI 1640 media supplemented with 5% FBS, 10% horse serum and 20 μg/ml of gentamycin sulfate and treated with VM26 for specified periods of time. The cells were then fixed with 3% paraformaldehyde in PBS, stained with 33258 Hoechst dye and photographed using Olympus Bmax Fluorescence Microscope and Photosystems.

Panel A: PC12 cells not treated with VM26 (0 h control)
A : Phase contrast
A': Hoechst staining showing corresponding fluorescence image

Panel B: PC12 cells treated with VM26 for 4 h
B : Phase contrast
B': Hoechst staining showing corresponding fluorescence image

Panel C: PC12 cells treated with VM26 for 8 h
C : Phase contrast photography
C': Hoechst staining showing corresponding fluorescence image

Panel D: PC12 cells treated with VM26 for 18 h
D : Phase contrast photography
D': Hoechst staining showing corresponding fluorescence image

Panel E: PC12 cell treated with VM26 for 24 h
E : Phase contrast photography
E': Hoechst staining showing corresponding fluorescence image

A′
B′
C′
D′
E′
E′

18 h post-treatment, there was a considerable amount of background debris from cells that were dying. The nuclear membrane in cells at this time point remained intact but the nuclei had become phase dark and smaller in size, brighter with Hoechst staining (panel D'), and showed the same characteristic pattern of chromatin condensation as seen in panel C. By 24 h after treatment with VM26 the formation of apoptotic bodies was apparent. Nuclear margination was clearly seen in both panels E and E', and in keeping with what is known about apoptosis, the nuclear membrane remained intact until a late stage followed by packaging of the regions of condensed chromatin into apoptotic bodies.

Again these experiments exhibited a clear temporal relationship between a decrease in cell viability, internucleosomal DNA cleavage (Fig. 1) and morphological changes (Fig. 2) which are all considered hallmarks of apoptosis. Taken together these results showed that VM26 induced cell death by apoptosis in PC12 cells.

PART 2: GENE EXPRESSION DURING APOPTOSIS

R. 2.0 INTRODUCTION

Apoptosis has been described as an active cellular process of gene-directed self-destruction (Kerr *et al.* 1972). As described in the introduction, a number of genes have been implicated in various model systems of apoptosis. The aim of this project was to determine if these genes also play a role in apoptosis occurring in PC12 cells induced to die with VM26. Using an RT-PCR technique developed by Guenette and Sridhar, the time course of expression of two genes previously shown to play a role in apoptosis; clusterin (Wilson *et al.* 1994; Wong *et al.* 1994), cathepsin B (Guenette *et al.* 1994), and three novel

genes to this process IGFBP2 (Guenette, R.S. unpublished), IGFBP5 (Guenette & Tenniswood, 1994) and RSG3 (Guenette, R.S. unpublished) were characterized. This technique involved the isolation of RNA, reverse transcription with a gene specific primer and amplification of the resulting cDNA using upstream and downstream gene-specific primers. The amplified product is specific for the gene of interest. By performing a southern hybridization, and probing with an internal primer specific for the gene the RT-PCR technique was even more specific and sensitive and was a good measure of gene expression over the time course of apoptosis.

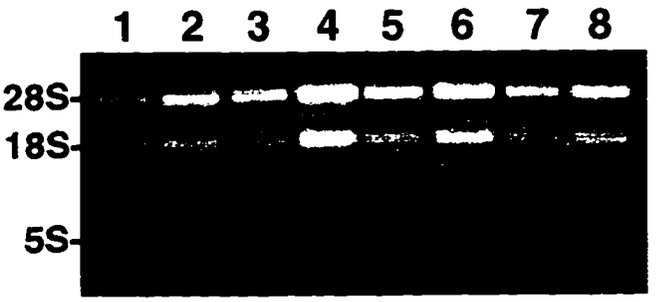
R. 2.1 RNA INTEGRITY

RNA for the purposes of RT-PCR was prepared as indicated in the Methods section. Its quality was assessed by agarose gel electrophoresis (Fig. 3). The ethidium bromide stained gel showed that the RNA preparation yielded good quality RNA since all three ribosomal RNA species 28S, 18S and 5S were intact. There was no sign of degradation in any of the samples which could potentially affect the RT-PCR process. This is a typical example of RNA extraction performed numerous times for the different experimental runs. The quantity of RNA isolated per cell, from experiment to experiment was approximately the same at each time point.

Figure 3. Analysis of RNA from PC12 cells treated with VM26

PC12 cells were grown to 70% confluency in RPMI 1640 medium in the presence of 5% FBS, 10% horse serum, and 20 μg/ml gentamycin as described in the Methods section. Cells were treated with 5 μM VM26 for specified periods of time and total RNA was recovered for each time point. Approximately 5 μg of total RNA was resolved on a 1.2% polyacrylamide gel containing 1.0M formaldehyde. Staining with ethidium bromide (1.5 μg/ml) allowed visualization of bands by UV (306nm) transillumination. The positions of the three ribosomal RNA species 28S, 18S and 5S are indicated.

Lane 1-RNA from control untreated PC12 cells
Lane 2-RNA from cells harvested at 1 h
Lane 3-RNA from cells harvested at 2 h
Lane 4-RNA from cells harvested at 4 h
Lane 5-RNA from cells harvested at 6 h
Lane 6-RNA from cells harvested at 8 h
Lane 7-RNA from cells harvested at 18 h
Lane 8-RNA from cells harvested at 24 h


1 2 3 4 5 6 7 8
28S
18S
5S

R. 2.2 HPRT GENE EXPRESSION (INTERNAL CONTROL)

The first step was to establish an internal control for the RT-PCR procedure. Initially α-tubulin was considered, however the expression of α-tubulin has been shown to be down regulated in some systems undergoing apoptosis. For this reason, it was not used as the internal control. The hypoxanthine phosphoribosyl transferase (HPRT) gene, a housekeeping gene, not expected to be altered during apoptosis was chosen instead. The negative control for the experiment was a reaction mixture lacking both RNA and reverse transcriptase and gave the expected negative result. (Fig. 4A, lane 11). This RT-PCR analysis demonstrated that the amplified product specific for the HPRT gene migrated as a tight band of the expected size of 220 bp, as indicated on Fig. 4A. HPRT showed a consistently high level of expression, with minimal change over the 24 h time course (Fig. 4, Panel A, lanes 2-10). This was verified further by densitometric analysis (Fig 4, Panel b), which showed only a 3% variation in levels of expression over the 24 h time course. Due to its relatively uniform level of expression, and role as a housekeeping gene in the cell, HPRT served as the internal control for all RT-PCR experiments conducted in this study.

R. 2.3 CLUSTERIN GENE EXPRESSION

The clusterin gene and its cognate protein have been characterized in a number of species and been implicated in apoptosis occurring in prostate tissue following hormone ablation. (Wong *et al.* 1994). It has also been shown to be expressed in the rat kidney (Guenette, R.S. unpublished). Rat ventral prostate 4 days post castration and rat kidney therefore served as positive controls. Yeast tRNA which consists of short nonspecific RNA

Figure 4: Effect of VM26 treatment on expression of the HPRT gene

Panel A: Electrophoresis of RT-PCR products using HPRT primers.

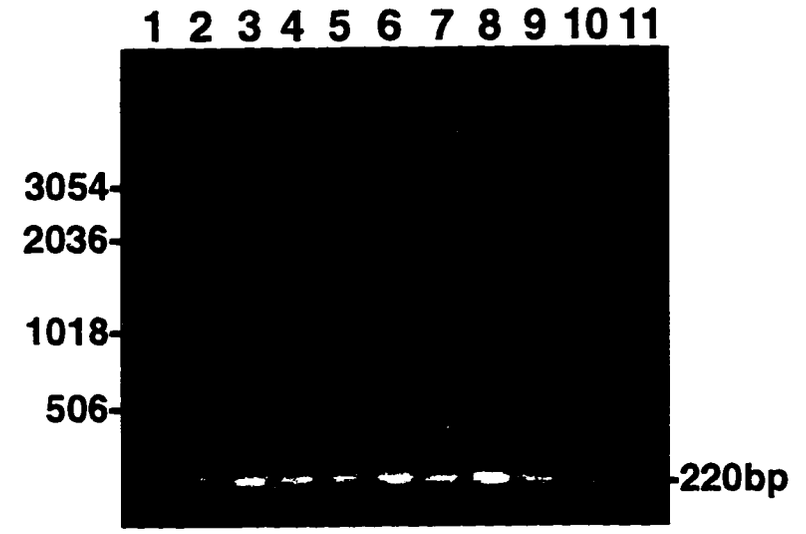
RT-PCR amplification of RNA from VM26 treated PC12 cells. 500 ng of RNA was reverse transcribed using downstream primers specific for the rat HPRT gene. Primers specific for HPRT were designed based on sequences from the GENBANK database and are listed in Table 1. 5 µl of this reverse transcriptase reaction was then amplified using upstream and downstream primers specific for rat HPRT, using Robocycler gradient 96 thermocycle (Stratagene, LaJolla, Ca), set for 30 cycles as outlined in the Methods section. PCR products were analyzed by electrophoresis on a 1% agarose gel at 70V for 3 h, and were visualized by ethidium bromide staining and photographed with Polaroid 55 film (Polaroid Corporation, Cambridge, MA). The 1 kbp marker in lane 1 indicates that the band specific to the HPRT gene migrates at 220 bp as is indicated beside the gel. The data is representative of 1 experiment of 4.

Lane 1- 1kbp DNA Ladder
Lane 2- RT-PCR on control untreated PC12 cells
Lane 3- RT-PCR on PC12 cells treated for 2 h
Lane 4- RT-PCR on PC12 cells treated for 4 h
Lane 5- RT-PCR on PC12 cells treated for 6 h
Lane 6- RT-PCR on PC12 cells treated for 8 h
Lane 7 -RT-PCR on PC12 cells treated for 14 h
Lane 8 -RT-PCR on PC12 cells treated for 18 h
Lane 9- RT-PCR on PC12 cells treated for 24 h
Lane 10-RT-PCR on rat ventral prostate (positive control))
Lane 11-RT-PCR on sample with no RT added (negative control)

Panel B: Graphic representation of level of HPRT gene expression over 24 h time course.

The level of expression indicated on the y axis, was determined from the pixel intensities of a digital image of the gel electrophoresis result (see Panel A). The corresponding treatment times of PC12 cells with VM26 is given on the x axis.

A


1 2 3 4 5 6 7 8 9 10 11
3054
2036
1018
506
220bp

B

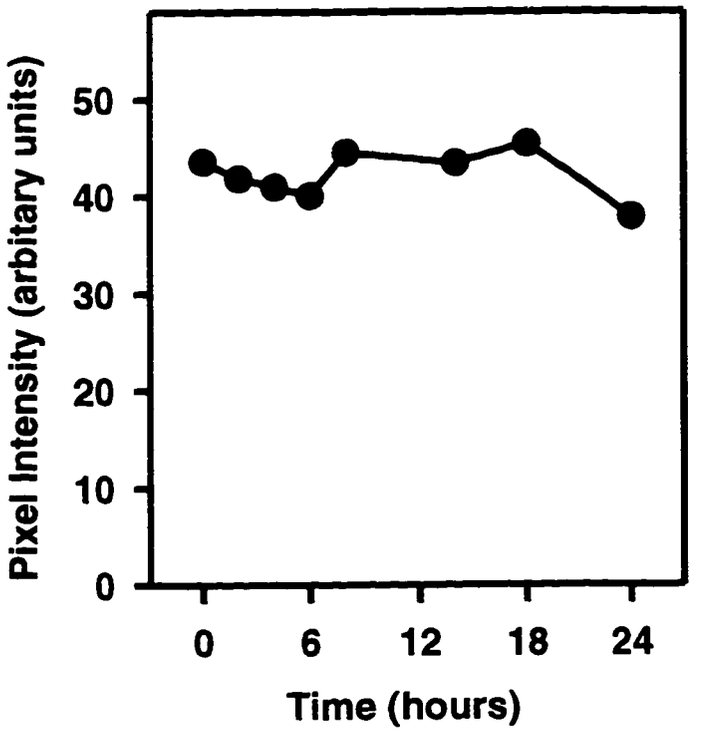

Pixel Intensity (arbitary units)
50
40
30
20
10
0
0
6
12
18
24
Time (hours)

Figure 5: Effect of VM26 treatment on expression of the Clusterin gene

Panel A: Electrophoresis of RT-PCR products using clusterin primers.

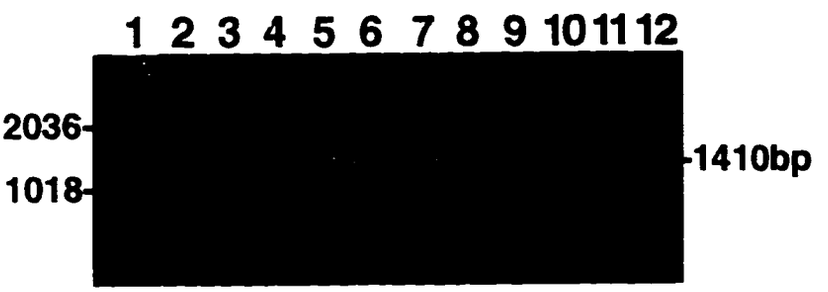
RT-PCR amplification of RNA from VM26 treated PC12 cells. 500 ng of RNA was reverse transcribed using downstream primers specific for the rat clusterin gene. Primers specific for clusterin were designed based on sequences from the GENBANK database and are listed in Table 1. 5 μl of this reverse transcriptase reaction was then amplified using upstream and downstream primers specific for rat clusterin, using Robocycler gradient 96 thermocycle (Stratagene, LaJolla, Ca), set for 30 cycles as outlined in the Methods section. PCR products were analyzed by electrophoresis on a 1% agarose gel at 70V for 3 h, and were visualized by ethidium bromide staining and photographed with Polaroid 55 film (Polaroid Corporation, Cambridge, MA). The 1 kbp marker in lane 1 indicates that the band specific to the clusterin gene migrates at 1410 bp as is indicated beside the gel. The data is representative of 1 experiment of 4.

