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Apoptosis is the genetically regulated death of retinal ganglion cells (RGC) in which there is a blockade of retrograde transport. This blockade results in the loss of neurotrophic growth factors that are essential for the survival of the RGCs. This study uses several different techniques to determine mechanisms underlying apoptosis in rat RGCs deprived of growth factors. An established line of transformed RGC was subjected to serum deprivation for 2-6 days and compared to RGC cells maintained in 10% FBS to study the cellular changes that occur as a result of the treatments. The results show that serum deprivation for 48 hours resulted in a 50% cell loss due to apoptosis. Apoptotic death was associated with activation of caspases 3, 8, and 9 along with increased levels of Bax and death receptors 3 & 4. These results indicate that serum deprivation results in RGC death via mitochondrial and also extrinsic pathways.

SERUM DEPRIVATION INDUCES APOPTOSIS OF RETINAL GANGLION CELLS UTILIZING MITOCHONDRIAL SIGNALING PATHWAYS

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Introduction and Background

Introduction to the project

Various studies have suggested that trophic factor withdrawal is one of the many causes of ganglion cell death in glaucoma (Aguayo, 1996; Peinado-Ramon, 1996). In glaucoma it is hypothesized that increased intraocular pressure (IOP) leads to injury of the optic nerve. This injury then is thought to block the transport of neurotrophic factors to the ganglion cells causing their death. Neurotrophins are trophic factors that support the survival of neurons during development as well as during adulthood. Neurotrophins interact with tropomyosin related kinase (Trk) receptor tyrosine kinases believed to be located at the axon terminals. The neurotrophin-receptor complexes are then internalized by endocytosis through a clathrin-mediated endocytic pathway (Howe, 2001). Continued association of the neurotrophin with the receptor supports Trk autophosphorylation as well as signaling. The endocytic vesicles that contain the neurotrophin bound to the receptor, evolve into specialized signaling vesicles (Weible, 2001). The neurotrophin/receptor endocytic vesicles are then retrogradely transported to the cell body via a microtubule-based mechanism (von Bartheld et al., 1996). It has been shown that blocking transport of neurotrophins in animal as well as human glaucoma models leads to ganglion cell death in the form of apoptosis (Pease, 2000; Fawcett, 1998;

von Bartheld, 2000). Because trophic withdrawal has been hypothesized as a primary cause of ganglion cell death in glaucoma (Wadia, 1998), we attempted to elucidate the pathways that retinal ganglion cells utilize when they undergo apoptosis during serum deprivation.

The Eye

The eye is an important sense organ since most of what we know about the external world comes through vision. It is composed of three basic layers or coats, often called tunics. The three layers are the fibrous layer (corneoscleral), the vascular pigmented layer (composed of choroids, ciliary body, and iris) and the neuronal layer (retina). The fibrous layer is composed of a posterior, opaque section, the sclera, and an anterior, transparent section, the cornea. The sclera functions to protect the intraocular contents as well as to maintain the shape of the eye. The cornea, which is transparent, is the main structure responsible for the refraction of light entering the eye. The vascular pigmented layer consists of, from anterior to posterior: the iris, ciliary body, and the choroids. The iris controls the size of the pupil which regulates the amount of light entering the eye. The ciliary body is composed of ciliary muscle and the ciliary processes. The processes are responsible for the production of aqueous humor, which is a clear, nutritive fluid, derived from a filtrate of plasma and secreted into the posterior chamber of the eye. Extending from the ciliary body anteriorly to the optic nerve posteriorly is the choroid. The main purpose of the choroid is to nourish the outer layers of the retina.

The retina is the innermost tunic of the eye. It is responsible for converting information from the external environment into neural impulses that are transmitted to the

brain for decoding and analysis. The retina is composed of ten layers: retinal pigmented epithelium, photoreceptor layer, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, optic nerve fiber layer, and inner limiting membrane. The types of cells present in the retina are photoreceptor, bipolar, ganglion, horizontal, amacrine, and müller. The nucleus of photoreceptor cells, rods and cones, are located in the outer nuclear layer of the retina. They contain pigments that are responsible for absorption of light and initiation of the neuroelectrical impulse. The cell bodies of bipolar cells reside in the inner nuclear layer and are oriented paralled to the photoreceptors. They are primarily responsible for transmitting signals from photoreceptors to ganglion cells. The cell bodies of most ganglion cells are located in the innermost nucleated layer of the retina located between the nerve fiber and plexiform layer. Ganglion cells are responsible for transmitting visual information to the brain. The cell bodies of amacrine cells are located in the inner nuclear layer. These cells are stimulated by bipolar cells which in turn stimulate ganglion cells. The horizontal cell bodies are located in the inner nuclear layer with their processes terminating in the outer plexiform layer. These cells respond to the neurotransmitter released by rods and cones following light excitation. They are then thought to release an inhibitory transmitter, gamma-aminobutyric acid (GABA), which inhibits the activity of bipolar cells, thereby sharpening contrast and increasing spatial resolution. The müller cells are the primary supporting glial cell of the retina which is considered analogous to oligodendroytes (Forrester, 1996). They surround neuronal cell bodies, processes, and blood vessels. These cells are believed to help nourish and maintain the outer retina.

Glaucoma

Glaucoma is a progressive optic neuropathy that is characterized by a typical excavated appearance of the optic nerve head and loss of retinal ganglion cells due to apoptosis (Pease, 2000). In non glaucomatous individuals, aqueous humor, which is a clear, nutritive fluid derived from a filtrate of plasma, is produced by the processes of the ciliary body and secreted into the posterior chamber of the eye at a rate of approximately 2.5 μl/minute. From the posterior chamber, the aqueous humor enters the anterior chamber of the eye through the pupil where circulation is driven by a convection current utilizing the temperature differences between the iris and cornea (Fingeret, 2001). The aqueous humor then exits the eye through the trabecular meshwork and canal of Schlemm into the venous system of the eye via a series of channels and venous pathways. But in individuals with glaucoma, there is a progressive increase in the resistance to the outflow of aqueous humor. This results in an increase in intraocular pressure. After a period of time, these increased levels may cause damage to the optic nerve head (Fingeret, 2001).

Damage to the nerve head is reflected as a loss of retinal ganglion cells and degeneration of the optic nerve (Kitazawa, 1989). The death of these neurons is due to a disruption of axonal transport (Minckler, 1989). The increased IOP that occurs with glaucoma leads to a compression or shearing of the axons that pass through the optic nerve head. The result of this compression is a blockage of neuronal cell transport. This blockage of transport leads to degeneration of the retinal ganglion cells. Because ganglion cells are lost, the axons are also lost which results in a loss of tissue at the optic

nerve (Radius, 1981). This loss creates prelaminar thinning of the optic nerve (Hernandez, 1990), which leads to the cupping that is observed in glaucoma.

In addition to the damage to the optic nerve, there is also damage that occurs in the central nervous system. The axons of the ganglion cells synapse with the lateral geniculate nucleus (LGN) located in the caudolateral region of the thalamus (Quigley, 1982). Primary neuron injury has profound effects on synaptically linked distant neurons through a process called transynaptic or transneuronal degeneration. Considerable evidence has accumulated suggesting that damage also is disseminated in this manner in glaucoma (Weber, 2000; Gupta, 2000). Central nervous system damage in glaucoma appears to be proportionate to the extent of optic nerve damage. Lesions of the optic nerve also cause lesions in the LGN (Quigley, 1998).

There are two main theories as to how ganglion cells die, a mechanical theory and a vascular theory. The vascular theory of glaucoma suggests that ischemia is the primary cause of ganglion cell death (Flammer, 1994). The basis for this theory is that a rise in intraocular pressure causes a reduction in blood flow in intraocular vessels (Gasser, 1989). One of the questions relating to the vascular theory is do autoregulatory mechanisms, mechanisms that restore blood flow in response to increases or decreases in pressure, exist for blood vessels that supply the optic nerve (Gasser, 1989)? The theory assumes that autoregulation exists in optic nerve vessels and therefore in glaucoma there is either a loss of autoregulation (Radius, 1987) or it is abnormal (Fechtner, 1994). In the mechanical theory, it is believed that elevated intraocular pressure leads to direct compression and/or shearing of axons resulting in subsequent death of neurons

(Osbourne, 1999). Whether the insult is mechanical or vascular the endpoints are the same. Ganglion cells die.

It has been hypothesized that withdrawal of trophic factor support is one of the primary causes of ganglion cell death in glaucoma (Wadia, 1998). Neurons are dependent on peptide factors and growth factors during growth and development. Neurons rely on a constant source of these factors throughout their lifetime in order to maintain their normal functioning. The neurotrophin most commonly linked with ganglion neurons is brain derived neurotrophic factor (BDNF) (Nickells, 1996). BDNF is released by neurons in the brain. Ganglion cell axons synapse with these neurons, take up the neurotrophin, and transport it in a retrograde direction to the cell bodies located in the retina (Fournier, 1997). During development, and also adulthood, the ganglion cells become dependent on the neurotrophin presence in order to survive (Johnson, 1986). Neurotrophins have been shown to activate pathways that are essential for cell survival such as the phosphoinositol 3 kinase and mitogen activated protein kinase pathways. Thus if anything happens to compromise the retrograde transport of neurotrophins, the ganglion cells will die. It is currently known that ganglion cells produce trophic factors, but it is unknown as to why the factors these cells produce are not enough to sustain the survival of the cells (Krishnamoorthy, 2001).

Retinal Ganglion Cells

The ganglion cells, named because they resemble cells found in nervous ganglia, compose the eighth layer of the retina (Figure 2). They vary from 10 to 30 μm in size and are multipolar cells whose dendrites synapse with the axons of bipolar and amacrine

cells. The ganglion cells have nonmyelinated axons that form the nerve fiber layer on the innermost surface of the retina and synapse with cells in the LGN of the thalamus. The axons of RGCs leave the eye forming the optic nerve. These fibers exit through the sclera at the lamina cribosa, the weakest section of the sclera, after which time the axons become myelinated with oligodendrocytes. Ganglion cells are morphologically characterized by large cell bodies, abundant Nissl substance, which consists of arrays of rough endoplasmic reticulum, and a large Golgi apparatus (Forrester, 1996). Ganglion cells are the output units of the retina (Xiang, 1996). Impulses are primarily received from bipolar and amacrine cells, with these impulses transported along the optic nerve fibers to the brain (Berne, 2000).

Until recently there was no established ganglion cell line to study the pathogenesis of glaucoma. For this reason, a transformed rat retinal ganglion cell line was established. The retina was isolated from postnatal day one non-pigmented Sprague-Dawley rats. The retinal cells were then transformed with the ψ2E1A virus (Krishnamoorthy, 2001). The cells were confirmed to be of retinal ganglion cell origin by using specific cell type markers. The cells expressed Thy-1, and Brn-3C which have been shown to be specific to retinal ganglion cells (Barnstable, 1984). The cells were negative for the expression of syntaxin/HPC-1, which is an amacrine cell marker. Negligible levels of GFAP, a Müller cell marker were detected. The RGC-5 cells did not express detectable levels of 8A1, which is used to detect horizontal cells (Krishnamoorthy, 2001).

Neurotrophins

Neurotrophic factors are endogenous soluble proteins (Thoenen, 1995) regulating survival (Korsching, 1993), growth (Lindsay,1996), morphological plasticity and synthesis of proteins for differentiated function of neurons (Snider, 1998). Neurotrophins bind to 2 types of receptors: tropomyosin related kinase (trk) receptors and the p75 receptor, which is a member of the Fas-tumor necrosis factor (TNF) receptor family (Chao, 1995). Binding of neurotrophins to Trk receptor tyrosine kinases initiates signaling cascades that promote cell survival and differentiation through Ras-MAP kinase and phosphotidylinositol 3-kinase activities (Greene, 1995). p75 has been shown to act as a co-receptor for the Trk tyrosine kinases or to signal independently. p75 can activate ceramide production, NF-κB, and c-jun kinase activity (Bothwell, 1996). The functions of these receptors vary markedly. While Trk receptors transmit positive signals, enhancing survival and growth, p75NTR transmits both positive and negative signals. The signals derived form the two receptors can either augment or oppose each other (Kaplan, 2000).