Lane 1- 1 kbp DNA Ladder
Lane 2- RT-PCR on control untreated PC12 cells
Lane 3- RT-PCR on PC12 cells treated for 2 h
Lane 4- RT-PCR on PC12 cells treated for 4 h
Lane 5- RT-PCR on PC12 cells treated for 6 h
Lane 6- RT-PCR on PC12 cells treated for 8 h
Lane 7 -RT-PCR on PC12 cells treated for 14 h
Lane 8 -RT-PCR on PC12 cells treated for 18 h
Lane 9- RT-PCR on PC12 cells treated for 24 h
Lane 10-RT-PCR on rat ventral prostate (positive control)
Lane 11-RT-PCR on rat kidney (positive control)
Lane 12-RT-PCR on sample with no RT added (negative control)

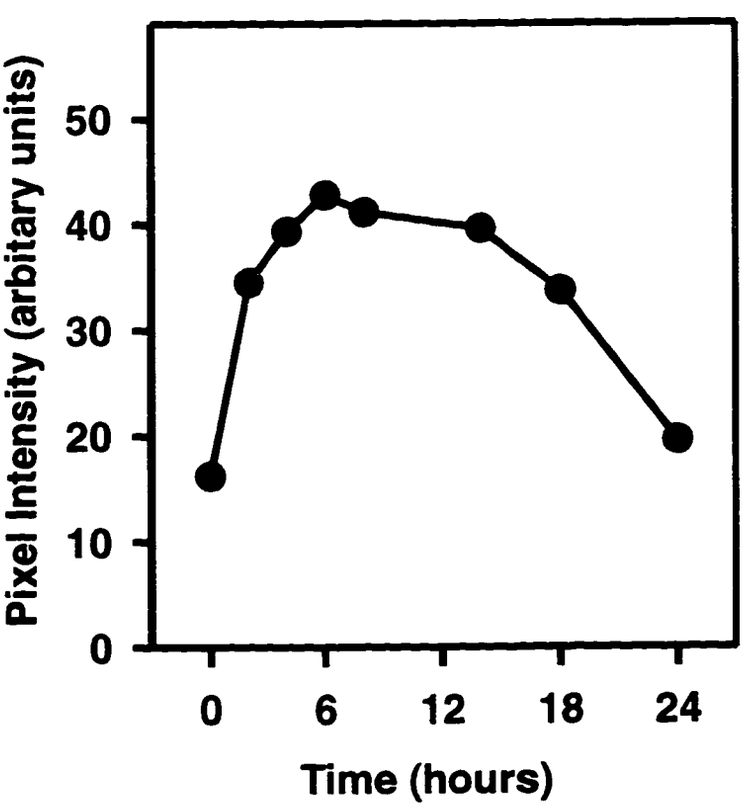
Panel B: Graphic representation of level of clusterin gene expression over 24 h time course.

The level of expression indicated on the y axis, was determined from the pixel intensities of a digital image of the gel electrophoresis result (see Panel A). The corresponding treatment times of PC12 cells with VM26 is given on the x axis.

A


1 2 3 4 5 6 7 8 9 10 11 12
2036
1018
1410bp

B


Pixel Intensity (arbitary units)
50
40
30
20
10
0
0
6
12
18
24
Time (hours)

sequences which lack a polyadenylated tail was used as the negative control for this experiment. RT-PCR analysis revealed a clusterin PCR product migrating as a tight, specific band at 1410 bp as indicated on Fig. 5A. Clusterin mRNA was expressed in untreated PC12 cells at the 0 h time point (lane 2) and was greatly upregulated by 6-8 h (lanes 5,6) after the induction of cell death with VM26. At 8 h (lane 6) peak levels of clusterin expression were approximately 3-fold above both positive controls. By 24 h (lane 9), mRNA levels had returned to values similar to those of the 0 h control. The kinetics of these changes were also illustrated by the densitometric analysis (Fig. 5, panel B) which showed a rapid 2 fold increase in expression by 2 h and a 3 fold increase by 8 h. Levels then decreased, somewhat more gradually than the initial increase. By 18 h, levels were similar to those seen at 2 h and by 24 h they were just above levels seen in the untreated PC12 cells. The positive (lane 10 prostate, lane 11 kidney) and negative (lane 12) controls showed the expected results.

R. 2.4 CATHEPSIN B GENE EXPRESSION

Numerous proteases are believed to play a role in the morphological changes associated with apoptosis. One of these, cathepsin B (RSG-2) a cysteine protease has been shown to be involved in the degradation of the cellular basement membrane (Guenette *et al.* 1994b). In regressing prostate, cathepsin B expression reached a maximum level 4 days post castration, and hence this was used as the positive control for RT-PCR. Figure 6 demonstrates that cathepsin B, migrating as a tight band at 570 bp, was expressed in untreated PC12 cells (lane 2). In panel B, a densitometric analysis showed a slight decrease in the level of expression in the first four hours following VM26 treatment (lane 3,4). After

Figure 6: Effect of VM26 treatment on expression of the CathepsinB gene

Panel A: Electrophoresis of RT-PCR products using cathepsin B primers.

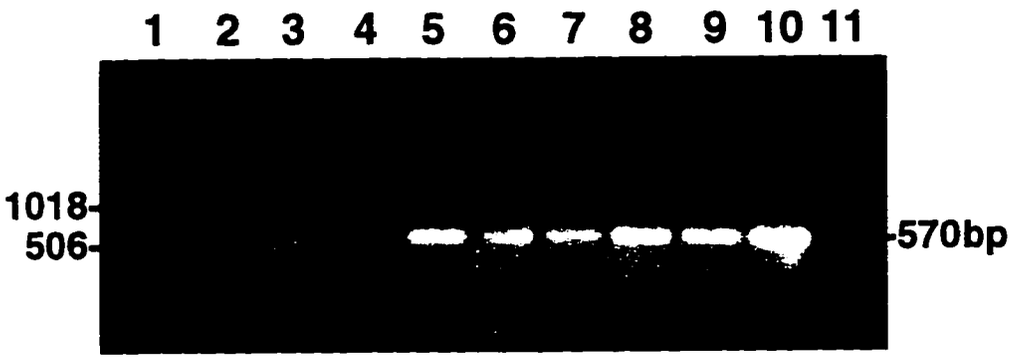
RT-PCR amplification of RNA from VM26 treated PC12 cells. 500 ng of RNA was reverse transcribed using downstream primers specific for the rat cathepsin B gene. Primers specific for cathepsin B were designed based on sequences from the GENBANK database and are listed in Table 1. 5 µl of this reverse transcriptase reaction was then amplified using upstream and downstream primers specific for rat cathepsin B, using Robocycler gradient 96 thermocycle (Stratagene, LaJolla, Ca), set for 30 cycles as outlined in the Methods section. PCR products were analyzed by electrophoresis on a 1% agarose gel at 70V for 3 h, and were visualized by ethidium bromide staining and photographed with Polaroid 55 film (Polaroid Corporation, Cambridge, MA). The 1 kbp marker in lane 1 indicates that the band specific to the cathepsin B gene migrates at 570 bp as is indicated beside the gel. The data is representative of 1 experiment of 4.

Lane 1- 1 kbp DNA Ladder
Lane 2- RT-PCR on control untreated PC12 cells
Lane 3- RT-PCR on PC12 cells treated for 2 h
Lane 4- RT-PCR on PC12 cells treated for 4 h
Lane 5- RT-PCR on PC12 cells treated for 6 h
Lane 6- RT-PCR on PC12 cells treated for 8 h
Lane 7 -RT-PCR on PC12 cells treated for 14 h
Lane 8 -RT-PCR on PC12 cells treated for 18 h
Lane 9- RT-PCR on PC12 cells treated for 24 h
Lane 10-RT-PCR on rat ventral prostate (positive control)
Lane 11-RT-PCR on sample with no RT added (negative control)

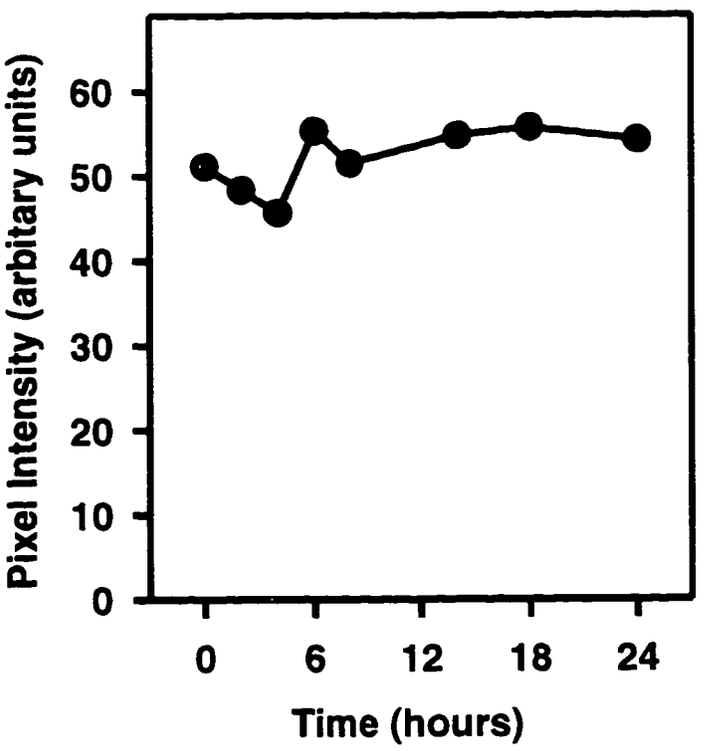
Panel B: Graphic representation of level of Cathepsin B gene expression over 24 h time course.

The level of expression indicated on the y axis, was determined by from the pixel intensities of a digital image of the gel electrophoresis result (see Panel A). The corresponding treatment times of PC12 cells with VM26 is given on the x axis.

A


1 2 3 4 5 6 7 8 9 10 11
1018
506
570bp

B


Pixel Intensity (arbitary units)
60
50
40
30
20
10
0
0
6
12
18
24
Time (hours)

6 h of treatment (lane 5), levels of cathepsin B expression showed a slight increase and then decreased slightly at 8 h. Levels stayed constant over the next 12 hours (lanes 8,9). Over the 24 h time course there was only a slight increase in expression of cathepsin B to levels just under those observed in the positive control (lane 10). The positive and negative controls (lanes 10,11) gave the expected results.

R. 2.5 IGFBP2 GENE EXPRESSION

IGFBP2, a member of the IGFBP family, is believed to act by modulating the mitogenic and metabolic effects of the IGFs (Guenette & Tenniswood, 1994). It has been detected in serum, seminal plasma and is secreted by various tumors including lung and prostate. It has also been shown to be expressed in high levels during fetal development. An RT-PCR-Southern experiment was used to show expression of IGFBP2 because low levels of RT-PCR product, and several bands (perhaps owing to PCR of other members of the IGFBP family) were present on the gel. For this reason, each time point with its corresponding -RT (negative) control was used. The background on the gels made the other lanes unreliable (Fig 7, Panel A). Again, using the rat ventral prostate as a positive control (lane 9), we were able to show that IGFBP2, which migrated at 504 bp, was expressed at low but detectable levels in normal untreated PC12 cells at 0 h (lane 3). The expression of IGFBP2 was dramatically upregulated to a peak at 8 h (lane 5) following treatment with VM26. IGFBP2 expression gradually decreased and by 24 h (lane 7) it was significantly less than the peak at 8 h but still higher than the levels recorded at 0 h. The graph shown in Fig. 7B illustrates that IGFBP2 expression started low, at about 28 pixel units, peaked at 8 h with

Figure 7: Effect of VM26 treatment on expression of the IGFBP2 gene

Panel A: Southern transfer of IGFBP2 RT-PCR products at 0, 8, 24 h after treatment.

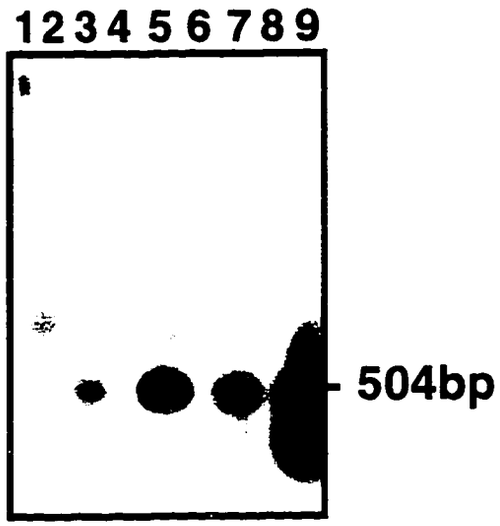
RT-PCR amplification of RNA from VM26 treated PC12 cells. 500 ng of RNA was reverse transcribed using downstream primers specific for the rat IGFBP2 gene. Primers specific for IGFBP2 were designed based on sequences from the GENBANK database and are listed in Table 1. 5 µl of this reverse transcriptase reaction was then amplified by PCR using upstream and downstream primers specific for rat IGFBP2, using Robocycler gradient 96 thermocycle (Stratagene, LaJolla, Ca), set for 30 cycles as outlined in the Methods section. PCR products which migrated at 504 bp were analyzed initially by electrophoresis on a 1% agarose gel at 70V for 3 h, then were transferred overnight on to a Pall Biodyne nylon membrane using 20X SSC as the transfer solution. The membranes were then probed with an internal primer specific for the IGFBP2 gene as outlined in the Methods section.The data is representative of 1 experiment of 4.

Lane 1-1 kbp ladder
Lane 2-RT-PCR on control untreated PC12 cells, no RT (negative control)
Lane 3-RT-PCR on control untreated PC12 cells
Lane 4-RT-PCR on PC12 cells treated for 8 h, no RT (negative control)
Lane 5-RT-PCR on PC12 cells treated for 8 h
Lane 6-RT-PCR on PC12 cells treated for 24 h, no RT (negative control)
Lane 7-RT-PCR on PC12 cells treated for 24 h
Lane 8-RT-PCR on rat ventral prostate, no RT (negative control)
Lane 9-RT-PCR on rat ventral prostate (positive control)

Panel B: Graphic representation of level of IGFBP2 gene expression over 24 h time course.

The level of expression indicated on the y axis, was determined from the pixel intensities of a digital image of the autoradiographic result (see Panel A). The corresponding treatment times of PC12 cells with VM26 is given on the x axis.