Trk is a single-chain member of the receptor tyrosine kinase superfamily (Barbacid, 1995). It was originally detected as a protooncogene fusion protein in which the N-terminal 392 residues of the normal Trk protein were replaced with tropomyosin sequences (Friedman, 1999). The extracellular domains contain 2 cysteine rich regions (domains 1 and 3) flanking a leucine rich repeat (domain 2) followed by 2 IgG like domains in the juxtamembrane region (domain 4 and 5) (Windisch, 1995). Domain 5 is responsible for neurotrophin binding (Urfer, 1998).

Binding of neurotrophin homodimers causes receptor dimerization, autophosphorylation on tyrosine residues within the activation loop (tyrosines 670, 674, and 675) followed by phosphorylation of additional tyrosine residues located on the receptor (Jing, 1992). These phoshorylated tyrosines, especially 490 and 785, act as docking sites for signaling molecules (Loeb, 1994) that regulate cell growth and survival through the Ras/Map kinase/Erk pathway, which regulates neuronal differentiation, phosphatidylinositol 3-kinase (PI3-K), that promotes cell survival, and the phospholipase C (PL-γC) pathway , which can control neurite outgrowth (Segal, 1996).

The other neurotrophin receptor, p75, was the first identified member of the tumor necrosis factor receptor (TNFR) superfamily (Baker, 1998). p75NTR contains an intracellular death domain, a ~ 80 amino acid association module initially identified in related pro-apoptotic TNFR superfamily members TNFR1 and FAS (Liepinsh, 1997). This receptor has several important roles in the nervous system. It can provide a positive modulatory influence on Trk receptors by increasing the number of binding sites (Verdi, 1994), regulate axon growth, mediate retrograde transport of neurotrophins, and help regulate survival and apoptosis of different neuronal populations. On the cellular level, binding to p75NTR results in activation of either the NF-κB pathway which appears to promote cell survival or the Jun kinase pathway which promotes apoptosis (Patapoutain, 2001).

While many growth factors have been shown to be important to retinal ganglion cells, (Quigley, 2000), BDNF has been shown to specifically retard ganglion cell loss after retinal hypoxia (Ikeda, 1999) and optic nerve transection (Mansour-Robaey, 1994). Adult retinal ganglion cell have been shown to synthesize (Perez, 1995) and depend on

neurotrophins (Peinado, 1996). Continued neurotrophin dependence among adult retinal ganglion cells was suggested by a study in which cat ganglion cells died after their target neurons, which supplied them with neurotrophins, were eliminated (Pearson, 1992).

Apoptosis

Apoptosis, also called Programmed Cell Death, is a genetically programmed pathway of cell death that does not significantly affect neighboring cells and tissues (Hacker, 2000). The process begins when a cell activates its own destruction by initiating a series of complex cascading events. Programmed cell death (PCD) can be divided into three distinct phases: initiation, execution, and degradation (Kroeme, 1997).

The first cascade phase of apoptosis is initiation. Iniation of PCD in neurons is triggered by several stimuli. For retinal ganglion cells these stimuli may include ischemia, excitotoxicity, and trophic insufficiency (Wadia, 1998). These stimuli cause cellular damage usually in the form of denatured proteins and early degradation of DNA (Nageta, 2000). In addition to DNA and protein damage, reactive oxygen species (ROS) can be generated and released from the mitochondria (Nicholls, 1999).

The second phase is execution. Execution consists of a proteolytic cascade involving a family of proteases called caspases (Reme, 2000). During this phase, p53, a tumor suppressor gene, functions as a transcription factor that causes the upregulation of Bax, which is a proapoptotic protein, and the down-regulation of Bcl-2, which is an anti-apoptotic protein. Normally the concentrations of bcl-2 and bax are in an equilibrium within the cell (Nickells, 1999). This prevents the cell from undergoing apoptosis. The increased levels of Bax in the cell alter the concentration equilibrium driving the cell

toward apoptosis. When there is no Bcl-2, Bax can then cause the permeability transition (PT), which is a breakdown in the transmembrane potential of the inner mitochondrial membrane. This occurs when a channel in the inner mitochondrial membrane opens forming the PT pore. The PT pore is a multiprotein complex that forms at points where the inner and outer mitochondrial membranes make contact (Green, 1998). This also causes the mitochondria to swell as water enters the matrix leading to the release of cytochrome c (Bernadi, 1999). Cytochrome c then activates a series of cytoplasmic caspases.

Cytochrome c combines with procaspase 9 and Apaf-1 along with dATP forming the apoptosome which stimulates the proteolytic cleavage of the caspase 9 prodomain, resulting in activation of caspase 9 (Li. 1999). Caspase 9 then causes cleavage of procaspase 3 (Zou, 1997), which is a 32 kDa precursor present in the cytosol, into 20 and 11 kDa fragments which then, along with cytochrome c and Apaf-1, form active caspase 3 (Liu, 1996). Caspase 3 cleaves a number of downstream targets such as Poly ADP-ribose polymerase (PARP) (Lazebnik, 1994), laminin B1 (Lazebnik, 1995), sterol-regulatory element-binding proteins (SREBPS) (Wang, 1996), and DNA fragmentation factor (DFF) (Liu, 1997) which lead to DNA fragmentation (Cellerino, 2000). This cleavage leads to the final phase of PCD which is degradation of genomic DNA. This phase is characterized by the disassembly of the cell into multiple membrane-enclosed vesicles that are engulfed by neighboring cells (Bright, 1994).

Death Receptors

Death receptors comprise a second pathway that utilizes specialized membrane receptors that are coupled to caspase activation, specifically Caspase 8 (Ashkenazi, 1998). Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily where they play a variety of key biological roles including regulation of apoptosis, cellular differentiation, and inflammation (Schulze-Osthoff, 1998; Tschopp, 1999; Ashkenazi, 1998; Gravestein, 1998). They are referred to as death receptors since one of their most prominent functions is to induce programmed cell death (Daniel, 2001). Members of the TNFR family are type 1 membrane proteins (Wang, 2001) whose common structural feature is the presence of cysteine- rich domains, containing three to six repeats of approximately six cysteines each, in their extracellular region. In their cytoplasmic region, they contain a 65-80 amino acid death domain named because it is required for the transmission of the cytoplasmic signal (Golstein, 1997; Griffith, 1998). The death domain, located in the cytoplasmic region, consists of a series of α-helices with many exposed charged amino acids that mediate self-association of the domain following ligand binding and receptor oligomerization (Huang, 1996).

Signaling in this pathway begins upon ligand binding. This causes receptor trimerization and aggregation of the intracellular death domains. The death domains of the receptors then associate with a similar death domain found in adaptor proteins (Chinnaiyan, 1995). The adaptor proteins also contain an effector domain that binds to a similar effector region in the prodomain of caspase-8 (Boldin, 1996). This complex is known as the death-inducing signaling complex (DISC) (Kischkel, 1995). Once the caspases are recruited, they are transproteolyzed which results in their activation. This

then leads to effector caspase initiation, mainly Caspase 3, which results in an irreversible commitment of the cells to apoptosis (Ashkenazi, 1998).

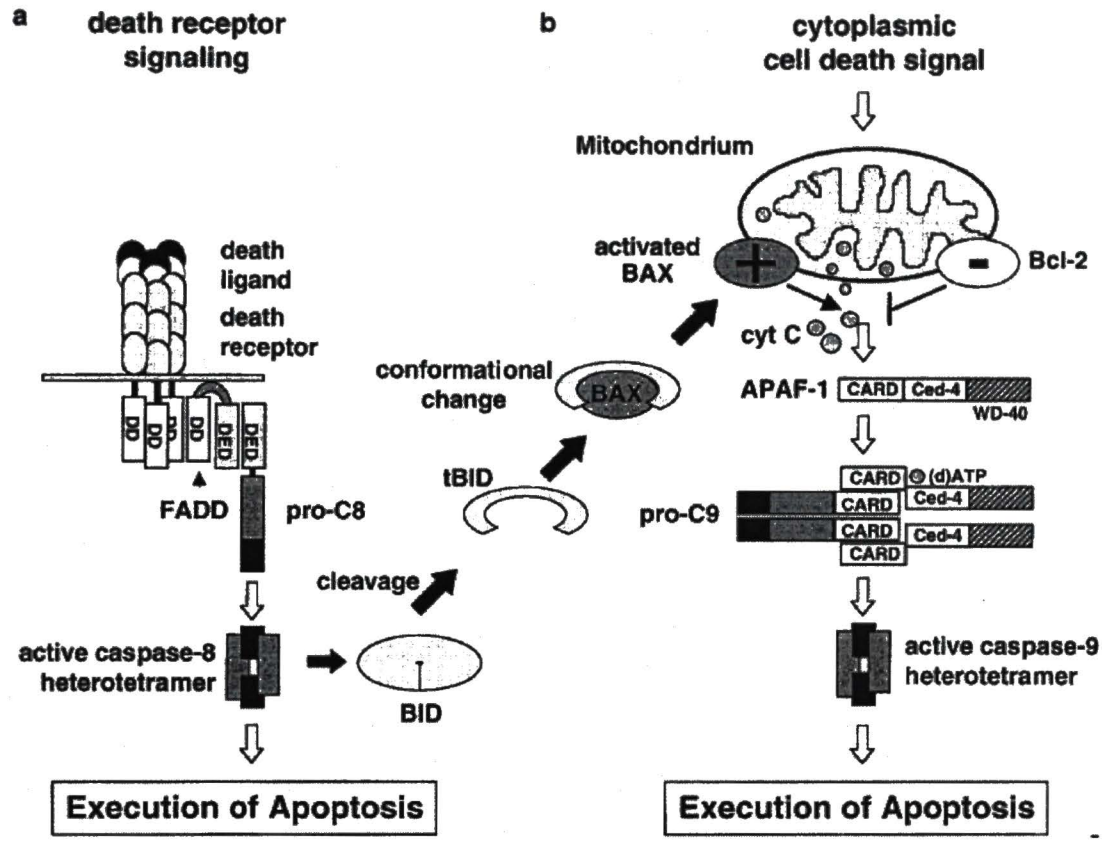
The death receptor pathway can also be connected to the mitochondrial apoptosis pathway (Figure 1). When caspase 8 is activated it can act on caspase 3 or it can also cleave Bid, which is a proapoptotic Bcl-2 family member (Li, 1998). Following cleavage, truncated Bid (tBid) translocates to the mitochondria and can induce the release of cytochrome-c (Luo, 1998). Caspase-3 can also cleave Bid or it can activate Caspase 6 which can feedback and cause more activation of caspase 8 amplifying the apoptotic signal (Daniel, 2001).

Significance of the project/Hypothesis

This study is being used to determine if the mitochondrial and extrinsic pathways are involved in apoptosis of retinal ganglion cells when they are deprived of trophic factors. The retinal ganglion cells play a key role in integrating visual information and relaying it to the cerebral cortex of the brain, thereby enabling vision. The retinal ganglion cells are sustained by neurotrophic factors that are retrogradely transported to the ganglion cells. This transport occurs using the optic nerve which is composed of axons of the ganglion cells. When the axons are damaged, as occurs with glaucoma, transport is disrupted and the ganglion cells die via apoptosis. This in vitro serum deprivation model is used to mimic the loss of neurotrophin support that is believed to occur in vivo with glaucoma. The ***hypothesis*** was that serum deprivation induces apoptosis of retinal ganglion cells through mitochondrial and extrinsic signaling pathways. We investigated the following specific aims to answer the hypothesis:

1. To determine is serum deprivation results in reduced viability of RGC-5 cells due to apoptosis.
2. To determine if serum deprived RGC-5 cells undergo apoptosis utilizing mitochondrial and/or death receptor signaling pathways.

Figure 1. Crosstalk between Apoptotic Pathways



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CHAPTER 2

MATERIALS & METHODS

This Chapter of the thesis is being included to provide more in depth methodology for the paper that is presented in Chapter 3.

RGC-5 Culture

The retinal ganglion cells used for this project are from a transformed cell line established from postnatal day one non-pigmented Sprague-Dawley rats . Rats were maintained and treated in accordance with NIH guidelines. Retinal cells were isolated as reported by Pang et al (1999). Retinal ganglion cells were selected by G418, (geneticin, Life technologies, Gibco-BRL) with infected cells expressing the viral neomycin/G418 resistance gene surviving. Cells were then grown to confluence and passaged every three days. Aliquots were frozen in 10% dimethylsulfoxide(DMSO)/10%FBS/80% DMEM in liquid N_2. Upon use the cells were thawed and plated on T25 flasks (Greiner Bio-One, Frickenhausen, Germany) with 10 mls of DMEM supplemented with 10% FBS. The cultures were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (Krishnamoorthy, 2001).

Sample Collection

RGC-5 cells were plated at a density of 100,000 cells/dish on a 94/16 mm tissue culture dish (Greiner Labortechnik) in basal medium (DMEM low glucose with 10 units/ml Penicillin, 10 μg/ml Streptomycin) supplemented with 10 % FBS. Cells were then allowed to attach for 3 hours at 37°C in 5% CO_2. After 3 hours, all cells were rinsed 3 times with basal medium. The cells to be deprived of serum were then incubated in basal medium for 48 hours at 37°C in 5% CO_2. The control cells were incubated in basal medium supplemented with 10% FBS for 48 hours at 37°C in 5% CO_2. Cell lysate was collected by washing the dish with 1x phosphate buffered saline (PBS). After the PBS was removed, 100-200 μl of protein lysis buffer was added. The protein lysis buffer consisted of 10 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 μg/ml aprotinin, leupeptin and pepstatin; 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 10% protein inhibitor cocktail. Cells were detached from tissue culture dishes using cell scrapers (Costar, Cambridge, MA) and placed in a 1.6 ml centrifuge tube (USA Scientific, Inc). The cell lysate was sonicated for 1 minute on ice. Before being used for Western Blot analysis, protein concentrations were measured using the Bradford Protein Quantitation Method. Cell lysate was stored at -20°C until use.

Cell Culture

RGC-5 cells were plated on a 24 well tissue culture plate (Becton Dickinson, Lincoln Park, NJ) containing 12 mm circle glass coverslips (Fisher Scientific, Pittsburgh, PA) at a density of 20,000 cells/well in basal medium supplemented with 10% FBS. Cells were allowed to attach for 3 hours at 37°C in 5% CO_2 after which time medium

supplemented with 10% FBS was removed from all the wells. The wells were then rinsed 3 times with basal medium and the experimental cells were incubated in basal medium for 48 hours at 37°C in 5% CO_2. The control cells were incubated in basal medium supplemented with 10% FBS for 48 hours at 37°C in 5% CO_2.

Propidium Iodide Nuclear Stain

The procedure was performed as described by Arndt-Jovin (1989) with slight modifications made to the procedure. The cells were treated as described in the cell culture section. After 48 hours, the cells were rinsed with 500 µl of 1x phosphate buffered saline (PBS). Cells were then rinsed with 500 µl of freshly prepared 1x Assay buffer (Santa Cruz Biotechnology). Propidium Iodide (Santa Cruz Biotechnology) was added to each well for a final dilution of 1:20 with a thirty minute room temperature incubation. The cells were rinsed 3 times with 1x PBS and fixed with 3.5% formalin for 30 minutes at 4°C. The cells were again rinsed 3 times with 1x PBS and the coverslips were mounted onto slides and viewed using a microscope with an epiflourescence attachment. The controls for this experiment were RGC-5 cells grown in basal medium supplemented with 10% FBS. At least 100 cells were examined for each condition and experiments were done 3 times to confirm results.

Neutral Red Survival Assay

The procedure was the same as described by Borenfreund et al (1985) with slight modifications. RGC-5 cells were plated as described under the cell culture section. At this time cells were treated as follows for the experiment:

Serum Deprivation of RGC-5 Cells

Columns 1-3- RGC-5 cells grown with 10% FBS Columns 4-6- RGC-5 cells grown without 10% FBS

The cells were allowed to grow for 48 hours. After 48 hours the medium was removed and the wells were washed 2 times with 1ml of HEPES assay buffer (pH 7.2). The composition of HEPES assay buffer was 125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2 2H_2O$, 2 mM $MgCl_2 6H_2O$, 0.5 mM $NaH_2PO_4 H_2O$, 5 mM $NaHCO_3$, 10 mM HEPES, and 10 mM D-glucose. After the final rinse, 500 µl of HEPES assay buffer was added to each well and incubated at room temperature for 20 minutes. After 20 minutes, 17 µl of 1% Neutral Red Dye was added to each well and incubated for 2 hours at room temperature. After 2 hours, the cells were again washed 2 times with 1 ml of HEPES assay buffer/well. The cells were allowed to dry for 20 minutes. Five hundred µl of ice cold solubilization buffer was added to each well and incubated for 20 minutes at room temperature on an orbit shaker. The composition of solubilization buffer was 1% glacial acetic acid and 50% anhydrous ethanol. The absorbance of each sample was read at 570 nm. Statistical analysis was done using SPSS ANOVA statistical software. Each of the buffers used were prepared using a protocol received from Yamini Patel at Alcon Laboratories.

Bax and Bcl-2 Quantitation of Messenger RNA Levels in RGC-5 Cells

RT-PCR was performed using cDNA made from RNA that was collected from RGC-5 cells grown with or without 10% FBS to determine the effect of serum deprivation on apoptotic message in RGC-5 cells. The procedure was the same as that used by Agarwal et al (1997).

Total RNA Extraction

RGC-5 cells were plated on 94/16 mm tissue culture dishes at a density of 100,000 cells/ dish in 12 ml of basal medium supplemented with 10% FBS. Cells attached for 3 hours at 37°C in 5% CO_2. The cells to be deprived of serum were rinsed 3 times with basal medium and 12 ml of basal medium was added to each dish. The cells were allowed to grow for 48 hours. After 48 hours, 11 ml of medium was removed, the cells were collected using a cell scraper, and transferred to a 1.6 ml microfuge tube. The lysate was centrifuged at 5000 rpm for 3 minutes. The medium was removed and 1 ml of RNAzol (Tel-Test Inc, Friendswood, TX) was used to resuspend the pellet with a 5 minute incubation on ice. Next, 200 µl of chloroform was added and the tubes were inverted 3 times to mix the lysate followed by a 5 minute incubation on ice. A 10 minute centrifugation at 14,000 rpm at 4°C was performed followed after the incubation. The homogenate separated into 2 phases; an organic phase and an aqueous phase. The aqueous phase is transferred to a new 1.6 ml microfuge tube and an equal volume of isopropanol was added. The samples were incubated on ice for 10 minutes, followed by a 14,000 rpm centrifugation for 10 minutes at 4°C. The supernatant was removed and 1 ml of 70% ethanol was added followed by a 10 minute centrifugation at 14,000 rpm. The supernatant was discarded and the pellet was allowed to air dry for 10 minutes. The RNA pellet was dissolved in 30 µl of autoclaved milli-q water.

Complementary DNA Synthesis

cDNA was synthesized from the isolated RNA using the random priming method. Five µg of total RNA was placed into a 1.6 ml microfuge tube with H_2O added to make a final volume of 27 µL. One µl of random primer (Promega, Madison, WI)

was added to the tube followed by a 3 minute incubation at 85°C. Ten µl of 5x reverse transcription buffer (Promega), 10 µl dNTP's (Promega), 1 µl RNasin (40 units/µl, Promega), and 1 µl AMV Reverse transcriptase (Promega) were added to the reaction mixture and incubated at 42°C for 30 minutes, followed by 85°C for 5 minutes. Samples were stored at -20°C until further use.

Reverse Transcriptase-Polymerase Chain Reaction

The analysis of the RGC-5 cells was performed using the apoptosis PCR bax/bcl-2 multiplex primer sets (Sigma-Aldrich, St. Louis, MO). Cycle parameters were 1 cycle at 95°C for 2 minutes; 94°C for 45 seconds, 53°C for 45 seconds, and 75°C for 1.5 minutes for 30 cycles; and a final extension of 72°C for 7 minutes. Control reactions were performed without cDNA. Amplified products were separated by a 1% agarose gel containing 10 mg/ml ethidium bromide to facilitate visualization using UV light. Experiments were performed three times to confirm results. The results were analyzed by Quantitation.

SDS-PAGE and Western Blot

Western Blot analysis was performed on cell lysates from RGC-5 cells grown with or without 10% FBS to determine the effect of serum deprivation on the protein levels of Bax, Bcl-2, cytochrome c, and Caspase 3 and 9. The procedure used was the same as that of Towbin et al (1979) with slight modifications made to the procedure. The samples were separated using SDS-PAGE with a 5% stacking gel and a 10%-12% resolving gel depending on the protein size. 40 µg of protein of lysate was loaded onto the stacking gel. Separation was performed using the Bio-Rad mini protean II Electrophoresis

Chamber using 1x SDS PAGE electrode buffer with a setting of 45 mAmps. Following electrophoresis, the proteins were transferred overnight at 4°C onto a nitrocellulose membrane (Micron Separations, Westborough, MA) at a setting of 19 volts. After the transfer the membrane was blocked with 5% milk (Carnation Instant Nonfat Dry) at room temperature for 1 hour and then incubated at 4°C with shaking in one of the following: goat polyclonal Bax (1:500 dilution, Santa Cruz Biotechnology), rabbit polyclonal Caspase 9 (1:200 dilution, Santa Cruz Biotechnology), rabbit polyclonal Bcl-2 (1:500 dilution, Santa Cruz Biotechnology), rabbit polyclonal cytochrome-c (1:200 dilution, Santa Cruz Biotechnology), or polyclonal rabbit anti-Caspase 3 (1:300 dilution, Pharmingen). The following day, membranes were washed in 1x tris buffered saline with tween 20 (TBST) 3 times for 10 minutes each. Membranes were then incubated in 1:10,000 dilutions of an appropriate species of horseradish peroxidase conjugated secondary antibodies (200 μg/ml, Santa Cruz Biotechnology) for 1 hour at room temperature on shaker. Membranes were then washed with 1x TBST 3 times for 10 minutes each. The proteins were visualized by chemiluminescent detection. Chemilumescent Detection reagents (Amersham Biosciences) were applied to the membrane for 1 minute. The membrane was exposed to x-ray film for varying amounts of time to visualize the protein bands. Film was developed using a Konica processor. The membranes were reprobed with 1:1000 dilution of monoclonal β-actin (Chemicon) to confirm equal loading. Western Blots for each protein were performed 3 times to confirm results.

Rhodamine 123 Assay

The procedure used was the same as that described by Darzynkiewicz et al (1981). RGC-5 cells were treated according to the cell culture section. After 48 hours, the cells were rinsed 2 times with 1x PBS and 1 μM Rhodamine 123 (Molecular Probes, Eugene, OR) was added to each well. The cells were incubated at 37°C for 30 minutes. After the incubation, the cells were rinsed 2 times with 1x PBS and then fixed with 3.5% Formaldehyde for 20 minutes at room temperature. The cells were then washed 3 times with 1x PBS and the coverslips were mounted onto slides and viewed using a microscope with an epiflourescence attachment. The controls for this experiment were RGC-5 cells grown in basal medium supplemented with 10% FBS. At least 100 cells were viewed under each condition and experiments were performed 3 times to confirm results.