A


1 2 3 4 5 6 7 8 9
504bp

B

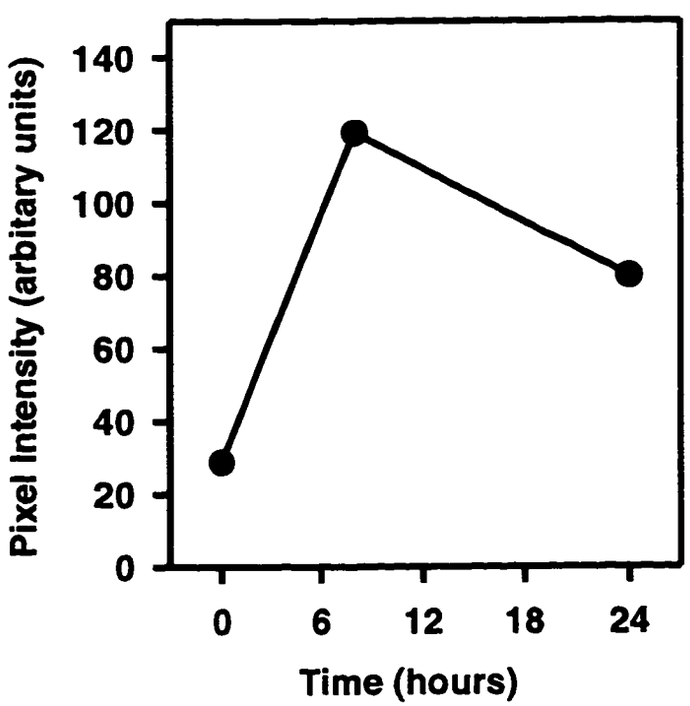

Pixel Intensity (arbitary units)
140
120
100
80
60
40
20
0
0
6
12
18
24
Time (hours)

Figure 8: Effect of VM26 treatment on expression of the IGFBP5 gene

Panel A: Southern transfer of IGFBP5 RT-PCR products at 0,8,24 h after treatment.

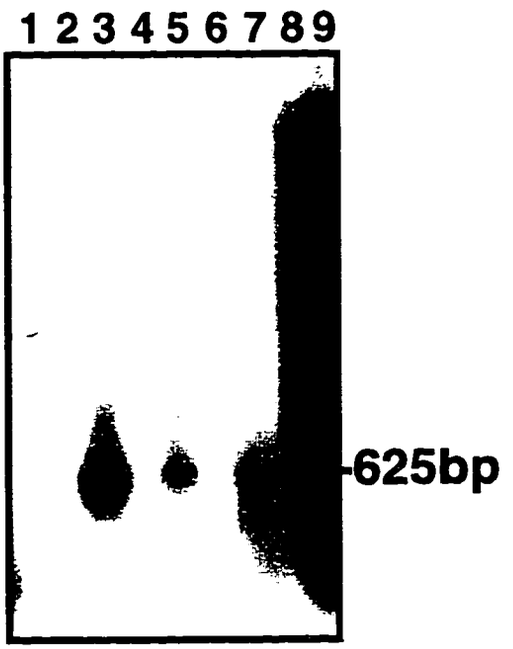
RT-PCR amplification of RNA from VM26 treated PC12 cells. 500 ng of RNA was reverse transcribed using downstream primers specific for the rat IGFBP5 gene. Primers specific for IGFBP5 were designed based on sequences from the GENBANK database and are listed in Table 1. 5 µl of this reverse transcriptase reaction was then amplified by PCR using upstream and downstream primers specific for rat IGFBP5, using Robocycler gradient 96 thermocycle (Stratagene, LaJolla, Ca), set for 30 cycles as outlined in the Methods section. PCR products (625 bp) were initially analyzed by electrophoresis on a 1% agarose gel at 70V for 3 h, and then were transferred overnight to a Pall Biodyne nylon membrane using 20X SSC as the transfer solution. The membranes were then probed with an internal primer specific for the IGFBP5 gene as outlined in the Methods section. The data is representative of 1 experiment of 4.

Lane 1-1 kbp ladder
Lane 2-RT-PCR on control untreated PC12 cells, no RT (negative control)
Lane 3-RT-PCR on control untreated PC12 cells
Lane 4-RT-PCR on PC12 cells treated for 8 h, no RT (negative control)
Lane 5-RT-PCR on PC12 cells treated for 8 h
Lane 6-RT-PCR on PC12 cells treated for 24 h, no RT (negative control)
Lane 7-RT-PCR on PC12 cells treated for 24 h
Lane 8-RT-PCR on rat ventral prostate no RT (negative control)
Lane 9-RT-PCR on rat ventral prostate (positive control)

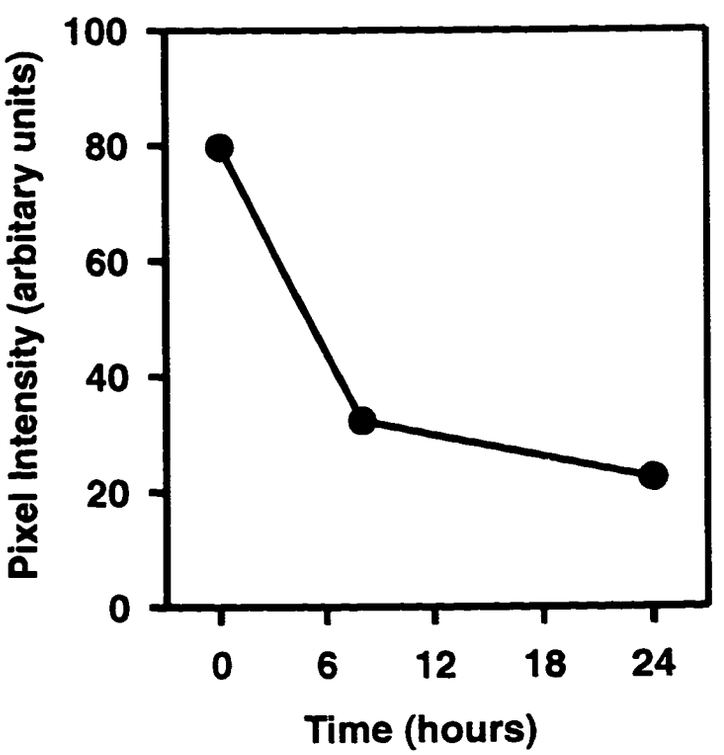
Panel B: Graphic representation of level of IGFBP5 gene expression over 24 h time course.

The level of expression indicated on the y axis, was determined from the pixel intensities of a digital image of the autoradiographic result (see Panel A). The corresponding treatment times of PC12 cells with VM26 is given on the x axis.

A


1 2 3 4 5 6 7 8 9
625bp

B


Pixel Intensity (arbitary units)
100
80
60
40
20
0
0
6
12
18
24
Time (hours)

a four fold induction to 100 pixel units and then decreased to about 80 pixel units at 24 hours. Peak levels in PC12 cells, however, do not reach those seen with the positive control of rat ventral prostate (lane 9).

R. 2.6 IGFBP5 GENE EXPRESSION

IGFBP5, another member of the IGFBP family, has been shown to be induced *de novo* during the process of apoptosis in the prostate (Guenette & Tenniswood, 1994). Thus, RNA from prostate tissue was used as the positive control for the RT-PCR in these experiments. The results presented here show that IGFBP5 levels, noted by the band migrating at 625 bp, was expressed in untreated cells at 0 h (lane 3) but decreased dramatically by 8 h (lane 5) and remained low after 24 h (lane 7) of incubation with VM26. Once again, peak levels of expression were well below those seen in the positive control (lane 9). A densitometric analysis was performed on the autoradiograph and the results are presented in graph form (panel B). A two fold decrease in the level of IGFBP5 expression can be seen by 8 h and an overall four fold decrease can be seen by 24 h. The positive (lane 9) and negative controls (lanes 2,4,6,8) gave the expected result.

R. 2.7 RSG3 GENE EXPRESSION

The RSG3 gene (embigin) is a member of the V_{κ} immunoglobin gene superfamily and plays an important role in cell surface interactions, cell-cell adhesion and as a cell surface receptor (Huang *et al.* 1990). Since RSG3 has been shown to be expressed in the normal rat kidney (lane 9) it was used as a positive control for this experiment (Guenette R.

Figure 9: Effect of VM26 treatment on expression of the RSG3 gene

Panel A: Electrophoresis of RT-PCR products using RSG3 primers.

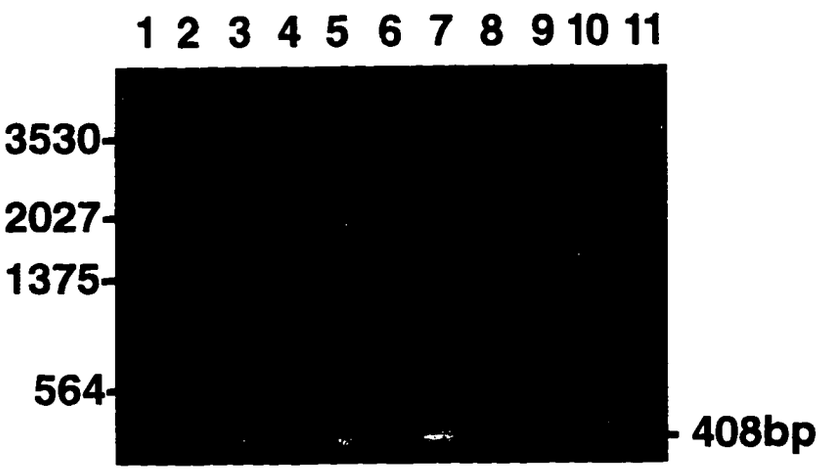
RT-PCR amplification of RNA from VM26 treated PC12 cells. 500 ng of RNA was reverse transcribed using downstream primers specific for the rat RSG3 gene. Primers specific for RSG3 were designed based on sequences from the GENBANK database and are listed in Table 1. 5 µl of this reverse transcriptase reaction was then amplified using upstream and downstream primers specific for rat RSG3, using Robocycler gradient 96 thermocycle (Stratagene, LaJolla, Ca), set for 30 cycles as outlined in the Methods section. PCR products were analyzed by electrophoresis on a 1% agarose gel at 70V for 3 h, and were visualized by ethidium bromide staining and photographed with Polaroid 55 film (Polaroid Corporation, Cambridge, MA). The 1 kbp marker in lane 1 indicates that the band specific to the RSG3 gene migrates at 408 bp as is indicated beside the gel. The data is representative of 1 experiment of 4.

Lane 1- 1 kbp DNA Ladder
Lane 2-RT-PCR on control untreated PC12 cells, no RT (negative control)
Lane 3-RT-PCR on control untreated PC12 cells
Lane 4-RT-PCR on PC12 cells treated for 8 h, no RT (negative control)
Lane 5-RT-PCR on PC12 cells treated for 8 h
Lane 6-RT-PCR on PC12 cells treated for 24 h, no RT (negative control)
Lane 7-RT-PCR on PC12 cells treated for 24 h
Lane 8-RT-PCR on rat kidney, no RT (negative control)
Lane 9-RT-PCR on rat kidney (positive control)
Lane 10-RT-PCR on tRNA (negative control)
Lane 11-RT-PCR on sample lacking both RNA and RT (negative control)

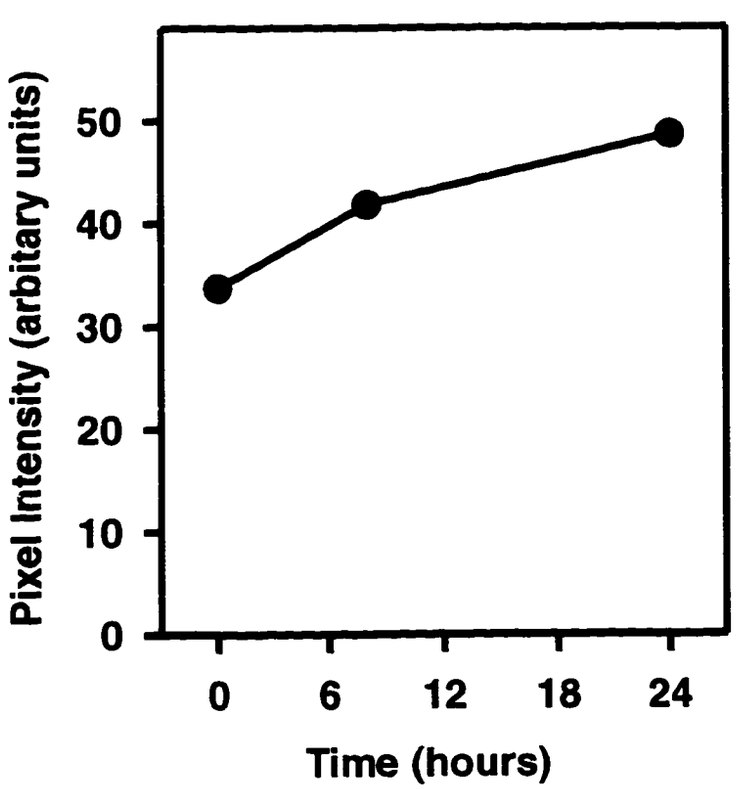
Panel B: Graphic representation of level of RSG3 gene expression over 24 h timecourse.

The level of expression indicated on the y axis, was determined from the pixel intensities of a digital image of the gel electrophoresis result (see Panel A). The corresponding treatment times of PC12 cells with VM26 is given on the x axis.

A


1 2 3 4 5 6 7 8 9 10 11
3530
2027
1375
564
408bp

B


Pixel Intensity (arbitary units)
50
40
30
20
10
0
0
6
12
18
24
Time (hours)

S. unpublished). RSG3, appearing as a PCR band migrating at 408 bp, was expressed in untreated PC12 cells (lane 3). It was also expressed at 8 h (lane 5) and 24 h (lane 7) after VM26 treatment, but showed no significant change in expression over the 24 hour time course when compared to IGFBP2 and IGFBP5. The graphic analysis demonstrated a gradual increase over the 24 h with a 1.2 fold increase in the first 8 h and a 1.5 fold increase by 24 h. This perhaps indicates RSG3 plays no real role or at best a very limited one, in apoptosis in this system. Positive (lane 9) and negative controls (lane 10,11) gave the expected results.

PART 3: IGF PROTECTION STUDIES

R. 3.0 INTRODUCTION

Having demonstrated that the IGFBPs in particular IGFBP2 and IGFBP5, may play a role in apoptosis, we decided to investigate the effects of IGF-I treatment on cells undergoing apoptosis. The survival of a cell population both *in vivo* and *in vitro* represents a balance of cell proliferation and cell death. Recent studies have shown that IGF-I, by interacting with the IGF receptor, is able to prevent VM26 induced apoptosis in BALB/c3T3 cells, in a fashion independent of the mitogenic actions of IGF-I (Sell *et al.* 1995). In a similar set of experiments on PC12 cells we determined the effects of increasing IGF-I concentration on VM26 induced apoptosis in PC12 cultures. As well, the effectiveness of IGF as a protective (anti-apoptotic) agent was determined by assessing cell viability following treatment with gradually increasing doses of VM26 and over increasing times of incubation. Finally, to rule out mitogenic effects of IGF-I, cell numbers were determined at

0 h and following 24 h of treatment in the presence and absence of IGF-I.