Electrophoretic Mobility Shift Assay (EMSA)

The procedure is the same as described by Krishnamoorthy (1999). The double stranded NF-κB oligonucletides (50 ng) (Santa Cruz Biotechnology, CA) were end labeled with (γ-^{32}P)-ATP (NEN) using T4 polynucleotide kinase. This labeled probe was then purified. A DNA binding reaction containing 10 μg cytoplasmic or nuclear extract, 10 mM Tris (pH 7.6), 60 mM NaCl, 1 mM DTT, 4 mM $MgCl_2$, 1 mM EDTA, 6 fmol of ^{32}P-labeled oligonucleotide and 5% glycerol, in a total volume of 20 μl was incubated in the presence and absence of excess unlabeled oligos and the binding reaction was carried out for 20 minutes at 37°C. The samples were then subjected to electrophoresis on a 4% native polyacrylamide gell using 0.25x TBE. The gel was dried and autoradiographed.

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Chapter 3

Serum Deprivation Induces Retinal Ganglion Cell Death via Intrinsic & Extrinsic Signaling Pathways

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ABSTRACT

The mechanism of apoptosis of retinal ganglion cells (RGC) due to blockade of retrograde transport of neurotrophins (NT) in glaucoma is currently not completely understood. In the present report, we determined mechanisms underlying apoptosis in rat RGCs deprived of growth factors in vitro following serum withdrawal in the growth medium. An established line of transformed rat retinal ganglion cells, RGC-5 was subjected to serum deprivation for 2-6 days and compared with RGC-5 cells maintained in growth medium containing 10% fetal calf serum. RGC-5 cells deprived of serum for two days resulted in about 50% cell loss due to apoptosis as established by DNA laddering and propidium iodide nuclear staining. Increased oxidative stress was observed in serum-deprived RGC-5 cells, as suggested by the increase in malonyldialdehyde (MDA) and a decrease in reduced glutathione (GSH) levels in cell lysates. The apoptotic cell death of RGC-5 cells was associated with activation of caspases-3, 8, and 9, and increased levels of Bax and death receptors-3 and 4 along with a decrease in Bcl-2 levels and NF-kB binding activity. Serum deprivation was also associated with a loss of mitochondrial function as revealed by cytochrome-c release and rhodamine-123 staining. Taken together, these results indicate that serum deprivation results in retinal ganglion cell death via both the intrinsic as well as extrinsic apoptotic pathways involving oxidative stress.

INTRODUCTION

Glaucoma is currently the second leading cause of blindness in the U.S. and its incidence is on the rise every year (Quigley, 1996). Because of this, glaucoma places a tremendous burden on the economy and the health services infrastructure, with over 3 million annual clinic visits in the U.S. alone (Klein et al., 1992). Glaucoma is a progressive optic neuropathy that is characterized by a typical excavated appearance of the optic nerve head and loss of RGCs due to apoptosis (Pease, 2000).

Glaucoma was originally characterized by an elevation in intra-ocular pressure (IOP), which remains one of the highest risk factors in glaucoma. While several other risk factors contribute to the etiology of glaucoma, the cause of vision loss in all cases is ultimately through apoptosis of RGCs (Quigley et al., 1995; Quigley, 1999; Levin, 1999; Hanninen et al., 2002). Apoptosis of RGCs was first shown by optic nerve transection in the rat (Berkalaar et al., 1994; Garcia-Valenzuela et al., 1994; Rabacchi et al., 1994 a, b). Other investigators including Quigley found apoptosis of RGCs in the monkey model of experimental glaucoma (Quigley et al., 1995; Garcia-Valenzuela et al., 1995). Studies in human primary open angle glaucoma (POAG) showed TUNEL positive labeling in the retinal ganglion cell layer in 50% of the patients as compared to less than 10% in the control group (Kerrigan et al. 1998). One of the hypotheses to explain apoptotic cell death of RGCs is that the elevated intra-ocular pressure results in disruption of axonal transport within the optic nerve head, leading to blockade of retrograde transport of neurotrophi ns (Quigley and Addicks, 1980; Aguayo, 1996; Peinado-Ramon, 1996;

Nickells, 1996; Fawcett, 1998; Pease et al., 2000; Johnson et al., 2000; von Bartheld, 2000).

The in vitro model proposed in this report involving serum deprivation- induced apoptotic cell death mimics the "blocked axonal transport of neurotrophins" paradigm of retinal ganglion cell death in glaucoma. Because trophic withdrawal has been hypothesized as a primary cause of ganglion cell death in glaucoma (Wadia, 1998) we attempted to elucidate the pathways that retinal ganglion cells utilize when they undergo apoptosis using serum deprivation of RGC-5 cells as an experimental model.

RGC-5 cells represent an established permanent transformed rat retinal cell line of retinal ganglion cells (Krishnamoorthy et al., 2001). These cultured RGCs could be subjected to serum deprivation induced cell death to study apoptotic mechanisms of retinal ganglion cell death, which could be an in vitro model of glaucoma. Our studies show that serum deprivation of RGC-5 cells for various durations resulted in activation of caspases-3, 8, and 9 and increased levels of Bax and death receptors-3, and 4. A decrease in both Bcl-2 levels and NF-kB binding activity was observed consistent with an apoptotic mode of cell death. Moreover, a decrease in cellular GSH levels were also observed in apoptotic RGC-5 cells suggestive of oxidative pathway in cell death. Furthermore, serum deprivation resulted in cytochrome-c release from the mitochondria with associated loss of mitochondrial membrane potential in RGC-5 cells. Taken together, these data suggest that serum deprivation results in oxidative damage induced apoptotic cell death of retinal ganglion cells involving mitochondrial and receptor mediated signaling pathway.

MATERIALS AND METHODS

Culture of Retinal Ganglion (RGC-5) Cells

Cultures of RGC-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 100 units/ml of penicillin and 100mg/ml of streptomycin (Sigma, St Louis, MO) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (Krishnamoorthy et al., 2001). The cells were passaged every three to four days with a doubling time of about 18-20 hours.

Serum Deprivation

RGC-5 cells were plated on a 24 well tissue culture plate (Becton Dickinson, Lincoln Park, NJ) either containing 12 mm circle glass coverslips (Fisher Scientific, Pittsburgh, PA) or no coverslips or in 100 mm tissue culture dishes at various densities in basal medium (DMEM low glucose with 10 units/ml Penicillin, 100 mg/ml Streptomycin) supplemented with 10% FBS. Cells were allowed to attach for 3 hours at 37°C in 5% CO_2 after which the medium supplemented with 10% FBS was removed from the experimental wells. The experimental wells were then rinsed 3 times with basal medium and cells were incubated in basal medium for 48-144 hours at 37°C.

Cell Viability Assays

The effects of serum deprivation on the survival of RGC-5 cells were evaluated using the Neutral red (Gibco/BRL) uptake viability assay (Borenfreund and Puerner, 1985). Neutral red dye was added to a final concentration of 0.033% in HEPES buffer (125mM

NaCl, 5mM KCl, 1.8mM $CaCl_2$, 2mM $MgCl_2$, 0.5mM NaH_2PO_4, 5mM $NaHCO_3$, 10mM D-glucose, 10mM Hepes, pH 7.2) after the cells were treated with the indicated conditions of serum deprivation conditions for 2 hours. Cells were then gently washed with 2 volumes of a HEPES buffer to wash off the neutral red dye not taken up by the live cells. The cells were then allowed to air dry for 20 minutes and treated with 500µl of ice-cold solubilization buffer (1% acetic acid/50% ethanol). Twenty minutes later, 100 µl aliquots were transferred to wells of flat-bottomed 96 well plates and optical densities of samples were read at 570 nm.

Propidium Iodide (PI) Nuclear Staining for Apoptosis

The PI staining was performed on RGC-5 cells grown with or without 10% fetal bovine serum to determine if serum deprivation of RGC-5 cells resulted in apoptosis. After 48 hours of serum deprivation, the cells were rinsed with 500 µl of 1x phosphate buffered saline (PBS). Cells were then rinsed with 500 µl of freshly prepared 1x Assay buffer as suggested by the supplier (Santa Cruz Biotechnology, Santa Cruz, CA). Propidium Iodide Solution (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each well for a final dilution of 1:20 followed by thirty minutes of incubation at room temperature. The cells were rinsed 3 times with 1x PBS and fixed with 3.5% Formaldehyde for 30 minutes at 4°C. The cells were again rinsed 3 times with 1x PBS and the coverslips were mounted onto slides and viewed using a microscope with an epiflourescence attachment.

Immunoblot Analysis

The lysates from control or serum deprived RGC-5 cells were subjected to SDS-PAGE and immunoblot analysis using ECL reaction utilizing a peroxidase labeled second antibody (Kirkegaard and Perry Laboratories Inc, Gaithersberg, MD) to compare the levels of the various caspases, Bcl-2/Bax; and death receptors proteins (Krishnamoorthy et al., 1999; Crawford et al., 2001). The antibodies for various caspases; Bcl-2/Bax; death receptors 3 and 4 were purchased from commercial sources (Santa Cruz Biotechnologies, Santa Cruz, CA). To ensure the equal loading of protein in each lane, the blots were reprobed with a housekeeping protein antibody such as β-actin. For cytochrome-c release assays, the mitochondria-free cytosolic extracts from control or serum deprived RGC-5 cells were prepared and subjected to immunoblot analysis using a commercially available antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) as described above.

Electrophoretic Mobility Shift Assays (EMSAs)

The nuclear and cytosolic extracts from control and serum deprived RGC-5 cells were prepared as described earlier (Krishnamoorthy et al., 1999; Crawford et al., 2001). A double stranded oligonucleotide containing the NF-κB DNA-binding consensus sequence- 5'-AGT TGA **GGG GAC TTT CCC** AGG C-3'-and a double stranded mutant oligonucleotide, 5'-AGT TGA **GGC GAC TTT CCC** AGG C-3' (Santacruz Biotechnology, SantaCruz, CA) were used to study the DNA binding activity of NF-κB by EMSA as described by Krishnamoorthy et al., 1999.

Effect of Serum Deprivation on Mitochondrial Membrane Potential

Cells were plated on sterile 12 mm glass coverslips and subjected to serum deprivation as described above. The cells were rinsed 2 times with 1x PBS and 1 mM Rhodamine 1,2,3 (Molecular Probes, Eugene, OR) was added to each well and the cells were incubated at 37°C for 30 minutes. After incubation, the cells were again rinsed 2 times with 1x PBS and then fixed with 3.5% buffered paraformaldehyde for 20 minutes at room temperature as described (Darzynkiewicz et al., 1981). The cells were then washed 3 times with 1x PBS and the coverslips were mounted onto glass slides using Fluoromount G (Southern Biotechnology, Birmingham AL). The cells were viewed using a microscope with an epiflourescence attachment. Control RGC-5 cells were grown in basal medium supplemented with 10% FBS.

Measurements of Reduced (GSH) and Oxidized Glutathione (GSSG) levels

The membrane lipid peroxidation of serum deprived RGC-5 cells was studied by measuring the malonyldialdehyde (MDA) levels by a colorimetric method involving thiobarbituric acid (TBA) adduct formation (Jain, 1989). The GSH levels in serum deprived RGC-5 cells was studied by using the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reagent (Beutler et al., 1963).