R. 3.1 IGF-I PROTECTION

Fig. 10 (Panel A) demonstrates the effect of increasing IGF-I concentrations on VM26-induced apoptosis in PC12 cells. Following 24 h of treatment with 5 μM VM26, in the absence of IGF-I, cell viability was 35.8% as assessed by trypan blue exclusion. With 50ng/ml IGF-I, cell viability rose to 44.8%, with 100 ng/ml it was 49.1% and with 200 ng/ml it was 52.1 %. Cell viability measurements therefore illustrated a concentration-dependent protective effect of IGF-I, although IGF-I did not block all apoptosis induced by VM26.

R. 3.2 EXTENT OF IGF-I PROTECTION

Fig 10 (Panel B) demonstrates the protective (anti-apoptotic) effects of IGF-I on PC12 cells at increasing concentrations of VM26 from 0 μM to 5 μM. Cell viability is shown following 24 h of treatment with the indicated concentrations of VM26, in the presence (●) or absence (○) of 100 ng/ml IGF-I. The difference in cell viability between IGF-I treated and untreated cells remained approximately uniform over the concentrations of VM26 used. IGF-I treatment increased cell viability by about 15%. However,with increasing concentrations of VM26, the protective effect of IGF-I decreased, as noted by a progressive drop in cell viability even in protected cells.

R. 3.3 TIME COURSE OF IGF-I PROTECTION

Fig. 10 (Panel C) demonstrates the protective (anti-apoptotic) effects of IGF-I on PC12 cells treated with 5 μM VM26 over 48 h. Cells protected with 100 ng/ml IGF-I (●) showed a more gradual decrease in cell viability over 48 h as compared with the cells not exposed to IGF-I (○). The upper curve on the graph demonstrates that over time IGF-I is not able to completely protect against apoptosis, but when compared with the unprotected cells IGF-I was able to significantly slow the rate of apoptosis. In the presence of IGF-I, a 1.8 fold decrease in cell viability was observed whereas in the absence of IGF-I there was a 2.8 fold decrease in cell viability.

R. 3.4 IGF-I EFFECTS ON PC12 PROLIFERATION

Fig. 10 (Panel D) demonstrates that IGF-I inhibited apoptosis in a manner independent of its mitogenic actions. Indeed, after 24 h of incubation, cells exposed to IGF-I, but not VM26, showed a smaller increase in cell number compared with the cells not exposed to IGF-I. The bar graph illustrates that IGF-I did not exert any proliferative effects on the PC12 cells. Thus, the decrease in cell death seen in the previous experiments could not be due to an effect of IGF-I on increasing cell numbers.

Figure 10. Effect of IGF-I protection on apoptosis

Panel A: Graphic representation of the effect of increasing IGF-I concentration on PC12 cells exposed to 5 μM VM26 for a 24 h period of time.

All flasks of PC12 cells were treated at the 0 h time point with 5 μM VM26 and varying concentrations of IGF-I, ranging from 0 to 200 ng/ml final concentration. After 24 h of incubation cell number and cell viability were assessed. Points are the means of triplicate determinations from a representative experiment. IGF-I concentration is plotted on the x-axis against % cell viability on the y axis.

Panel B: Graphic representation of the effect of increasing concentration of VM26, on PC12 cells exposed to 100 ng/ml of IGF-I for a 24 h time period.

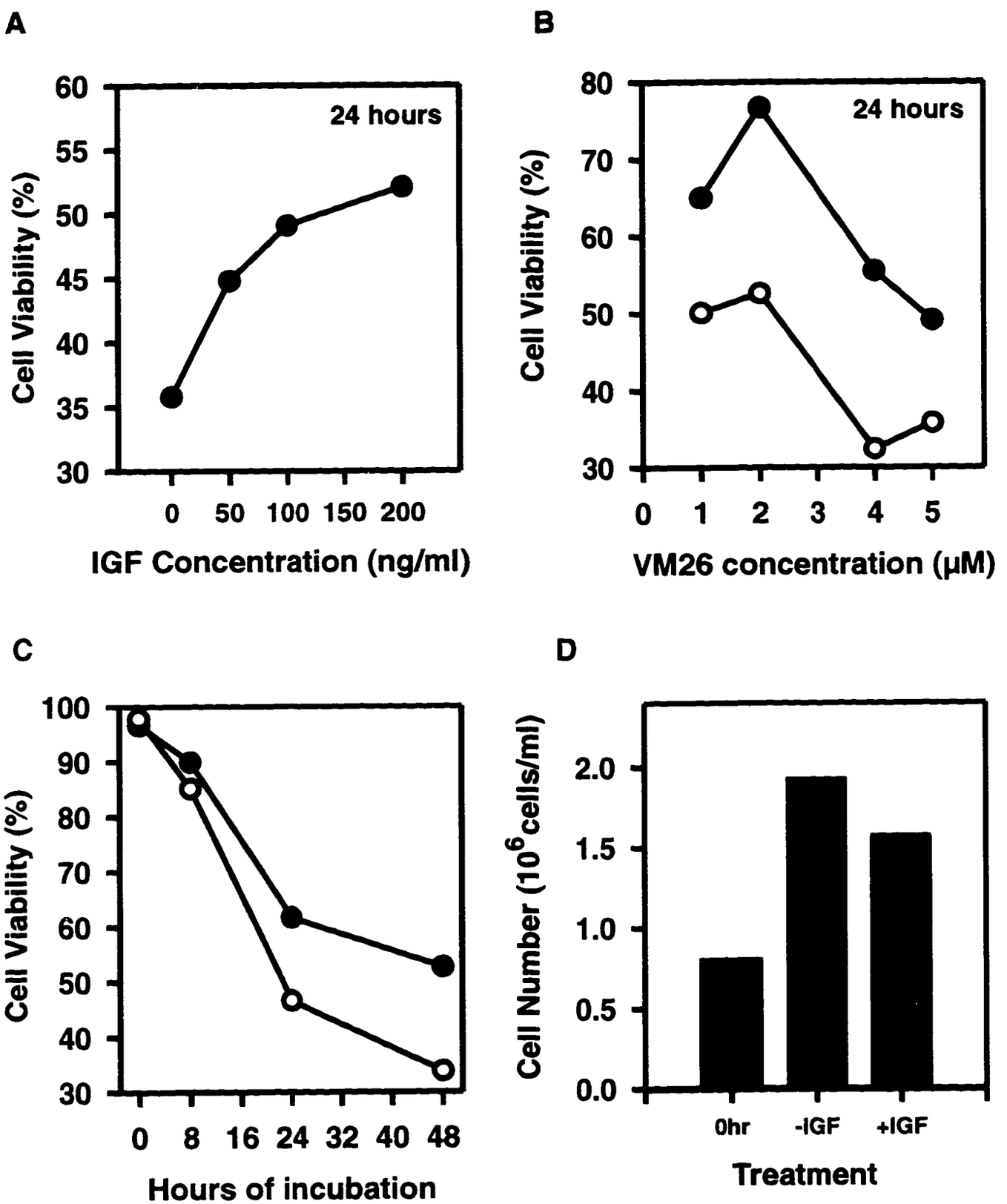
PC12 flasks were divided into two groups. The first group were treated with 100 ng/ml of IGF-I, and exposed to VM26 concentrations of 0 to 4 μM (●). The second group were not treated with 100 ng/ml of IGF-I, but were exposed to VM26 concentrations of 0 to 4 μM (○). At 24 h, cell numbers and cell viability were asssessed. Points are the means of triplicate determinations from a representative experiment. VM26 concentration is plotted on the x-axis against % cell viability on the y-axis.

Panel C: Graphic representation of the protective effect of 100 ng/ml IGF-I on PC12 cells treated over 24 h with 5μM VM26.

PC12 flasks were divided into two groups. The first group were treated with 100ng/ml IGF-I, and 5μM VM26 for 0 h, 8 h, 24 h, and 48 h at which time cell counts and viability were assessed (●). The second group were not treated with IGF-I but were exposed to 5 μM VM26, cell counts and viability were determined at 0 h, 8 h, 24 h, and 48 h (○) Points are the means of triplicate determinations from a representative experiment. Hours of incubation are plotted on the x-axis against % cell viability on the y-axis.

Panel D: Graphic representation of the effect of 100 ng/ml IGF-I on PC12 number after 24 h of incubation.

PC12 cell numbers were assessed using a haemocytometer at the 0 h time point and at 24 h following treatment with and without IGF-I. The bar graph illustrates the cell number determined for each of the three categories. Points are the means of triplicate determination from a representative experiment. The treatment and length of time of incubation is indicated on the x-axis and the cell number on the y-axis.


A
60
55
50
45
40
35
30
24 hours
Cell Viability (%)
0
50
100
150
200
IGF Concentration (ng/ml)
B
80
70
60
50
40
30
24 hours
Cell Viability (%)
0
1
2
3
4
5
VM26 concentration (μM)
C
100
90
80
70
60
50
40
30
Cell Viability (%)
0
8
16
24
32
40
48
Hours of incubation
D
2.0
1.5
1.0
0.5
0.0
Cell Number (10^6cells/ml)
0hr
-IGF
+IGF
Treatment

DISCUSSION

It is now widely accepted that apoptosis is a controlled form of cell death that serves as a genetic control point for a number of biological processes. The involvement of gene expression in apoptosis is a recently recognized phenomenon, that came to light after several experiments which demonstrated that apoptosis can be effectively blocked by the presence of inhibitors of macromolecular synthesis (Martin *et al.* 1988; Oppenheim *et al.* 1990). This has subsequently led to the search for genes that are required for, and play an important role in apoptosis. In the regressing rat ventral prostate, for example, several remarkable changes in gene expression have been demonstrated following the induction of apoptosis. Similarly, other model systems of apoptosis have also shown a dependence on specific gene expression and it is now becoming increasingly evident that the apoptotic pathway is under tight genetic control. The understanding of the role of specific genes regulating the process is therefore key to expanding our knowledge in this field.

The question that was addressed in this study was the roles of the clusterin, cathepsin B, IGFBP2, IGFBP5 and RSG3 genes in PC12 neuronal cells undergoing apoptosis. These genes were shown previously in the regressing rat ventral prostate to have varied levels of expression during apoptosis, consistent with a putative role in apoptosis in the prostate and a potential role in other model systems of apoptosis. In this work, neuronal PC12 cell cultures were used to simulate an *in vitro* model of neuronal apoptosis because these cells have been used widely in the study of the response of neuronal cells to a variety of toxic and non-toxic stimuli (Peunova & Enikolopov, 1993). For example, PC12 cells

have been successfully used for studying the survival promoting actions of NGF (nerve growth factor) and IGF-I (Insulin-like growth factor) and the mechanisms of neuronal cell death (Batistatou & Greene, 1991). To induce apoptosis in the PC12 cell cultures, the chemotherapeutic drug VM26 was used. VM26 belongs to a family of topoisomerase inhibitors that stabilizes topoisomerase-DNA cleavable complexes by blocking the DNA relegation step of the catalytic reaction, which results eventually in DNA cleavage and cell death. Consistent with other studies on PC12 cells (Nakajima *et al.* 1994), thymocytes (Onishi *et al.* 1993; Walker *et al.* 1991), IL-3 dependent pre-murine B-cells (Ascaso *et al.* 1994), ALL leukemic cells (Klumper *et al.* 1995), unstimulated mature murine lymphocytes (Roy *et al.* 1992), and MCF-7 cells (Orr *et al.* 1995) the topoisomerase-II inhibitor, VM26, induced cell death in the PC12 cells with classical characteristics of apoptosis.

The degree of apoptosis, assessed by trypan blue uptake, increased over the 24 h incubation period. This is similar to another study on PC12 cells where exposure to a topoisomerase II inhibitor lead to apoptosis within 24 h (Nakajima *et al.* 1994). In our PC12 cells, there was a dramatic increase in cell death 8 h after addition of 5 μM VM26 which may reflect the time required for cell signalling to effect specific induction or inhibition of gene expression necessary for apoptosis. This hypothesis is supported by findings in MCF-7 cells treated with VM26, in which a concentration dependent downregulation of c-Myc expression accompanied apoptosis suggesting that VM26 is capable of altering gene expression in a way that promotes apoptosis (Orr *et al.* 1995). It is also conceivable that cells undergoing apoptosis earlier than 8 hours are more sensitive to the effects of VM26 and have already induced some of the genes necessary for apoptosis, while those which take longer than 8 h,

are more resistant to the effects of VM26 on gene induction.

Not only was VM26 able to induce changes in gene expression but also cause DNA fragmentation, a widely used biochemical indicator of apoptosis. Using pulse-field gel electrophoresis to detect fragments of 200bp-1Mb, progressive DNA fragmentation in the PC12 cells was observed and shown to occur coincidently with an increase in cell death determined by the loss of trypan blue exclusion. A similar pattern has been seen in other studies in which PC12 cells were induced to die following serum withdrawal or treatment with calcium ionophores (Joseph *et al.* 1993). In addition, VM26 has been shown to induce DNA fragmentation and apoptosis in both thymocytes as well as unstimulated and mature murine lymphocytes (Walker *et al.* 1991; Onishi *et al.* 1993). In our system, like others, DNA fragmentation progressed in a stepwise manner producing fragments of 300 kbp and 50 kbp. These two cleavage steps have been observed in almost every cell type undergoing apoptosis, and thus serves as a good indicator of the early stages of apoptosis. Temporally, DNA fragmentation to the 50 kbp size, became apparent 6-8 h into the incubation, and appeared to occur concomitantly with the sharp increase in the level of cell death at 8h, suggesting that 6-8 h was required for the gene induction or activation necessary for DNA fragmentation and apoptosis. Such dependence on gene induction or activation can explain why inhibitors of RNA and protein synthesis can block or delay apoptosis. This effect has been shown in PC12 cells, which upon exposure to topoisomerase II inhibitors in the presence of cycloheximide and actinomycin D, did not undergo apoptosis (Nakajima *et al.* 1994). The same phenomenon has also been seen in other model systems of apoptosis, and is a key feature distinguishing apoptosis from necrosis. The ability to promote or block

apoptosis with various agents such as transcriptional and translational inhibitors and growth factors indicates that complex signalling systems must be in place to allow the cell to respond to its environment and undergo apoptosis when necessary. It is also likely that the signals that regulate apoptosis are as varied as the systems in which apoptosis occurs.