Results

Serum Deprivation of RGC-5 Cells Resulted in Cell Loss Via Apoptosis

To determine the effect of serum deprivation on the viability of RGC-5 cells, the RGC-5 cells were cultured in serum-free medium for 48 hrs along with the control cells, which were grown in medium supplemented with 10% fetal calf serum. Cell viability was determined by neutral red dye uptake assay. Serum deprivation of RGC-5 cells for 48 hrs resulted in about 50-60% cell loss (Figure 1A). Serum deprived RGC-5 cells were also tested for genomic DNA laddering and the results showed a typical ladder comprising of nucleosomal fragments of genomic DNA in serum deprived cells but not in the control cells (Figure 1B). To further establish that the cell loss is due to apoptosis, serum deprived RGC-5 cells were subjected to propidium iodide staining and compared with the control cells. The results showed that the nuclei of RGC-5 cells were condensed, a hallmark of apoptosis (arrows, Figure 1C). Taken together these results suggested that serum deprivation resulted in apoptosis of RGC-5 cells.

Serum Deprivation of RGC-5 Cells Resulted in Release of Mitochondrial Cytochrome-C and Oxidative Damage:

It is becoming increasingly evident that neurotrophin deprivation of neurons activates oxidative processes such as release of cytochrome-c from mitochondria. To model neurotrophin deprivation, RGC-5 cells were grown in serum free medium. To determine the involvement of mitochondria in apoptosis of serum deprived RGC-5 cells, we measured the release of cytochrome c in the cytoplasm of serum deprived cells (-) as compared with the control cells (+) grown in full growth medium. Our results showed

(Figure 2 A) considerable release of cytochrome-c from mitochondria of serum deprived RGC-5 cells (-), thus confirming that serum deprivation results in release of cytochrome-c, which could be involved in retinal ganglion cell death.

Since damage to mitochondria occurs in the apoptotic pathway, it is conceivable that reactive oxygen species are released in the process causing oxidation of proteins and membrane damage. We observed that serum deprivation of RGC-5 cells resulted in oxidative damage as shown by the increased levels of MDA and a decrease in reduced GSH levels (Figure 2B). Since the results suggested that oxidative damage might be occurring on serum deprivation in these cells, the effect of inclusion of the N-acetyl cysteine (NAC) and thiourea was studied in serum-deprived cells (Figure 2C). The results showed that the two antioxidants did not reverse the effects of serum deprivation.

Serum Deprivation of RGC-5 Cells Results in Upregulation of bax and Down-regulation of bcl-2 mRNA and Their Proteins:

Bcl-2 is an anti-apoptotic protein, whereas Bax is a pro-apoptotic protein belonging to bcl-2 gene family. Together, these anti- and pro-apoptotic proteins determine a cell's fate for survival or cell death. Based on this assumption, we measured the mRNA levels of bcl-2 and bax in the serum deprived RGC-5 cells as compared with the control RGC-5 cells grown in complete growth medium by using semi-quantitative RT-PCR analysis. The results showed a decrease in the bcl-2 with a concomitant increase in bax mRNA levels in serum deprived cells for 48-72 hrs (Figures 3A). The densitometry of the RT-PCR product showed a significant decrease in bcl-2/bax ratio in serum deprived RGC-5 cells (Figure 3B). The results were further confirmed by

immunoblot analysis using specific antibodies against Bcl-2 and Bax showing increase in Bax with a concomitant decrease in Bcl-2 levels with increase in number of days of serum deprivation as compared with the control cells (Figure 3C).

Effect of Serum Deprivation of RGC-5 Cells on Caspases and Death Receptors:

To elucidate the apoptotic signaling pathway(s) involved in RGC-5 cell death from serum deprivation, caspases 3, 8, and 9 activation was determined by immunoblot analysis of RGC-5 cells deprived of serum for various time points in comparison with the RGC-5 cells grown in growth medium containing 10% fetal calf serum. The results of this analysis showed that there was a time dependent increase in caspases activation in serum deprived RGC-5 cells as compared with the control RGC-5 cells (Figure 4A). To determine if the serum deprivation results in activation of death receptors 3 and 4, we performed immunoblot analysis. The results of β-actin was used as a control of loading the protein in each lane and the results showed that the levels of β-actin did not change during the course of serum deprivation of RGC-5 cells (Figure 4A). These findings established that there was indeed an activation of caspases 3, 8 and 9 along with an activation of DR-3 and -4.

Effect of Serum Deprivation of the RGC-5 cells on NF-kB Binding Activity:

RGC-5 cells were subjected to serum deprivation for 1-3 days in culture along with control RGC-5 cells for similar time intervals. After serum deprivation, nuclear and cytoplasmic extracts were prepared and subjected to electrophoretic mobility shift assays (EMSA) using end-labeled double stranded oligos having the consensus binding sequence of NF-κB. A time course of serum deprivation of RGC-5 cells showed a

decrease in NF-κB activity in both the nucleus (Figure 5, lanes 8, 10, and 12) and cytoplasm (Figure 5, lanes 4 and 6) as compared to control cells grown in complete growth medium (Figure 5, lanes 7, 9, and 11, for nucleus and lanes 1, 3, and 5 for cytoplasm, respectively). These results indicated that the RGC-5 cells expressed NF-κB constitutively and that the activity of NF-κB decreased following serum deprivation.

Effect of Serum Deprivation on Mitochondrial Membrane Potential in RGC-5 Cells:

RGC-5 cells were subjected to serum deprivation for 3 days in culture along with control RGC-5 cells for similar time intervals. After serum deprivation, the RGC-5 cells were subjected to rhodamine 123 staining in live cells and examined under a fluorescent microscope. The results showed that there was barely any detectable level of rhodamine 123 in RGC-5 cells, which were serum deprived for a period of 3 days as compared with the control RGC-5 cells (Figure 6). These results indicate that serum deprivation resulted in a disruption of mitochondrial membrane potential of RGC-5 cells.

DISCUSSION

Retinal ganglion cells play a key role in integrating visual information and relaying it to the cerebral cortex of the brain via the optic nerve, thereby enabling vision. The retinal ganglion cells are sustained by neurotrophic factors that are retrogradely transported to the ganglion cells. When the axons are damaged, as occurs in glaucoma, retrograde transport is disrupted and the ganglion cells die via apoptosis. This study determines the pathways that are involved in apoptosis of ganglion cells when they are deprived of trophic factors in a serum deprivation paradigm in vitro. In this paper we hypothesized that serum deprivation induces apoptosis of retinal ganglion cells via mitochondrial as well as receptor mediated signaling pathways. This serum deprivation model may mimic the loss of neurotrophin support of retinal ganglion cells as seen during glaucoma. Serum deprivation of RGC-5 cells for various durations resulted in typical characteristics of apoptosis such as nuclear condensation, DNA laddering associated with activation of caspases-3, 8, and 9, and increased levels of Bax and death receptors-3, and -4 with a decrease in Bcl-2 levels and NF-kB binding activity and cellular GSH levels. Furthermore, serum deprivation also resulted in cytochrome-c release from the mitochondria with a loss of mitochondrial membrane potential of RGC-5 cells. Thus these findings suggest that serum deprivation results in apoptotic cell death of RGC-5 cells via receptor mediated as well as mitochondrial pathways.

Glaucoma is an optic neuropathy with characteristic ONH and associated visual field changes that effects more than 67 million people worldwide. Primary open angle glaucoma (POAG) is the most common form of glaucoma, accounting for nearly 70% of

all cases (Liesegang, 1996). Both mechanical stress and ischemia are thought to contribute to optic nerve damage in glaucoma, but the lamina cribrosa (LC) region of the optic nerve head (ONH) appears to be a major site of damage (Hernandez and Ye, 1993).

Oxidative stress plays an important role in the pathophysiology of glaucoma (Polansky et al., 1997; Levine et al., 1999; Zhou et al., 1999; Kourteum et al., 2000; Wang et al., 2001; Geiger et al., 2002; Paul et al., 2002; 2003). Ischemia, excitotoxicity, and/or trophic insufficiency have been suggested to play an important role in retinal ganglion cell death in glaucoma (Wadia, 1998). These stimuli cause cellular damage usually in the form of cleavage of structural and repair proteins and early degradation of DNA (Nageta, 2000) leading to apoptosis. In addition to DNA and protein damage, reactive oxygen species (ROS) can be generated and released from the mitochondria (Nicholls, 1999). It is clear from these studies that oxidative damage may play an important role in the pathophysiology of glaucoma, not only in the trabecular meshwork region but also in the back of the eye in the retinal ganglion cells of the retina. In this paper we tested if oxidative damage played a role in serum deprivation induced cell death and addressed its possible mechanism(s). The levels of MDA were increased with a concomitant lowering of GSH levels on serum deprivation of RGC-5 cells. Recent reports suggest that NF-κB is also activated during oxidative signaling (Schulze-Osthoff et al., 1995; Pahl and Baeuerle, 1996; Pinkus et al., 1996; Ginn-Pease and Whisler, 1996). It has been suggested in many of these studies that reactive oxygen intermediates (ROI) may be involved in the activation of NF-κB. NF-κB signaling is also implicated in the regulation of apoptosis. One of the earliest significant observations in this direction was made by Beg et al. (1995), who demonstrated extensive apoptosis of liver cells

leading to embryonic death of mice lacking the Rel A subunit. Subsequent work by Beg and Baltimore (1996), demonstrated that treatment of RelA-deficient ($RelA^{-/-}$) mouse fibroblasts and macrophages with TNF-α resulted in a significant reduction in cell viability. Along similar lines, showed a role of NF-κB in suppression of TNF-α induced apoptosis (Wang et al., 1996; Van Antwerp et al., 1996; Liu et al., 1996). There is also evidence of pro-apoptotic aspects of RelA activity. For instance, it was shown that serum starvation of 293 cells causes cell death accompanied by the activation of RelA containing NF-κB (Grimm et al., 1996). Redox changes in RGC-5 cells due to serum deprivation resulted in lowering of the NF-κB binding activity and apoptosis of RGC-5 cells. Similar to these results, lowering of the p65 subunit of NF-κB binding activity has been shown to be associated with apoptosis in photo-oxidative induced insult of photoreceptor cells (Krishnamoorthy et al., 1999; Crawford et al., 2000). It is thought that activation or induction of NF-κB is usually associated with cell survival signals (Ling et al., 2003; Brummelkamp et al., 2003). Pharmacological studies using NF-kB inhibitors such as aspirin, results in neuronal cell death, consistent with its role in cell survival (Grilli et al., 1996). Thus it is not surprising that a lowering of NF-κB binding activity was observed in serum deprivation induced cell death of RGC-5 cells.

The execution of apoptosis consists of a proteolytic cascade involving a family of proteases called caspases (Reme, 2000). This phase is usually associated with an upregulation of bax, which is a proapoptotic gene. The increased levels of Bax in the cell then combines with Bcl-2 already present in the cell. This lowers the amount of free Bcl-2 in the cell driving the cell toward apoptosis. Bax and Bcl-2 proteins then closely interact with mitochondria and affect the permeability of mitochondrial membranes. Bax

binds to the outer membrane of the mitochondria. This binding causes the opening of the permeability transition pore, which is a multiprotein complex that forms at points where the inner and outer mitochondrial membranes make contact (Green, 1998). This also causes the mitochondria to release of cytochrome-c (Bernadi, 1999). Cytochrome-c then activates a series of caspases. The current studies show that serum deprivation of RGC-5 cells resulted in loss of mitochondrial membrane potential with a release of cytochrome-c and activation of caspases 3, 8, and 9 and Bax with a decrease in Bcl-2 protein levels. These results are not surprising since release of cytochrome-c has been shown to activate caspase-9 (Li, 1999), which then activates caspase-3 (Liu 1996) leading to DNA fragmentation (Liu 1997). Caspase-9 activation is activated as demonstrated by immunocytochemistry in the retinal ganglion cell layer in a rat model of experimental glaucoma (Hanninen et al., 2002). This pathway represents the classical mitochondrial or intrinsic pathway for apoptosis. Reactive oxygen species have been shown to result in activation of caspases 8 and 3, which represent the key players in most extrinsic (death receptor) mediated pathways of apoptosis (Zhang et al., 2003).