Apoptosis can also be characterized by specific morphological criteria including chromatin condensation and margination and formation of apoptotic bodies. VM26-induced changes in the morphology of PC12 cells became evident at around 8 h when the chromatin marginated to the nuclear membrane, and some cells began to look visibly different from their neighbors under phase contrast. Interestingly, the time point of these changes correlated well with the increase in DNA fragmentation suggesting that nuclear morphological changes may thus begin to occur only after a threshold level of DNA fragmentation has occurred, suggesting that the progression of DNA cleavage may be reflected in the increasing morphological changes in the apoptotic cell. Once genes have been activated, all the necessary changes for apoptosis can follow in an ordered manner. By 18 h following VM26 treatment many cells were smaller in size and showed phase differences indicative of changes within the cytoplasm. By 24 h chromatin was marginated against the well preserved nuclear membrane in most cells. A similar pattern of morphological change has been observed in DU145, 5123tc and PC12 cells undergoing apoptosis following growth factor deprivation (Pandey, S.R., personal communciation). These morphological changes are also similar to those seen in thymocytes incubated *in vitro* with VM26, in which the condensed chromatin masses displayed a characteristic reticular and fibrillated substructure. It has been suggested that chromatin must be detached from the nuclear scaffold attachment points before

chromatin condensation can occur. This process is likely mediated by the joint actions of nucleases and proteases, making the process susceptible to the effects of inhibitors of transcription, translation and enzyme activation (Walker & Sikorska, 1994, Weaver *et al*, 1996) which may be able to effectively block or delay apoptosis by preventing gene induction, gene activation, DNA fragmentation and the associated chromatin condensation. Another interesting morphological feature that was noted was the accumulation of abundant debris in the cell cultures by 18 h. *In vivo*, it is likely that this debris would be phagocytosed by neighboring healthy cells or support cells such as astrocytes, thereby averting an inflammatory response by the body. By examining both the biochemical and morphological changes induced by VM26 we were able to conclude that VM26 induced apoptosis in the PC12 cells in a process likely requiring gene induction and gene activation.

Having established a neuronal model system of apoptosis we then sought to understand the role of clusterin, cathepsin B, IGFBP2, IGFBP5, and RSG-3 in this model system. There is already evidence in the literature suggesting that these genes are involved in the regulation of apoptosis in the regressing prostate and in other model systems of apoptosis. There is also the suggestion that these genes may work in concert with each other and cell signalling systems to control different aspects of the apoptotic process. The changes in the levels of expression of these genes was compared with that of HPRT, a housekeeping gene not likely to change during apoptosis. As expected, the levels of HPRT were relatively constant allowing it to serve as a good internal control for the gene expression experiments which were conducted in this study.

The first gene to be studied in the PC12 model of neuronal cell death was clusterin.

Clusterin and its cognate protein has been characterized in a number of species and has been implicated in apoptosis occurring in the prostate tissue following hormone ablation (Tenniswood *et al.*, 1996). In the PC12 neuronal model system, clusterin was expressed in untreated cells then was dramatically upregulated to peak at 8 h, followed by an equally dramatic drop by 24 h. This time course of expression suggests that clusterin may be synthesized early and be actively involved in the later stages of apoptosis at a time when cytoplasmic changes and membrane remodelling are readily occurring. These expression kinetics also support findings from other studies that suggests that clusterin is involved in controlling cholesterol efflux from the membranes of dying cells, and facilitating the transfer of cholesterol from these membranes to ApoA-1/HDL, a key process that must occur before fragmentation of the dying cell into apoptotic bodies (Wilson *et al.*, 1994). This function would generally operate at low levels in healthy cells, explaining clusterin expression in the untreated PC12 cells. During tissue damage or death, it has been suggested that enhanced clusterin expression is required to cope with the increased lipid load from the membranes of dying cells and to protect the cells from complement mediated lysis. The observed increase at 8 h likely reflects this increase in requirements for clusterin . In addition, peak levels of clusterin at 8 h, which coincides with the sharp increase in the numbers of apoptotic cells, also supports the putative role of clusterin in preventing large numbers of dying cells from eliciting a complement-based response. After the dying cells have become apoptotic bodies, they no longer undergo membrane remodelling, and are rapidly phagocytosed by neighboring cells. Clusterin expression is thus no longer required, and can account for the drop in clusterin expression at 24 hours, back to baseline levels. In addition many of the cells

expressing clusterin have died by this stage.

Studies suggest that clusterin likely plays a role in a number of physiological processes requiring membrane remodelling and inhibition of an inflammatory response. Change in the expression of clusterin has not only been seen in the neuronal system but also in the prostate, retina and kidney cells in which apoptosis is occurring. Overexpression of clusterin has also been noted in Alzheimer's disease and several other neurodegenerative diseases (Michel *et al.* 1992). The widespread changes in expression of this gene across different model systems of apoptosis therefore highlights its role as an important modulator of apoptosis.

Another gene named cathepsin B which is a member of a family of cysteine proteases, has also shown potential for being involved in apoptosis. Studies have shown that degradation of components of the ECM *in vivo* is a key step involved in the cytoplasmic condensation of apoptotic cells (Sloane & Honn, 1984) and this degradation may in fact be mediated by the effects of specific proteases. In the PC12 cells undergoing apoptosis, cathepsin B was expressed in untreated cells then showed a slight transient increase at 6 h but in general did not show a significant increase in expression over the 24 h time course. This pattern of expression is similar to that seen in apoptotic cortical cultures (Hill I.E., personal communication), but contrasts to cathepsin B expression patterns found in the regressing prostate and mammary tissue (Guenette *et al.* 1994).

The similarity between apoptotic PC12 cells and apoptotic cortical cultures could be explained by the fact that both systems are dealing with neuronal models of apoptosis. Immunocytochemistry studies on cortical cells undergoing apoptosis suggest that levels of

cathepsin B may not actually be increasing significantly, but instead there is a redistribution of cathepsin B from a lysosomal location in normal cells to a nuclear location in apoptotic cells (Hill, I.E. personal communication). In addition, the expression of cathepsin B in untreated PC12 cells suggests that cathepsin B may have a role in normal cellular function, and then is recruited to perform a specific role during apoptosis. This is similar to findings in the regressing prostate and mammary gland (Guenette *et al.* 1994b) where there is expression in normal cells but unlike the regressing prostate and mammary models, there is no significant induction of cathepsin B . Induction appears to be necessary in the prostate and mammary tissues to ensure that the dying cell is released from the basement membrane and the ECM of the prostate and mammary tissues. Since the PC12 cells are grown in culture the ECM may not be as extensive or contain the same components that would be expected *in vivo*, or in a complex tissue such as the prostate, and hence may not require a significant activation of cathepsin B to disrupt associations. In addition, hormone-dependent tissues may exhibit a dependency upon different factors for apoptosis compared with the neuronal system. It is also important to bear in mind that changes in the protein level of cathepsin B, in terms of expression and localization, may not be reflected at the mRNA level and would, therefore, be an important future direction of the study of this gene product, especially owing to the fact that cathepsin B may play a role in several neurodegenerative diseases.

The IGFBP2 gene is a gene whose potential role in modulating apoptosis has only recently been recognized following studies on the regressing prostate (Nordqvist *et al.* 1996) In general, peptide growth factors have been studied by determining the response of cells to

soluble growth factors that diffuse rapidly through a liquid culture medium. However, *in vivo*, cells are embedded in a complex extracellular matrix, and diffusible growth factors may be exposed to the ECM before binding to their cognate receptors. Access to the receptors may therefore involve intermediate binding to components of the matrix or specific binding proteins which can stabilize the growth factor, protect it from proteolytic degradation, increase the local concentrations of the growth factor and/or alter the rate of diffusion through the matrix. As a result, it is becoming increasingly clear that the cellular response to growth factors is greatly dependent upon the presence of binding proteins and the components of the ECM (Jones *et al.* 1993). In support of these findings, sequence analysis of IGFBP2 has revealed RGD sequences which raises the possibility that IGFBP2s may bind to integrin-type receptors at the cell surface and thus be able to target IGF-I to particular sites or participate in the local organization of ligands at the cell surface. Conversely, IGFBP2 may sequester IGF-I and prevent its interaction with the IGF-I receptor.

IGFBP2 was expressed in untreated PC12 cells, then dramatically upregulated four fold by 8 h. This pattern of expression is similar to that seen with clusterin and peak levels of IGFBP2 at 8 hours were coincident with the onset of DNA fragmentation, appearance of cytoplasmic changes and sharp increases in the levels of cell death. During apoptosis, IGFBP2 expression was significantly induced which could potentially attenuate the cellular response to IGF-I through the high affinity binding of IGF-I to IGFBP2. The increase in IGFBP2 could therefore sequester IGF-I away from its receptor, and prevent IGF-I from exerting any survival effects on the dying cell. Other studies in the neuronal system, have shown that IGFBP2 is able to inhibit IGF-I promoted DNA synthesis in cultured astroglia

(Han *et al.* 1988). In this system IGFBP2 appears to be produced in order to shield glial cells against the mitogenic effects of IGF-I so that glia can continue to perform their differentiated functions. In the setting of CNS injury, where there is reactive gliosis, or proliferation of astrocytes, IGF-I is produced in abundance, but IGFBP2 is not expressed, suggesting that IGF-I, in the absence of IGFBP2 is able to stimulate astrocytic proliferation. In another study IGFBP2 expression has been shown to be temporally and anatomically coordinated with that of IGF-I, during cerebellar and retinal development, thus implicating IGFBP2 as a key modulator of IGF-I action in CNS development (Lee *et al.* 1992). Studies also suggest that IGFBP2, as a major component of the CSF, may play a major role in changes occurring in the brain in normal and pathological conditions (Greene & Tischler, 1976).

Another binding protein which plays a role in modulating IGF actions is IGFBP5. This binding protein has been found in many tissues and particularly high levels have been found in the CSF, bone and regressing prostate and bone. Studies have also demonstrated that matrix-associated IGFBP5 has physiological significance in mediating the mitogenic effects of IGF-I by localizing and stabilizing the concentration of the hormone in the vicinity of the IGF-I receptors facilitating receptor interactions and promoting cell survival (Jones *et al.* 1993). In contrast, it has been shown that IGFBP5 is not expressed constitutively in the rat ventral prostate and mammary gland but is greatly upregulated following induction of apoptosis by androgen ablation (Guenette & Tenniswood, 1994). IGFBP5 expression occurs early in the apoptotic process in the regressing prostate leading to the suggestion that it may serve as a trigger for apoptosis. It was therefore studied in the neuronal system to determine if it had similar expression kinetics in the apoptotic PC12 cells.

In sharp contrast to IGFBP2, IGFBP5 mRNA levels were relatively high in untreated PC12 cells but decreased significantly by 8 h. This dramatic decrease in expression suggests a number of possibilities. First, that IGFBP5 expression is not involved in apoptosis, or secondly, that the sharp drop in the steady state levels signals the cell to enter a death pathway. In addition, IGFBP5 has been shown to have an intimate relationship with the ECM, and since the PC12 cells are grown in culture they may not have an extensive matrix to which to bind and IGFBP5 therefore might not be expressed. Also, it is conceivable that if IGFBP5 does in fact promote growth, that it is unlikely that a large population of dying cells would secrete a growth factor unless of course it was part of a stress response.

When compared to the regressing prostate where IGFBP5 was induced *de novo*, IGFBP5 expression in the PC12 cells dropped off dramatically following VM26 treatment. This could be reflective of differences in the role of IGFBP5 in different systems or differences between *in vivo* conditions and *in vitro* conditions. In addition, it has been suggested that in the prostate IGFBP5 levels are repressed by the presence of androgens and following androgen ablation IGFBP5 mRNA is induced. In contrast, androgens do not play as large a role in the neuronal system, which could further explain differences in gene expression patterns between these two systems. Differences in the composition of the ECM between the two systems may also account for differential functions of the binding proteins in different systems.

The final gene to be studied in this thesis was RSG-3, a gene that was initially isolated form the prostate undergoing apoptosis and was subsequently also shown to be expressed in the regressing mammary tissue (Sridhar, 1993). RSG-3 is a cell surface

molecule that may play a role in targeting apoptotic bodies to neighboring cells for phagocytosis. In the apoptotic PC12 cells, there appeared to be a slight increase in expression over the 24 h period, with levels peaking at 24 h. This is in keeping with the suggestion that RSG-3 is involved in targeting apoptotic bodies to neighboring cells for phagocytosis to avoid an inflammatory response. This event is expected to occur in the final stages of apoptosis. It is also possible that an *in vivo* experiment may show an increased levels of RSG-3 expression because more surrounding healthy cells are present and targeting of apoptotic bodies to these cells may be more effective. The expression kinetics of RSG-3 were similar to those seen in the regressing prostate and mammary tissues. The gene shows expression in normal cells, and is gradually induced in apoptotic cells. These observations indicate that the gene may play a role in a variety of systems, to ensure that the apoptotic process is completed, ensuring that apoptotic cells are phagocytosed to prevent an inflammatory response.

This study into genetic involvement in apoptosis has shown that following induction of apoptosis with VM26, PC12 cells show differential gene expression. It appears that clusterin and IGFBP2 play a significant role in neuronal apoptosis given that the timing and level of their induction is quite significant. In addition these gene products have been found in the brain and CSF respectively, with some association to neurodegenerative conditions. The expression of the RSG-3 gene showed a gradual increase over the 24 h time course. This pattern of induction, suggests that RSG-3 may play an important role in the final stages of apoptosis, when apoptotic bodies are being phagocytosed. Unlike RSG-3, cathepsin B did not show a significant increase in expression over the 24 hour time course indicating that the

apoptotic process may require relocation of the protein rather than an increase in expression. Finally the IGFBP5 gene showed a substantial drop in expression, early in apoptosis, suggesting that apoptotic cells may not require this gene or alternatively the dramatic drop may serve as a trigger for apoptosis. The relative changes in IGFBP2 and IGFBP5 observed in this study are consistent with a role of IGFBP2 in cell death and IGFBP5 in cell survival. It may well be that the ratio of these two binding proteins decide the overall balance between survival and death. The observations of Lee *et al.* (1996), Nordqvist *et al.* (1996) and Zhou *et al.* (1996) are consistent with this view. However, the patterns of expression of IGFBPs is cell and tissue specific and may be affected by the nature of the cell death signal, so it is not surprising that different results are obtained in other models of cell death (Guenette & Tenniswood, 1994).