On the other hand, serum deprivation was also associated with activation of caspase-8 along with the DR-3 and –4. Members of tumor necrosis factor (TNF) receptor represents a superfamily of receptors including Fas, TNFR1, TNF-related apoptosis inducing ligand (TRAIL) R1, TRAILR2, and DR-3/4 (Al-Lamki et al, 2003). All of these members contain a homologous intracellular region called death domain (DD). The DD represents a protein-protein interaction domain that allows these receptors to interact with their respective adaptor molecules, which reside in the cytosol and contain a DD. Examples of cytosolic DD are Fas-associated DD protein (FADD), TNFR-associated DD

protein (TRADD) and receptor associated protein (RIP) (Berglund et al., 2000; Al-Lamki et al., 2003). The DD are capable of initiating the death inducing signaling complex (DISC) that catalyzes caspase activation and apoptosis (Al-Lamki et al., 2003). A number of ligands for DR-3 have been suggested in the literature (Kitson et al., 1996; Chicheportiche et al., 1997; Ashkenazi and Dixit, 1998; Migone et al., 2002). There are both membrane bound as well as secreted ligands have been proposed (Chicheportiche et al., 1997). TWEAK, a secreted protein has been shown to be a ligand for TNF receptor family (Chicheportiche et al., 1997). The secreted signals may affect the distant targets as compared to the membrane bound ligands. In the present study we do not know about the ligand for DR-3/4. It is proposed that serum deprivation of RGC-5 cells results in secretion of a certain undetermined factor, which in turn binds to DR-3/4 resulting in activation of an extrinsic apoptotic pathway. Furthermore, NF-kB has been shown to result in induction of pro-apoptotic molecules such as DR-4, -5, -6 and Fas (Karin and Lin, 2002). Activation of caspase-8 along with DR-3 and -4, has been shown to represent extrinsic or receptor mediated apoptotic pathway (Zhang et al., 2003; Harper et al., 2003).

It was interesting to note that both NAC and thiourea did not reverse the effects of serum deprivation suggesting either total serum deprivation insult is far greater to be reversed by the anti-oxidants or the reduced GSH with increase in MDA levels may not be a direct effect of serum deprivation. Thus suggesting that these changes may be down stream of the activation of DR-3, and –4 and NF-kB activity changes. Further studies are needed to delineate the exact mechanism of extrinsic pathway of apoptosis in serum deprivation induced cell death paradigm. These studies further suggest that maintenance of redox state of the cell is extremely important in neuroprotection of retinal ganglion

cells and therapeutics pertaining to NF-kB may be valuable. Taken together the results presented in this paper suggest that both a mitochondrial as well as receptor mediated pathways may be involved in serum deprivation induced apoptotic cell death of retinal ganglion cells. In vivo studies will be needed to extrapolate these results in a rat model of glaucoma.

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FIGURE LEGENDS

Figure 1. Effect of Serum Deprivation on Viability of RGC-5 Cells. RGC-5 cells were deprived for serum for various time durations after which they were subjected to cell viability assays along with the control cells. A. neutral red dye uptake assay after 48 hrs of serum deprivation; B. DNA laddering for genomic DNA of RGC-5 cells after 48, 96, and 144 hrs of serum deprivation; and C. propidium iodide staining of serum deprived RGC-5 cells after 48 hrs. The upper panel represents the DIC of the lower panel representing the propidium iodide staining of the same cells (compare the black arrows vs white arrows).

Figure 2: Effect of Serum Deprivation on Oxidative State of the RGC-5 cells. RGC-5 cells were deprived for serum for 48 hrs after which they were subjected either to determine release of cytochrome-c in the cytosol by immunoblot analysis (A) or measurements of MDA and GSH by biochemical methods along with the control cells (B). In figure A, lanes 1, 3, and 5 represent the cytosol from the serum deprived (-) RGC-5 cells and lanes 2, and 4 represent the cytosol of RGC-5 cells grown in full medium (+). There was a release of mitochondrial cytochrome-c (A) and the levels of GSH were reduced with an increase in the MDA levels on serum deprivation of RGC-5 cells (B). Inclusion and preincubation of cells with antioxidants N-acetyl cysteine (2mM) and thiourea (7 mM) did not protect serum deprivation induced cell death (C). Bars: 1-represent control cell; 2-cells with NAC; 3-cells with thiourea; 4-serum deprived cells for 48 hrs; 5-serum deprived cells with NAC; and 6-serum deprived cells with thiourea.

Fig. 3. Effect of Serum Deprivation of RGC-5 cells on bcl-2 and bax mRNA Expression and Protein Levels. RGC-5 cells were deprived for serum for various time

durations after which they were subjected either to RT-PCR analysis of bcl-2 and bax mRNA expression (A) or their protein levels by immunoblot analysis (C). Following 3 days of serum deprivation, the bcl-2 message was down-modulated as compared with the control RGC-5 cells maintained in complete growth medium (A). On the other hand, serum deprivation resulted in an increase in bax mRNA expression as compared with the control cells (A). b-actin was included as a control to compare the levels of cDNA synthesis in both treatments (A). The band density of bcl-2 and bax was determined by densitometry using NIH IMAGE program and the ratio of bcl-2/bax was plotted (B). There was a significant decrease (*) in the ratio of bcl-2/bax in serum deprived RGC-5 cells (B). Immunoblot analysis of control and serum deprived RGC-5 cells showed a time dependent increase of Bax protein levels with a concomitant increase in Bcl-2 protein levels (C). b-actin immunoblot was included as a control to compare the equal loading of proteins in all treatments (not shown).

Fig. 4. Effect of Serum Deprivation of RGC-5 cells on Activation of Caspases (8, 9, and 3) and Death Receptors (3, and 4). RGC-5 cells were deprived of serum for various time durations after which they were subjected to immunoblot analysis for caspases (A) and death receptors (B). b-actin immunoblot was included as a control to compare the equal loading of proteins in all treatments (A). A time dependent increase was observed in serum deprived RGC-5 cells for caspases-3, -8, & -9 (A) and DR-3 & -4 (B) as compared to the control cells. The arrowheads represent the smaller subunits of 20 kDa and 12 kDa for caspase-9 and 20 kDa for caspases-3 and –8.

Figure 5: Effect of a Time Course of Serum Deprivation on NF-κB Binding Activity in RGC-5 cells. RGC-5 cells express NF-κB constitutively (lanes 1 and 7, for

cytoplasmic and nuclear fractions respectively). Lanes 2, 4 and 6, and 8,10,and 12 represent NF-κB binding activity in cytoplasm and nucleus respectively after 1, 2, and 3 days of serum deprivation of the retinal ganglion cells. Clearly, upon serum deprivation of RGC-5 cells, NF-κB binding activity in the cytoplasm as well as the nucleus is reduced as compared to control samples, at all stages of serum deprivation.

Fig. 6. Effect of Serum Deprivation of RGC-5 cells on Mitochondrial Membrane Potential. RGC-5 cells were deprived of serum for 48 hrs (B) along with the controls (A) after which they were subjected to rhodamine 123 labeling in live cells prior to fixation with buffered 4% paraformaldehyde and visualized under a fluorescent microscope. The control cells showed nice uptake of rhodamine 123 indicating intact mitochondria (A) as compared with the serum deprived RGC-5 cells (B), which were devoid of rhodamine 123 indicating loss of mitochondrial membrane potential.

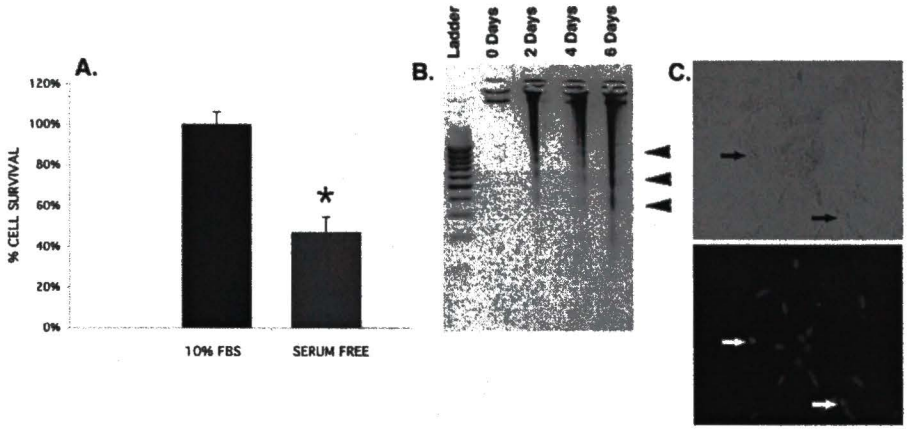

A.
120%
100%
80%
60%
40%
20%
0%
% CELL SURVIVAL
*
10% FBS
SERUM FREE
B.
Ladder
0 Days
2 Days
4 Days
6 Days
C.

Figure-1

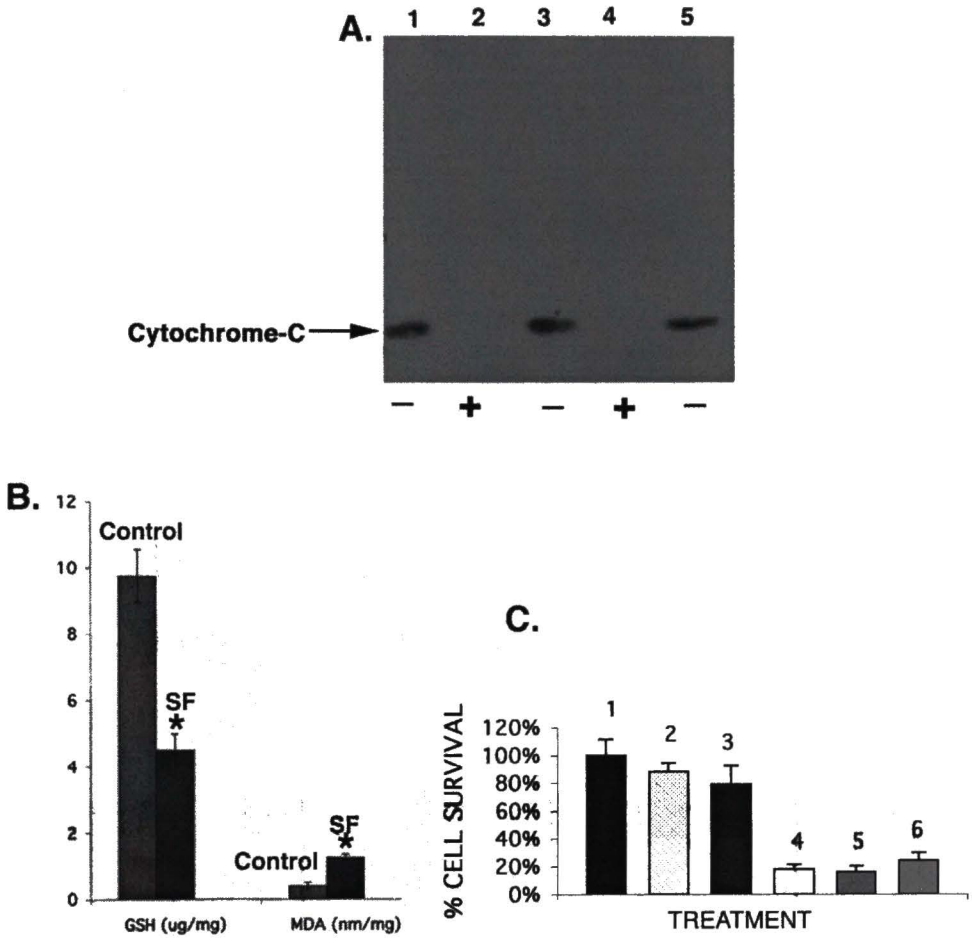

A.
1
2
3
4
5
Cytochrome-C
–
+
–
+
–
B.
12
10
8
6
4
2
0
Control
SF
*
Control
SF
*
GSH (ug/mg)
MDA (nm/mg)
C.
% CELL SURVIVAL
120%
100%
80%
60%
40%
20%
0%
1
2
3
4
5
6
TREATMENT