A significant amount of data has been accumulated recently characterizing the expression of IGFBPs in a variety of different cell lines (Clemmons, 1993). The emerging data suggests that the regulation of IGF is a complex process, involving IGF receptors, IGF binding proteins and proteases that can proteolyze both the growth factor and the binding proteins and likely other as yet unidentified factors. This complex network can work in conjunction with binding proteins to regulate apoptosis in a number of systems. Specifically in the neuronal system, IGF-I has been shown to prevent the loss of choline acetyltransferase activity in embryonic spinal cord cultures, as well as reduce the level of apoptosis *in vivo* in motor neurons during normal development or following axotomy or spinal transection (Lewis *et al.* 1993). Consistent with these findings, our data showed that treatment of PC12 cells with IGF-I exerts a protective effect on the PC12 cells as noted by the decreased levels

of cell death. Furthermore, we have demonstrated that the protective effect of IGF-I increases in a concentration-dependent manner, indicating that cells are capable of responding to a protective signal even in the presence of a death-inducing signal. A similar result was shown in BALB/c3T3 cells treated with a topoisomerase inhibitor in the presence of IGF-I. IGF-I treatment in this system, inhibited apoptosis in a manner independent of the mitogenic actions of IGF-I (Sell *et al.* 1995). This supports our findings, that showed that IGF-I treatment does not in fact lead to an increased cell number but increased survival, suggesting that IGF-I must operate through its receptor and specific cell signalling pathways to exert survival effects upon the cell. Finally we can conclude in the neuronal system that an upregulation of IGFBP2 may be interfering with IGF-I signalling, leading to apoptosis. Exogenous IGF-I appears to be able to overcome the effects of increased IGFBP2, and partially inhibit apoptosis. It also introduces the potential for using IGF or IGFBPs as a treatment for progressive neuronal apoptosis associated with a number of neurodegenerative conditions.

The findings in this thesis offer exciting information in the area of neuronal apoptosis which is believed to result in many neurodegenerative diseases currently under intense study. IGF-I therapy in the future may become an important treatment strategy for a number of these conditions. Further studies should be aimed at understanding the role of the complex IGF signalling pathways, particularly the IGFBPs in regulating apoptosis.

BIBLIOGRAPHY

AMATI, B., LITTLEWOOD, T. D., EVAN, G. I. & LAND, H. (1993). The c-myc protein induces cell cycle progression and apoptosis through dimerization with max. *EMBO Journal* **12**, 5083-5087.

ARENDS, M. J., MORRIS, R. G. & WYLLIE, A. H. (1990). Apoptosis: The role of the endonuclease. *American Journal of Pathology* **136**, 593-608.

ARTEAGA, C. L. (1992). Interference of the IGF systems as a strategy to inhibit breast cancer growth. *Breast Cancer Research and Treatment* **22**, 101-106.

ASCASO, R., MARVEL, J., COLLINS, M. K. L. & LOPEZRIVAS, A. (1994). Interleukin-3 and bcl-2 cooperatively inhibit etoposide- induced apoptosis in a murine pre-B cell line. *European Journal of Immunology* **24**, 537-541.

ASKEW, D. S., ASHEMN, R. A., SIMMONS, B. C. & CLEVELAND, J. L. (1991). Constitutive c-myc expression in an IL-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915-1922.

AUFFRAY, C. & ROUGEON, F. (1979). Purification of mouse immunoglobulin heavy chain messenger RNA's from total myeloma tumor RNA. *European Journal of Biochemistry* **107**, 303-314.

BARRY, M. A. & EASTMAN, A. (1993). Identification of Deoxyribonuclease-II as an endonuclease Involved in apoptosis. *Archives of Biochemistry and Biophysics* **300**, 440-450.

BARRY, M. A. & EASTMAN, A. (1992). Endonuclease activation during apoptosis: The role of cytosolic Ca^{2+} and pH. *Biochemical and Biophysical Research Communcications* **186**, 782-789.

BATISTATOU, A. & GREENE, L. A. (1993). Internucleosomal DNA cleavage and neuronal cell survival/death. *Journal of Cell Biology* **122**, 523-532.

BATISTATOU, A. & GREENE, L. A. (1991). Aurintricarboxylic acid rescues PC12 cells and sympathetic neurons from cell death caused by nerve growth factor deprivation: correlation with suppression of endonuclease activity. *Journal of Cell Biology* **115**, 461-471.

BATISTATOU, A., MERRY, D. E., KORSMEYER, S. J. & GREEN, L. A. (1993). Expression of Bcl-2 proto-oncogenes rescues PC12 cells from death caused by withdrawal of trophic of support. *Journal of Neuroscience* **13**, 4422-4428.

BENNETT, M. R., EVAN, G. I. & SCHWARTZ, S. M. (1995). Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *Journal of Clinical Investigation* **95**, 2266-2274.

BETUZZI, S., HIIPAKKA, R. A., GILNA, P. & LIAO, S. (1989). Identification of an androgen-repressed mRNA in rat ventral prostate as coding for sulphated glycoprotein 2 by cDNA cloning and sequence analysis. *Biochemical Journal* **257**, 293-296.

BEVER, C. T. J. & GARVER, D. W. (1995). Increased cathepsin B activity in multiple sclerosis brain. *Journal of Neurological Sciences* **131**, 71-73.

BISSONNETTE, R. P., EXHEVERRI, F., MAHBOUBI, A. & GREEN, D. R. (1992). Apoptotic cell death induced by c-myc is inhibited by bcl-2. *Nature* **359**, 552-554.

BOISE, L. H., GONZALEZGARCIA, M. & POSTEMA, C. E. (1993). Bcl-x, a bcl-2 related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597-608.

BUERKE, M., MUROHARA, T., SKURK, C., NUSS, C., TOMASELLI, K. & LEFER, A. M. (1995). Cardioprotective effect of insulin-like growth factor I in myocardial ischemia followed by reperfusion. *Proceedings of the National Academy of Sciences USA* **92**, 8031-8035.

BURSCH, W., PAFFE, S., PUTZ, B., BARTHEL, G. & SCHULTE-HERMANN, R. (1990b). Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. *Carcinogenesis* **11**, 847-853.

CAIN, K., INAYATHUSSAIN, S. H., WOLFE, J. T. & COHEN, G. M. (1994). DNA fragmentation into 200-250 and/or 30-50 kilobase pair fragments in rat liver nuclei is stimulated by Mg2+ alone and Ca2+/Mg2+ but not by Ca2+ alone. *FEBS Letters* **349**, 385-391.

BURSCH, W., KLEINE, L. & TENNISWOOD, M. P. (1990a).The biochemistry of cell death by apoptosis. *Biochemistry and Cell Biology* **68**, 1071-1074.

BURSCH, W., GLEESON, T. G., KLEINE, L. & TENNISWOOD, M. P. (1995). Expression of TRPM-2/Clusterin mRNA during growth and regression of rat liver. *Archives of Toxicology* **69**, 253-258.

CATALDO, A. M. & NIXON, R. A. (1990). Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain. *Proceedings of the National Academy of Sciences, USA.* **87**, 3861-3865.

CHITTENDEN, T., HARRINGTON, E. A., OCONNOR, R., FLEMINGTON, C., LUTZ, R. J., EVAN, G. I. & GUILD, B. C. (1995). Induction of apoptosis by the Bcl-2 homologue Bak. *Nature* **374**, 733-736.

CHUN, S. Y., EISENHAUER, K. M., MINAMI, S., BILLIG, H., PERLAS, E. & HSUEH, A. J. W. (1996). Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. *Endocrinology* **137**, 1447-1456.

CLARKE, A. R., PURDIE, C. A., HARRISON, D. J., MORRIS, R. G., BIRD, C. C., HOOPER, M. L. & WYLLIE, A. H. (1993). Thymocyte apoptosis induced by p53 dependent and independent pathways. *Nature* **362**, 849-852.

CLARKE, A. R., GLEDHILL, S., HOOPER, M. L., BIRD, C. C. & WYLLIE, A. H. (1994). p53 dependence or early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. *Oncogene* **9**, 1767-1773.

CLARKE, P. G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anatomy and Embryology* **181**, 195-213.

CLEMMONS, D. R. (1993). IGF Binding Proteins and their functions. *Molecular Reproduction and Development* **35**, 368-375.

COHEN, J. J. & DUKE, R. C. (1984). Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *Journal of Immunology* **132**, 38-42.

COHEN, J. J. (1993). Apoptosis. *Immunology Today* **14**, 126-130.

COHEN, P., PEEHL, D. M., LAMSON, G. & ROSENFELD, R. G. (1991). Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins in primary cultures of prostate epithelial cells. *Journal of Clinical Endocrinology and Metabolism* **73**, 401-407.

COLOMBEL,M., OLSSON, C. A., NG, P.-Y. & BUTTYAN, R. (1992). Hormone-regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle. *Cancer Research* **52**, 4313-4319.

COLOMBEL, M., SYMMANS, F., GIL, S., OTOOLE, K. M., CHOPIN, D., BENSON, M., OLSSON, C. A., KORSMEYER, S. & BUTTYAN, R. (1993). Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. *American Journal of Pathology* **143**, 390-400.

CONOVER, C. & POWELL, D. R. (1991). IGFBP3 blocks IGF-1 induced receptor down regulation and cell desensitization in cultured bovine fibroblasts. *Endocrinology* **129**,

710-716.

COWAN, W. M., FAWCETT, J. W., O'LEARY, D. D. & STANFIELD, B. B. (1984). Regressive events in neurogenesis. *Science* **225**, 1258-1265.

DAMSKY, C. & WERB, Z. (1996). Signal transduction by integrin receptors for extracellular matrix: co-operative processing of extracellular information. *Current Opinions in Cell Biology* **4**, 772-781.

DELUCA, A., WELLER, M. & FONTANA, A. (1996). TGF-beta-induced apoptosis of cerebellar granule neurons is prevented by depolarization. *Journal of Neuroscience* **16**, 4174-4185.

DUSENBURY, C. E., DAVIS, M. A., LAWRENCE, T. S. & MAYBAUM, J. (1991). Induction of megabase DNA fragments by 5-Fluorodeoxyuridine in human colorectal tumor (HT29) cells. *Molecular Pharmacology* **39**, 285-289.

EASTMAN, A. & BARRY, M. A. (1992). The origins of DNA breaks: A consequence of DNA damage, DNA repair, or Apoptosis? *Cancer Investigation* **10**, 229-240.

EISENHAUER, K. M., CHUN, S. Y., BILLIG, H. & HSUEH, A. J. W. (1995). Growth hormone suppression of apoptosis in preovulatory rat follicles and partial neutralization by insulin-like growth factor binding protein. *Biology of Reproduction* **53**, 13-20.

ELKON, K. B. (1994). Apoptosis in SLE - Too little or too much? *Clinical and Experimental Rheumatology* **12**, 553-559.

ENARI, M., HUG, H. & NAGATA, S. (1995). Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* **375**, 78-81.

ENGLISH, H. F., DRAGO, J. R. & SANTEN, R. J. (1985). Cellular response to androgen depletion and repletion in the rat ventral prostate: autoradiography and morpholmetric analysis. *Prostate* **7**, 41-51.

ENGLISH, H. F., KYPRIANOU, N. & ISSACS, J. T. (1989). Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. *Prostate* **15**, 233-250.

EVAN, G. I., WYLLIE, A. H., GILBERT, C. S., LITTLEWOOD, T. D., LAND, H., BROOKS, M., WATERS, C. M., PENN, L. Z. & HANCOCK, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128.

EVAN, G. I. & LITTLEWOOD, T. D. (1993). The role of c-myc in cell growth. *Current Opinions in Genetics and Development* **3**, 44-49.

FANIDI, A., HARRINGTON, E. A. & EVAN, G. I. (1992). Cooperative interaction between c-myc and bcl-2 proto-oncogenes. *Nature* **359**, 554-556.

FAVOLORO, J., FREISMAN, R. & KAMEN, R. (1980). Transcription maps of polyoma virus-specific RNA: analysis of two dimensional nuclease S1 gel mapping. *Methods in Enzymology* **65**, 718-749.

FEDORCSAK, I. & EHRENBERG, L. (1966). Effects of diethyl pyrocarbonate and methyl methanesulfonate on nucleic acids and nucleases. *Acta Chemica Scandinavica* **20**, 107-112.

FILIPSKI, J., LEBLANC, J., YOUDALE, T., SIKORSKA, M. & WALKER, P. R. (1990). Periodicity of DNA folding in higher order chromatin. *EMBO Journal* **9**, 1319-1327.

FRISCH, S. M. & FRANCIS, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *Journal of Cell Biology* **124**, 619-626.

FURUYA, Y., LUNDMO, P., SHORT, A. D., GILL, D. L. & ISAACS, J. T. (1994). The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. *Cancer Research* **54**, 6167-6175.

GARCIA, I., MARTINOU, I., TSUJIMOTO, Y. & MARTINOU, J. C. (1992). Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. *Science* **258**, 302-304.

GEIER, A., HEMI, R., HAIMSOHN, M., BEERY, R., MALIK, Z. & KARASIK, A. (1994). Epidermal growth factor and insulin-like growth factor-1 protect MDA-231 cells from death induced by actinomycin D: The involvement of growth factors in drug resistance. *In Vitro Cell Developmental Biology-Animal* **30A**, 336-343.

GEIER, A., WEISS, C., BEERY, R., HAIMSOHN, M., HEMI, R., MALIK, Z. & KARASIK, A. (1995). Multiple pathways are involved in protection of MCF-7 cells against death due to protein synthesis inhibition. *Journal of Cell Physiology* **163**, 570-576.

GRANERUS, M., SCHOFIELD, P., BIERKE, P. & ENGSTROM, W. (1995). Growth factors and apoptosis in development. The role of insulin like growth factor I and TGF beta 1 in regulating cell growth and cell death in a human teratocarcinoma derived cell line. *International Journal of Developmental Biology* **39**, 759-764.

GREENE, L. A. & TISCHLER, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the National Academy of Sciences, USA* **73**, 2424-2428.

GREENE, L. A. & REIN, G. (1977). Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. *Brain Research* **129**, 247-263.

GRISWOLD, M. D., ROBERTS, K. & BISHOP, P. (1986). Purification and characterization of a sulfated glycoprotein secreted by Sertoli cells. *Biochemistry* **25**, 7265-7270.

GUENETTE, R. S. & TENNISWOOD, M. (1994a). The role of growth factors in the suppression of active cell death in the prostate: An hypothesis - Review. *Biochemistry and Cell Biology* **72**, 553-559.

GUENETTE, R. S., MOOIBROEK, M., WONG, P. & TENNISWOOD, M. (1994b). Cathepsin B, a cysteine protease implicated in metastatic progression, is also expressed during regression of the rat prostate and mammary glands. *European Journal of Biochemistry* **226**, 311-321.

GUENETTE, R. S., CORBEIL, H. B., LEGER, J. G., WONG, K., MEZL, V., MOOIBROEK, M. & TENNISWOOD, M. P. (1994c). Induction of gene expression during involution of the lactating rat mammary gland. *Journal of Molecular Endocrinology* **12**, 47-60.

HAN, V. K. M., LAUDER, J. M. & D'ERCOLE, A. J. (1988). Rat astroglial somatomedin/insulin-like growth factor binding proteins: characterization and evidence of biological function. *Journal of Neuroscience* **8**, 3135-3143.

HARRINGTON, E. A., BENNETT, M. R., FANIDI, A. & EVAN, G. I. (1994). c-myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO Journal* **13**, 3286-3295.

HOCKENBERRY, D. M., NUNEZ, G., MILLIMAN, C., SCHREIBER, R. D. & KORSMEYER, S. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-336.

HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B. & HARRIS, C. C. (1991). p53 mutations in human cancers. *Science* **253**, 49-53.

HUANG, R., OZAWA, M., KADOMATSU, K. & MURAMATSU, T. (1990). Developmentally regulated expression of embigin, a member of the immunoglobulin

superfamily found in embryonal carcinoma cells. *Differentiation* **45**, 76-83.

IWAMURA, M., SLUSS, P. M., CASAMENTO, J. B. & COCKETT, A. T. (1993). Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. *Prostate* **22**, 243-252.

JACKSON, R. L., BUSCH, S. J. & CARDIN, A. D. (1991). Glycosaminoglycans: Molecular properties, protein interactions, and role in physiological processes. *Physiological Reviews* **71**, 481-522.

JACOBSON, M. D., BURNE, J. F., KING, M. P., MIYASHITA, T., REED, J. C. & RAFF, M. C. (1993). Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* **361**, 365-369.

JACOBSON, M. D. & EVAN, G. I. (1994). Apoptosis. Breaking the ICE. *Current Opinion in Cell Biology* **4**, 337-340.

JONES, I. J., GOCKERMAN, A., BUSBY, W. H. J., CAMACHO-HUBNER, C. & CLEMMONS, D. R. (1993). Extracellular matrix contains Insulin-like Growth Factor Binding Protein-5: potentiaiton of the effects of IGF-1. *Journal of Cell Biology* **121**, 679-687.

JOSEPH, R., LI, W. & HAN, E. (1993). Neuronal death, cytoplasmic calcium and internucleosomal dna-fragmentation - evidence for DNA-fragments being released from cells. *Molecular Brain Research* **17**, 70-76.

KELLEY, K. W., ARKINS, S., MINSHALL, C., LIU, Q. & DANTZER, R. (1996). Growth hormone, growth factors and hematopoiesis. *Hormone Research* **45**, 38-45.

KENNEDY, W. A., STENBERG, A., LACKGREN, G., HENSLE, T. W. & SAWCZUK, I. S. (1994). Renal tubular apoptosis after partial ureteral obstruction. *Journal of Urology* **152**, 658-664.

KERR, J. F. R., WYLLIE, A. H. & CURRIE, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* **26**, 239-257.

KERR, J. F. R. & HARMON, B. V. (1991). Definition and incidence of Apoptosis: An historical perspective. In *Apoptosis: The molecular basis of cell death*, eds. TOMEI, L. D. & COPE, F. O. pp. 5-29. Cold Spring Harbor,NY: Cold Spring Harbor Laboratory Press.

KERR, J. F. R. & SEARLE, J. (1973). Deletion of cells by apoptosis during castration

induced involution of the rat prostate. *Virchows Arch. Zellpath.* **13**, 87-102.

KLUCK, R. M., MCDOUGALL, C. A., HARMON, B. V. & HALLIDAY, J. W. (1994). Calcium chelators induce apoptosis - Evidence that raised intracellular ionised calcium is not essential for apoptosis. *Biochimica et Biophysica Acta* **1223**, 247-254.

KLUMPER, E., GIACCONE, G., PIETERS, R., BROEKEMA, G., VANARKOTTE, J., VANWERING, E. R., KASPERS, G. J. L. & VEERMAN, A. J. P. (1995). Topoisomerase II alpha gene expression in childhood acute lymphoblastic leukemia. *Leukemia* **9**, 1653-1660.

KOFOED, J. J., TUMILASCI, O. R., CURBELO, H. M., FERNANDEZ LEMOS, S. M., ARIAS, N. H. & HOUSSAY, A. B. (1990). Effects of castration and androgens upon prostatic proteoglycans in rats. *Prostate* **16**, 93-102.

KOMINAMI, E., TSUKAHARA, Y., BANDO, Y. & KATUNUMA, N. (1985). Distributions of cathepsins B and H in rat tissues and peripheral blood cells. *Journal of Biological Chemistry* **98**, 87-93.

KYPRIANOU, N. & ISAACS, J. T. (1988). Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology* **122**, 552-562.

LAFERLA, F. M., TINKLE, B. T., BIEBERICH, C. J., HAUDENSCHILD, C. C. & JAY, G. (1995). The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nature Genetics* **9**, 21-30.

LAZEBNIK, Y. A., KAUFMANN, S. H., DESNOYERS, S., POIRIER, G. G. & EARNSHAW, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346-347.

LEE, W. H., JAVEDAN, S. & BONDY, C. (1992). Coordinate Expression of Insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. *Journal of Neuroscience* **12**, 4737-4744.

LEGER, J. G., MONTPETIT, M. L. & TENNISWOOD, M. P. (1987). Characterization and cloning of androgen repressed mRNAs from rat ventral prostate. *Biochemical and Biophysical Research Communications* **147**, 196-203.

LEWIS, M. E., NEFF, N. T., CONTRERAS, P. C., STONG, D. B., OPPENHEIM, R. W., GREBOW, P. E. & VAUGHT, J. L. (1993). Insulin-Like Growth Factor-I - potential for treatment of motor neuronal disorders. *Experimental Neurology* **124**, 73-88.

LI, S. W., RESNICOFF, M. & BASERGA, R. (1996). Effect of mutations at serines

1280-1283 on the mitogenic and transforming activities of the insulin-like growth factor I receptor. *Journal of Biological Chemistry* **271**, 12254-12260.

LISTON, P., ROY, N., TAMAI, K., LEFEBVRE, C., BAIRD, S., CHERTONHORVAT, G., FARAHANI, R., MCLEAN, M., IKEDA, J., MACKENZIE, A. & KORNELUK, R. G. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379**, 349-353.

LITTLE, S. A. & MIRKES, P. E. (1995). Clusterin expression during programmed and teratogen- induced cell death in the postimplantation rat embryo. *Teratology* **52**, 41-54.

LOTEM, J. & SACHS, L. (1993). Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and differentiation-defective myeloid leukemic cells. *Cell Growth and Differentiation.* **4**, 41-47.

LOWE, S. W., SCHMITT, E. M., SMITH, S. W., OSBORNE, B. A. & JACKS, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-849.

MARTIN, D. P., SCHMIDT, R. E., DISTEFANO, P. S., LOWRY, O. H., CARTER, J. G. & JOHNSON, J. ,E.M. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *Journal of Cell Biology* **106**, 829-844.

MCCONKEY, D. J., HARTZELL, P., NICOTERA, P. & ORRENIUS, S. (1989). Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB Journal* **3**, 1843-1849.

MCCUSKER, R. H., BUSBY, W. H., DEHOFF, M. H., CAMACHO-HUBNER, C. & CLEMMONS, D. R. (1996). Insulin-like growth factor (IGF) binding to cell monolayers is directly modulated by the addition of IGF-binding proteins. *Endocrinology* 939-949.

MCKHANN, G., DRACHMAN, D., FOLSTEIN, M., KATZMAN, R., PRICE, D. & STADLAN, E. M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Dept. of Health and Human services task force on Alzheimer's disease. *Neurology* **34**, 939-944.

MERRITT, A. J., POTTEN, C. S., KEMP, C. J., HICKMAN, J. A., BALMAIN, A., LANE, D. P. & HALL, P. A. (1994). The Role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53- deficient mice. *Cancer Research* **54**, 614-617.

MESNER, P. W., WINTERS, T. R. & GREEN, S. H. (1992). Nerve Growth Factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. *Journal of Cell Biology* **119**, 1669-1680.

MICHEL, D., CHABOT, J. G., MOYSE, E., DANIK, M. & QUIRION, R. (1992). Possible functions of a new genetic marker in central nervous system: the sulfated glycoprotein-2 (SGP-2). *Synapse* **11**, 105-111.

MIGHELI, A., CAVALLA, P., PIVA, R., GIORDANA, M. T. & SCHIFFER, D. (1994). bcl-2 protein expression in aged brain and neurodegenerative diseases. *Neuroreport* **5**, 1906-1908.

MILLIGAN, C. E., OPPENHEIM, R. W. & SCHWARTZ, L. M. (1994). Motoneurons deprived of trophic support in vitro require new gene expression to undergo programmed cell death. *Journal of Neurobiology* **25**, 1005-1016.

MINSHALL, C., ARKINS, S., FREUND, G. G. & KELLY, K. W. (1996). Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *Journal of Immunology* **156**, 939-947.

MIYASHITA, T., KRAJEWSKI, S., KRAJEWSKA, M., WANG, H. G., LIN, H. K., LIEBERMANN, D. A., HOFFMAN, B. & REED, J. C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* **9**, 1799-1805.

MONTAGUE, J. W., GAIDO, M. L., FRYE, C. & CIDLOWSKI, J. A. (1994). A calcium-dependent nuclease from apoptotic rat thmocytes is homologous with calcium-dependent nuclease from apoptotic rat thymocytes is homologous with cyclophilin. Recombinant cyclophilins A, B and C have nuclease activity. *Journal of Biological Chemistry* **269**, 8877-8880.

MONTPETIT, M. L., LAWLESS, K. R. & TENNISWOOD, M. (1986). Androgen-repressed messages in the rat ventral prostate. *Prostate* **8**, 25-36.

MUTA, K. & KRANTZ, S. B. (1993). Apoptosis of human erythroid colony-forming cells is decreased by stem cell factor and Insulin-Like Growth Factor-I as well as erythropoietin. *Journal of Cell Physiology* **156**, 264-271.

NAGATA, S. & GOLSTEIN, P. (1995). The Fas Death Factor. *Science* **267**, 1449-1455.

NAKAJIMA, M., KASHIWAGI, K., OHTA, J., FURUKAWA, S., HAYASHI, K., KAWASHIMA, T. & HAYASHI, Y. (1994). Nerve growth factor and epidermal growth factor rescue PC12 cells from programmed cell death induced by etoposide: distinct modes

of protection against cell death by growth factors and a protein-synthesis inhibitor. *Neuroscience Letters* **176**, 161-164.

NEUENSCHWANDER, S., SCHWARTZ, A., WOOD, T. L., ROBERTS, C. T., HENNINGHAUSEN, L. & LEROITH, D. (1996). Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model. *Journal Clinical Investigation* **97**, 2225-2232.

NICHOLSON, D. W., ALI, A., THORNBERRY, N. A., VAILLANCOURT, J. P., DING, C. K., GALLANT, M., GAREAU, Y., GRIFFIN, P. R., LABELLE, M., LAZEBNIK, Y. A., MUNDAY, N. A., RAJU, S. M., SMULSON, M. E., YAMIN, T. T., YU, V. L. & MILLER, D. K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43.

NUNEZ, G., LONDON, L., HOCKENBERRY, D., ALEXANDER, M., MCKEARN, J. P. & KORSMEYER, S. (1990). Deregulated BCL-2 gene expression selectively prolongs survival of growth factor deprived hemopoietic cell lines. *Journal of Immunology* **144**, 3602-3610.

OHMORI, T., PODACK, E. R., NISHIO, K., TAKAHASHI, M., MIYAHARA, Y., TAKEDA, Y., KUBOTA, N., FUNAYAMA, Y., OGASAWARA, H. & OHIRA, T. (1993). Apoptois of lung cancer cells caused by some anti-cancer agents (MMC,CPT-11,ADM) is inhibited by bcl-2. *Biochemical and Biophysical Research Communcications* **192**, 30-36.

OLTVAI, Z. N., MILLIMAN C.L & KORSMEYER, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619.

ONISHI, Y., AZUMA, Y., SATO, Y., MIZUNO, Y., TADAKUMA, T. & KIZAKI, H. (1993). Topoisomerase Inhibitors Induce Apoptosis in Thymocytes. *Biochimica Biophysica Acta* **1175**, 147-154.

OPPENHEIM, R. W., PREVETTE, D., TYTELL, M. & HOMMA, S. (1990). Naturally occurring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role of cell death genes. *Developmental Biology* **138**, 104-113.

OPPENHEIM, R. W. (1991). Cell death during development of the nervous system. *Annual Reviews of Neurosciences* **14**, 453-501.

ORR, M. S., FORNARI, F. A., RANDOLPH, J. K. & GEWIRTZ, D. A. (1995). Transcriptional down-regulation of c-myc expression in the MCF-7 breast tumor cell line by the topoisomerase II inhibitor, VM-26. *Biochemica Biophysica Acta* **1262**, 139-145.

PANDEY, S., WALKER, P. R. & SIKORSKA, M. (1994). Separate pools of endonuclease activity are responsible for internucleosomal and high molecular mass DNA fragmentation during apoptosis. *Biochemistry and Cellular Biology* **72**, 625-629.

PAULSSON, M. (1992). Basement membrane proteins: structure, assembly, and cellular interactions. *Critical Reviews in Biochemistry and Molecular Biology* **27**, 93-127.

PEITSCH, M. C., MULLER, C. & TSCHOPP, J. (1993). DNA Fragmentation during apoptosis is caused by frequent single-strand cuts. *Nucleic Acids Research* **21**, 4206-4209.