Figure-2

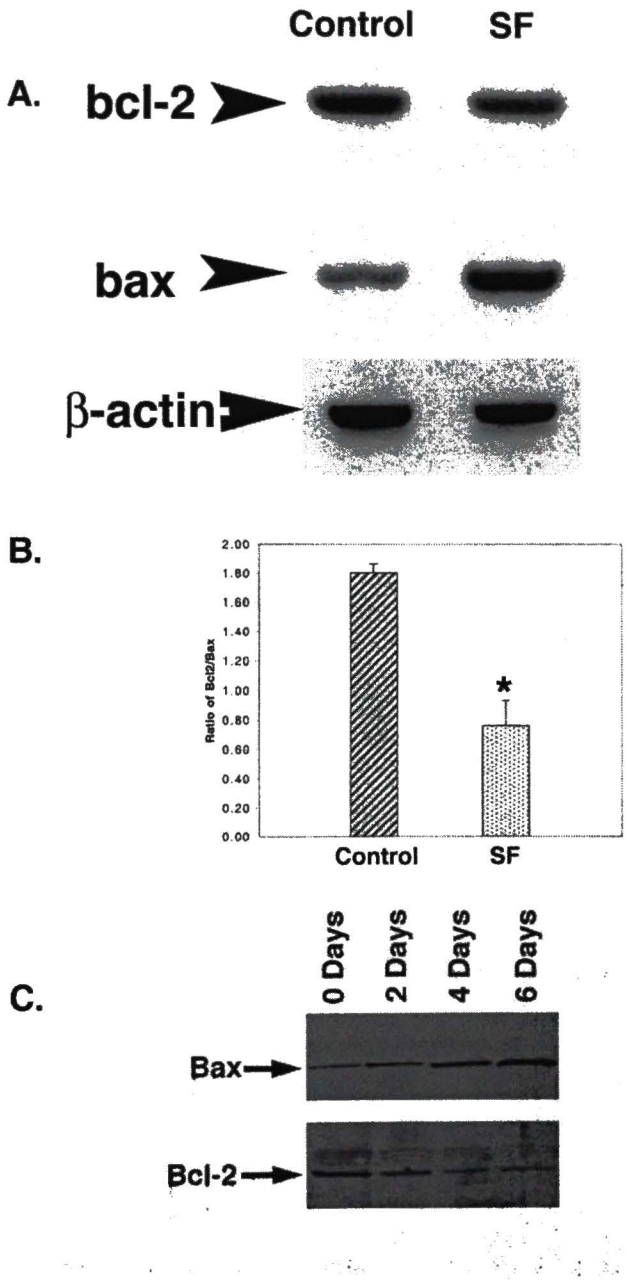

A.
Control
SF
bcl-2
bax
β-actin
B.
Ratio of Bcl2/Bax
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
Control
SF
C.
0 Days
2 Days
4 Days
6 Days
Bax
Bcl-2

Figure-3

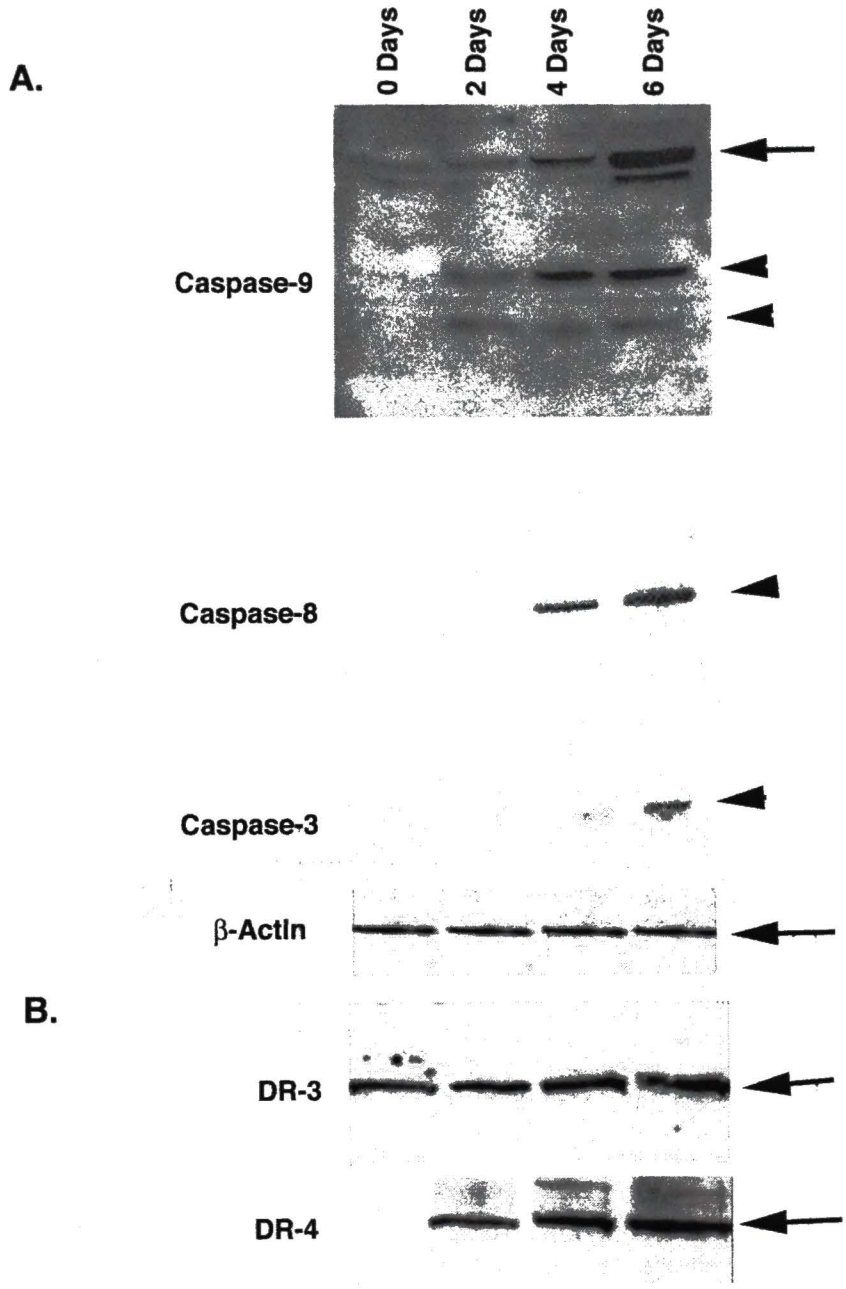

A.
0 Days
2 Days
4 Days
6 Days
Caspase-9
Caspase-8
Caspase-3
β-Actin
B.
DR-3
DR-4

Figure-4

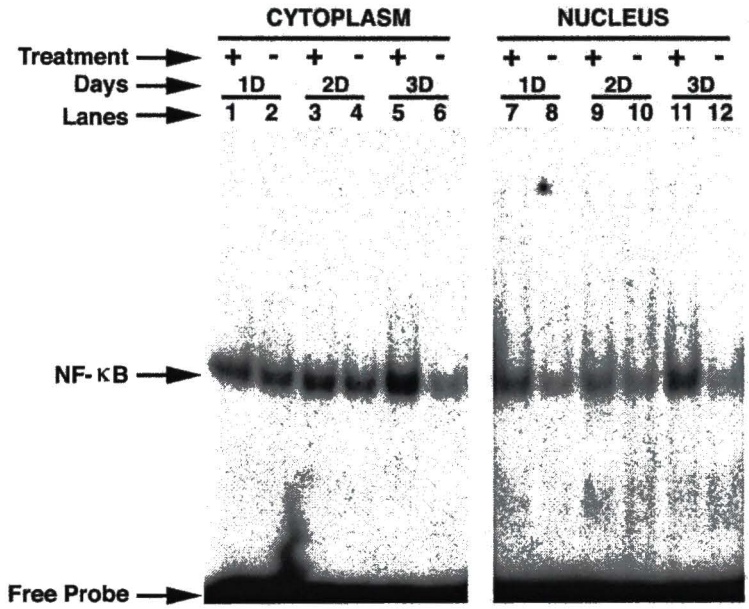

CYTOPLASM
NUCLEUS
Treatment
+ - + - + - + - + - + -
Days
1D 2D 3D 1D 2D 3D
Lanes
1 2 3 4 5 6 7 8 9 10 11 12
NF-κB
Free Probe

Figure-5

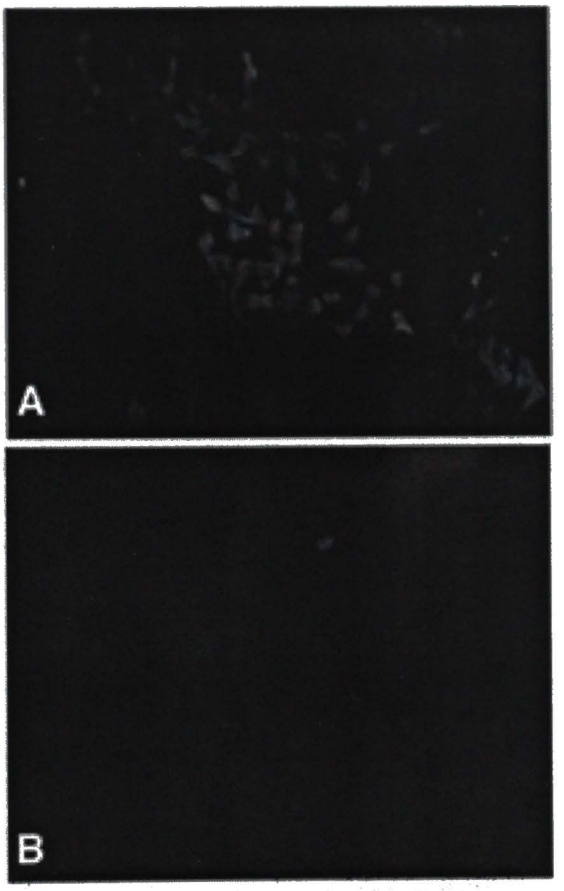


Figure-6

CHAPTER 4

An Alternate Signaling Pathway

Neurotrophins, while binding to their cognate Trk receptors, activate several signal transduction cascades that regulate cell death machinery (Segal, 1996; Friedman, 1999). The ERK/MAPK cascade mediates trophic factor survival in differentiated PC12 cells and other neurons (Meyer-Franke, 1995; Parrizas, 1997; Hetman, 1999). The PI3K pathway was originally shown to be involved in Nerve growth factor-dependent and serum dependent survival of PC12 cells (Yao, 1995).Additional experiments also showed that PI3K is involved in the NGF dependent survival of dorsal root and superior cervical ganglion neurons (Crowder,1998; Mazzoni 1999) as well as BDNF protection of spinal cord motor neurons after serum deprivation (Encinas, 1999). Since the PI3K and MAPK pathways are activated by neurotrophins we began to explore whether these pathways have a role in retinal ganglion cell survival. This study consists of the preliminary data to study the survival signaling pathways when retinal ganglion cells are deprived of serum.

The susceptibility of cells to apoptosis appears to be dependent on the balance between pro-apoptotic and survival signals. Two pathways that help with this balance are the Phosphoinositide 3-kinase pathway and the Mitogen Activated Protein Kinase Pathway. These pathways have been shown to phosphorylate the pro-apoptotic Bcl-2 family member Bad (Jin, 1992; Vanhaesebroeck, 2000), and also activate Nuclear Factor Kappa B (Adams, 2000; Lee, 1997).