PEUNOVA, N. & ENIKOLOPOV, G. (1993). Amplification of calcium induced gene transcription by nitric oxide in neuronal cells. *Nature* **364**, 450-453.

PIETRZKOWSKI, Z., WERNICKE, D., PORCU, P., JAMESON, B. A. & BASERGA, R. (1992). Inhibition of cellular proliferation by peptide analogues of insulin-like growth factor 1. *Cancer Research* **52**, 6447-6451.

RAFF, M. C. (1992). Social controls on cell survival and cell death. *Nature* **356**, 397-399.

RECKLIES, A. D. & MORT, J. S. (1985). Characterization of a cysteine proteinase secreted by mouse mammary gland. *Cancer Research* **45**, 2302-2307.

REEVE, J. G., MORGAN, J., SCHWANDER, J. & BLEEHEN, N. M. (1993). Role for membrane and secreted insulin-like growth factor binding protein 2 in the regulation of insulin-like growth factor action in lung tumors. *Cancer Research* **53**, 4680-4685.

RODRIGUEZ-TARDUCHY, G., COLLINS, M. K. L., GARCIA, I. & LOPEZ-RIVAS, A. (1992). Insulin-like growth factor-1 inhibits apoptosis in IL-3-dependent hemopoietic cells. *Journal of Immunology* **149**, 535-540.

ROSENBERG, M. E. & SILKENSEN, J. (1995). Clusterin: Physiologic and pathophysiologic considerations. *International Journal of Biochemistry and Cell Biology* **27**, 633-645.

ROY, C., BROWN, D. L., LITTLE, J. E., VALENTINE, B., WALKER, P. R., SIKORSKA, M., LEBLANC, J. & CHALY, N. (1992). The topoisomerase II inhibitor (VM-26) induces apoptosis in unstimulated mature murine lymphocytes. *Experimental Cell Research* **200**, 416-424.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). Molecular Cloning: *A Laboratory Manual*, New York: Cold Spring Harbor Laboratory Press.

SANDBERG, A., ENGBERG, C., LAKE, M. V., HOSLT, H. & SARA, V. R. (1988). The expression of insulin-like growth factor 1 and insulin like growth factor II genes in the human fetal and adult brain and in glioma. *Neuroscience Letters* **93**, 114-119.

SANDFORD, N. L., SEARLE, J. W. & KERR, J. F. R. (1984). Successive waves of apoptosis in the rat prostate after repeated withdrawal of testosterone. *Pathology* **16**, 406-410.

SELL, C., BASERGA, R. & RUBIN, R. (1995). Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. *Cancer Research* **55**, 303-306.

SELLINS, K. S. & COHEN, J. J. (1987). Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *Journal of Immunology* **139**, 3199-3206.

SELVAKUMARAN, M., LIN, H. K., MIYASHITA, T., WANG, H. G., KRAJEWSKI, S., REED, J. C., HOFFMAN, B. & LIEBERMANN, D. (1994). Immediate early up-regulation of bax expression by p53 but not TGF beta 1: A paradigm for distinct apoptotic pathways. *Oncogene* **9**, 1791-1798.

SENSIBAR, J. A., SUTKOWSKI, D. M., RAFFO, A., BUTTYAN, R., GRISWOLD, M. D., SYLVESTER, S. R., KOZLOWSKI, J. M. & LEE, C. (1995). Prevention of cell death induced by tumor necrosis factor alpha in LNCaP cells by overexpression of sulfated glycoprotein-2 (Clusterin).*Cancer Research* **55**, 2431-2437.

SENTMAN, C. L., SHUTTER, J. R., HOCKENBERY, D., KANAGAWA, O. & KORSMEYER, S. J. (1991). Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67**, 879-888.

SHI, Y., GLYNN, J. M., GUILBERT, L. J., COTTER, T. G., BISSONETTE, R. P. & GREEN, D. R. (1992). Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. *Science* **257**, 212-214.

SHIMASAKI, S., SHIMONAKA, M., ZHAN, H. P. & LING, N. (1991). Identification of five different insulin-like growth factor binding proteins (IGFBPs), from adult rat serum and molecular cloning of a novel IGFBP5, in rat and human. *Journal of Biological Chemistry* **266**, 10646-10653.

SLOANE, B. F. & HONN, K. V. (1984). Cystein proteinases and metastasis. *Cancer Metastasis Reviews* **3**, 249-263.

SMITH, S. B., BORA, N., MCCOOL, D., KUTTY, G., WONG, P., KUTTY, R. K. & WIGGERT, B. (1995). Photoreceptor cells in the vitiligo mouse die by apoptosis

-TRPM-2/Clusterin expression is increased in the neural retina and in the retinal pigment epithelium. *Investigative Ophthalmologic Visual Science* **36**, 2193-2201.

SRIDHAR, S. S. (1993). The characterization of RSG-3 (embigin) in regressing prostate and mammary gland. Honours Thesis, Department of Biochemistry, University of Ottawa.

STRANGE, R., LI, F., SAURER, S., BURCKHARDT, A. & FRIIS, R. R. (1992). Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* **115**, 49-58.

STRASSER, A., HARRIS, A. W. & CORY, S. (1991). Bcl-2 trangene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67**, 888-899.

SYLVESTER, S. R., MORALES, C., OKO, R. & GRISWOLD, M. D. (1991). Localization of sulfated glycoprotein-2 (Clusterin) on spermatozoa and in the reproductive tract of the male rat. *Biology of Reproduction* **45**, 195-207.

TENNISWOOD,M.P., GUENETTE,R.S., MORRISSEY,C., O'SULLIVAN,J., WANG,Z., ZHAN,P., SRIDHAR,S., LAKINS,J. AND TANG,H. (1996) Apoptosis and Tumor Invasion in Hormone dependent Cancers:In *Serono Symposia USA*, Inc. Eds. TILLY,J.L., STRAUSS,J.F. AND TENNISWOOD, M.P. Springer-Verlag New York, (in press)

TENNISWOOD, M. P., MONTPETIT, M. L., LEGER, J. G., WONG, P., PINEAULT, J. M. & ROULEAU, M. (1990). Epithelial-stromal interactions and cell death in the prostate. In *The Prostate as an endocrine gland*, eds. FARNSWORTH, W. E. & ABLIN, R. J. pp. 187 Boca Raton, FL: CRC press.

TENNISWOOD, M. & SIMPSON, A. J. G. (1982). The extraction and characterization and in vitro translation of RNA from adult Schistosoma mansoni. *Parasitology* **84**, 253-261.

TEWARI, M., BEIDLER, D. R. & DIXIT, V. M. (1995). CrmA-inhibitable cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein during Fas- and tumor necrosis factor-induced apoptosis. *Journal of Biological Chemistry* **270**, 18738-18741.

THOMPSON, C. B. (1995). Apoptosis in the Pathogenesis and Treatment of Disease. *Science* **267**, 1456-1462.

TSUJIMOTO, Y., FINGER, L., YUNIS, J., NOWELL, P. & CORCE, C. (1984). Involvement of the bcl-2 gene in human follicular lymphomas. *Science* **226**, 1097-1099.

TUNG, P. S. & FRITZ, I. B. (1985). Immunolocalization of Clusterin in the ram testis, rete testis, and excurrent ducts. *Biology of Reproduction* **33**, 177-186.

VAUX, D. L., CORY, S. & ADAMS, J. M. (1988). bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**, 440-442.

VOGELSTEIN, B. & KINZLER, K. W. (1992). p53 function and dysfunction. *Cell* **70**, 523-526.

WADEWITZ, A. G. & LOCKSHIN, R. A. (1988). Programmed cell death: dying cells synthesize a co-ordinated, uniquie set of proteins in two different episodes of cell death. *FEBS Letters* **241**, 19

WALICKE, P. A. (1989). Novel neurotrophic factors, receptors and oncogenes. *Annual Reviews of Neuroscience* **12**, 103-126.

WALKER, N. I., BENNETT, R. E. & KERR, J. F. R. (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. *American Journal of Anatomy* **185**, 19-32.

WALKER, P. R., PANDEY, S. & SIKORSKA, M. (1995). Degradation of chromatin in apoptosis. *Cell Death & Differentiation* **2**, 97-104.

WALKER, P. R., KOKILEVA, L., LEBLANC, J. & SIKORSKA, M. (1993). Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques* **15**, 1032-1041.

WALKER, P. R., SMITH, C., YOUDALE, T., LEBLANC, J., WHITFIELD, J. F. & SIKORSKA, M. (1991). Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Research* **51**, 1078-1085.

WALKER, P. R. & SIKORSKA, M. (1994). Endonuclease activities, chromatin structure, and DNA degradation in apoptosis - Review. *Biochemistry and Cell Biology* **72**, 615-623.

WALKINSHAW, G. & WATERS, C. M. (1994). Neurotoxin-induced cell death in neuronal PC12 cells is mediated by induction of apoptosis. *Neuroscience* **63**, 975-987.

WILLIAMS, G. T., SMITH, C. A., SPOONCER, F., DEXTER, T. M. & TAYLOR, D. R. (1990). Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**, 76-79.

WILLIAMS, G. T. & SMITH, C. A. (1993). Molecular regulation of apoptosis: Genetic controls on cell death. *Cell* **74**, 777-779.

WILLIAMS, A. F. & BARCLAY, A. N. (1988). The immunoglobulin superfamily-domains for cell surface recognition. *Annual Reviews of Immunology* **6**, 381-405.

WILLIAMS, A. F. & GAGNON, J. (1982). Neronal cell Thy-1 glycoprotein: Homology with immunoglobulin. *Science* **216**, 696-703.

WILSON, M. R., EASTERBROOK-SMITH, S. B., LAKINS, J. & TENNISWOOD, M. (1994). Mechanism of induction and function of Clusterin at sites of cell death. In *Clusterin: Function in vertebrate organ development, function and adaption*, ed. HARMONY, J. pp. 75-100. Austin,TX: Landes,R.G.

WONG, P., BORST, D. E., FARBER, D., DANCIGER, J. S., TENNISWOOD, M., CHADER, G. J. & VANVEEN, T. (1994). Increased TRPM-2/Clusterin mRNA levels during the time of retinal degeneration in mouse models of retinitis pigmentosa. *Biochemistry and Cell Biology* **72**, 439-446.

WONG, P., PINEAULT, J., LAKINS, J., TAILLEFER, D., LEGER, J. G., WANG, C. & TENNISWOOD, M. P. (1993). Genomic organization and expression of the rat TRPM-2 (Clusterin) gene, a gene implicated in apoptosis. *Journal of Biological Chemistry* **268**, 5021-5031.

WONG, P., TAILLEFER, D., LAKINS, J., PINEAULT, J., CHADER, G. & TENNISWOOD, M. (1994). Molecular characterization of human TRPM-2/Clusterin , a gene associated with sperm maturation, apoptosis and neurodegeneration. *European of Journal of Biochemistry* **221**, 917-925.

WU, X. P., FAN, Z., MASUI, H., ROSEN, N. & MENDELSOHN, J. (1995). Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell cine and its delay by insulin. *Journal of Clinical Investigation* **95**, 1897-1905.

WYLLIE, A. H., BEATTIE, G. J. & HARGREAVES, A. D. (1981). Chromatin changes in apoptosis. *The Histochemical Journal* **13**, 681-692.

WYLLIE, A. H. (1987). Apoptosis: cell death in tissue regulation. *Journal of Pathology* **153**, 313-316.

WYLLIE, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555-556.

WYLLIE, A. H., MORRIS, R. G., ARENDS, M. J. & WATT, A. E. (1986). Nuclease activation in programmed cell death. In *Co-ordinated regulation of gene expression*, eds. CLAYTON, R. M. & TRUMAN, D. pp. 33-41. New York: plenum Press.

YUAN, J. & HORVITZ, H. R. (1990). The Caenorhabditis elegans genes ced-3 and ced-4

act autonomously to cause programmed cell death. *Developmental Biology* **138**, 33-41.

ZAKERI, Z., BURSCH, W., TENNISWOOD, M. & LOCKSHIN, R. (1995). Cell Death: programmed, apoptosis, necrosis, or other. *Cell Death and Differentiation* **2**, 83-92.

ZAKERI, Z., CURTO, M., HOOVER, D. M., WIGHTMAN, K., ENGELHARDT, J., SMITH, F. F., KIERSZENBAUM, A. L., GLEESON, T. G. & TENNISWOOD, M. (1992). Developmental expression of the S35-S45/SGP-2/TRPM-2 gene in rat testis and epididymis. *Molecular Reproduction and Development* **33**, 373-384.

ZAWADA, W. M., KIRSCHMAN, D. L., COHEN, J. J., HEIDENREICH, K. A. & FREED, C. R. (1996). Growth factors rescue embryonic dopamine neurons from programmed cell death. *Experimental Neurology* **140**, 60-67.

ZHIVOTOVSKY, B., CEDERVALL, B., JIANG, S., NICOTERA, P. & ORRENIUS, S. (1994). Involvement of Ca^{2+} in the formation of high molecular weight DNA fragments in thymocyte apoptosis. *Biochemical and Biophysical Research Communications* **202**, 120-127.

ZHOU, Y. H., MOHAN, S., LINKHART, T. A., BAYLINK, D. J. & STRONG, D. D. (1996). Retinoic acid regulates insulin-like growth factor-binding protein expression in human osteoblast cells. *Endocrinology* **137**, 975-983.

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Publications:

1. Sridhar, S.S. (1993). The characterization of RSG-3 (embigin) in regressing prostate and mammary gland. Honours thesis, Department of Biochemistry, University of Ottawa.

2. Sridhar, S., Walker, P.R., Guenette, R.S. Tenniswood, M.P. (1996) The role of IGFBPs in VM-26 induced apoptosis in PC12 cells (in prep).

Book chapters and Reviews:

3. Tenniswood, M.P., Guenette, R.S., Morrissey, C., O'Sullivan, J., Wang, Z., Zhan, P., Sridhar, S., Lakins, J., and Tang, H. (1996) Apoptosis and Tumor Invasion in Hormone Dependent Cancers In: Serono Symposia USA, Inc. (Eds. Tilly, J.L., Strauss, J.F. and Tenniswood, M.P.) Springer-Verlag new York, (in press)

4. Tenniswood, M.P., Guenette, R.S., Morrissey, C., O'Sullivan, J., Wang, Z., Zhan, P., Sridhar, S., Lakins, J., Bennett, S and Tang, H. (1996) Apoptosis in Prostate. Endocrine Reviews (in prep)