Mitogen activated protein kinases (MAPKs) are membrane to nucleus signaling modules that transduce extracellular signals from tyrosine-kinase receptors and G protein coupled receptors to cytoplasmic and nuclear effectors (Schaeffer, 1999; Chang, 2001). Once these proteins translocate to the nucleus they phosphorylate a large number of nuclear proteins. With the MAPK pathway, an extracellular signal binds to the tyrosine kinase receptor causing dimerization of the receptor (Emaduddin, 1999). The dimerization causes the intracellular tyrosine kinases(s) to become phosphorylated, which are then bound by the SH2 domains of the adaptor proteins, like Grb2 (Buday, 1993). Grb2 also contains an SH3 domain that binds proline-rich motifs of other peptides such as son of sevenless (SOS), which is a guanine dissociation stimulator which binds Ras after receptor activation (Moodie, 1993). Ras, which is a membrane-bound guanosine triphophate/diphosphate binding G protein, becomes activated by exchanging GDP for GTP and recruits Rad to the cell membrane enabling its activation (Voitek, 1993). Raf activates MEK by phosphorylating serines 218 and 222 (Zhng, 1994). MEK can then phosphorylate ERK 1/2 on threonine 183 and tyrosine 185 (Crews, 1992) which causes nuclear translocation (Khokhlatchev,1998; Canagarajah,1997) of ERK activating transcription factors that are necessary for cell survival (Cobb, 2000; Shapiro,1999).

The second pathway is the Phosphatidyinositide-3-pathway (PI3K). PI3K is found in the cytoplasm of cells as a p85 regulatory subunit combined with a p110 catalytic subunit (Hiles, 1992; Klippel,1992). When a tyrosine kinase is activated, there is autophosphorylation and binding of the SH2 domain of p85 to the phosphorylated tyrosines (Shoelson,1993). PI3K generates several phosphorylated lipids that recruit the phosphatidylinositol-dependent kinases (PDKs) and AKT (Whitman,1988,

Anderson,1998), which is phosphorylated by the PDKs (Alessi,1996; Alessi, 1997). Activated AKT then can phosphorylate several downstream substrates that enable the cell to proliferate including but not limited to the pro-apoptotic moleculre BAD and Caspase-9.

The PI3K pathway is one that often transduces signals that are similar to that of the MAPK pathway. It is believed that some crosstalk exists between the pathways, i.e. between AKT and raf (Lee,2002). Certain studies suggest that PI3K activity is essential for induction of MAPK activity (King, 1997; Wennstrom, 1999; Sheng,2001). Additional studies suggest PI3K activation enhances MAPK signaling to provide a stronger signal through the lower components of the cascade, ie ERK (McCubrey, 2001).

Due to these pathways being so important to neuronal cell survival, we decided to look at the effect blocking specific kinases in the PI3K and also MAPK pathways would have on the survival of serum deprived retinal ganglion cells. For these studies we used Cell Survival Assays with RGC-5 Cells treated with inhibitors to specific kinases. The first inhibitor used was PD098059 (Alexis Biochemicals) which is a commercially available MEK inhibitor that functions by binding to and preventing activation of the dephosphorylated form of MEK-1 (Alessi, 1995, Dudley,1995). For the PI3K Pathway , an inhibitor of PI3k, LY294002 (Alexis Biochemicals), was used to inhibit the activity of phosphatidyinositide-3-kinase. An AKT inhibitor (Calbiochem) was also used to block the effects of AKT. These two were used to study the differences in survival when you block the activation of the pathway early as opposed to late in the cycle.

Methods:

Survival assays were performed on RGC-5 cells grown with or without 10% FBS and treated with appropriate drugs to determine if these drugs could prevent ganglion cell death. The procedure was the same as described by Borenfreund et al (1985) with slight modifications. RGC-5 cells were plated as described under the cell culture section in Chapter 2. At this time cells were treated as follows for each separate experiment:

Inhibitor Study with AKT and JNKII

1- RGC-5 cells grown with 10% FBS
2- RGC-5 cells grown with 10% FBS & 10 µl of DMSO
3- RGC-5 cells grown without 10% FBS
4- RGC-5 cells grown with 10 µl of DMSO & without 10% FBS
5- RGC-5 cells grown with 10 µM AKT Inhibitor & without 10% FBS
6- RGC-5 cells grown with 1 µM JUN Inhibitor & without 10% FBS

Inhibitor Study with PD 98059

1- RGC-5 cells grown with 10% FBS
2- RGC-5 cells grown with 10% FBS & 10 µl DMSO
3- RGC-5 cells grown without 10% FBS
4- RGC-5 cells grown with 10 µM PD98059 & without 10% FBS
5- RGC-5 cells grown with 20 µM PD98059 & without 10% FBS
6- RGC-5 cells grown with 50 µM PD98059 & without 10% FBS

Inhibitor Study with LY 294002

1- RGC-5 cells grown with 10% FBS
2- RGC-5 cells grown with 10% FBS & 10 µl DMSO
3- RGC-5 cells grown without 10% FBS
4- RGC-5 cells grown with 10 µM LY294002 & without 10% FBS
5- RGC-5 cells grown with 20 µM LY294002 & without 10% FBS
6- RGC-5 cells grown with 50 µM LY294002 & without 10% FBS

The cells were allowed to grow for 48 hours. After 48 hours the medium was removed and the wells were washed 2 times with 1ml of HEPES assay buffer (pH 7.2). The

composition of HEPES assay buffer was 125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2 2H_2O$, 2 mM $MgCl_2 6H_2O$, 0.5 mM $NaH_2PO_4 H_2O$, 5 mM $NaHCO_3$, 10 mM HEPES, and 10 mM D-glucose. After the final rinse, 500 µl of HEPES assay buffer was added to each well and incubated at room temperature for 20 minutes. After 20 minutes, 17 µl of 1% Neutral Red Dye was added to each well and incubated for 2 hours at room temperature. After 2 hours, the cells were again washed 2 times with 1 ml of HEPES assay buffer/well. The cells were allowed to dry for 20 minutes. Five hundred µl of ice cold solubilization buffer was added to each well and incubated for 20 minutes at room temperature on an orbit shaker. The composition of solubilization buffer was 1% glacial acetic acid and 50% anhydrous ethanol. The absorbance of each sample was read at 570 nm. Statistical analysis was done using using SPSS ANOVA statistical software . Each of the buffers used were prepared using a protocol received from Yamini Patel from Alcon Laboratories.

Results:

Figure 10. Effect of AKT and JNK Inhibitors on Serum Deprived RGC-5 Cells.

RGC-5 cells were serum deprived and treated with 1 μM concentration JNK Inhibitor or 10 μM AKT Inhibitor for 48 hours. There was a significant decrease ($p<0.05$) observed between the serum deprived cells and the serum deprived cells treated with the JNK Inhibitor. Bars represent mean ± SD. Statistical analysis was performed with SPSS using ANOVA. Each Experiment was performed three times to confirm results. Cells grown for 48 hours in basal medium supplemented with 10% FBS were used as a control.

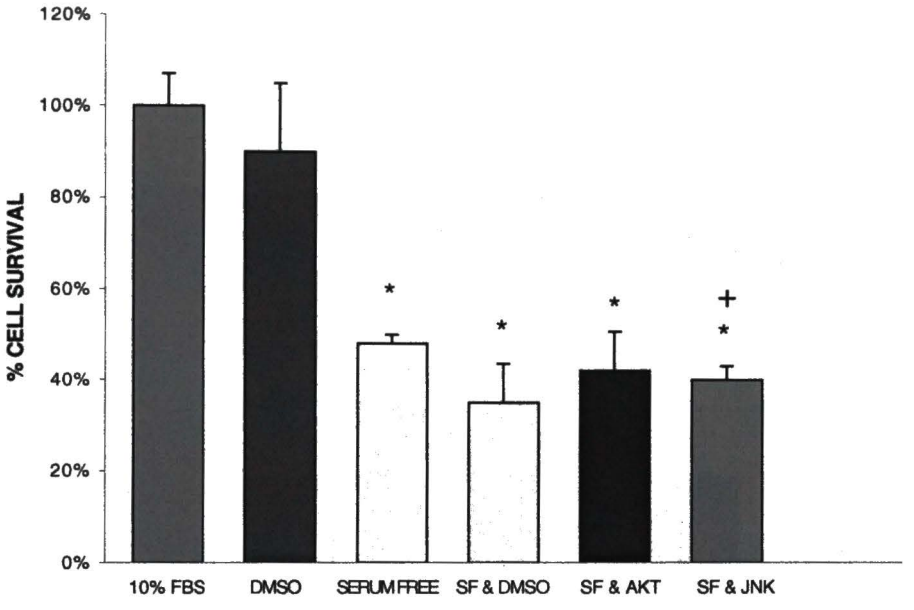
Figure 11. Effect of PD 098059 on Serum Deprived RGC-5 cells.

RGC-5 cells were serum deprived and treated with 10, 20 and 50 μM concentrations of PD 098059 for 48 hours. There were no significant difference ($p<0.05$) observed between serum deprived RGC-5 cells and those that were treated with 10 μM concentrations of PD 098059. There was a significant difference between the serum deprived cells and those treated with 20 and 50 μM concentrations of PD 098059. Bars represent mean ± SD. Statistical analysis was performed with SPSS using ANOVA. Each experiment was performed three times to confirm results. Cells grown for 48 hours in basal medium supplemented with 10% FBS were used as a control.

Figure 12. Effects of LY 294002 on Serum Deprived RGC-5 Cells.

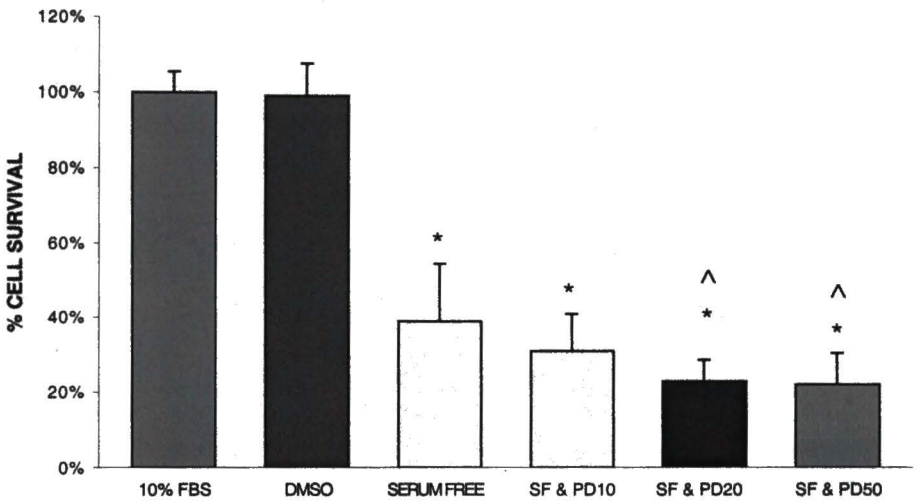
RGC-5 cells were serum deprived and treated with 10, 20, and 50 μM concentrations of LY 294002 for 48 hours. There was a significant decrease ($p<0.05$) between the serum deprived cells and the cells that were treated with 10, 20 and 50 μM concentrations of LY 294002. There was also a significant decrease between the serum deprived cells treated with 10 μM LY294002 and those treated with 20 and 50 μM LY 294002. Bars represent mean ± SD. Statistical analysis was performed with SPSS using ANOVA. Experiments were performed three times to confirm results. RGC-5 cells grown 48 hours in basal medium supplemented with 10% FBS were used as a control.

Figure 10. Effects of JNK and AKT Inhibitors on Serum Deprived RGC-5 Cells.



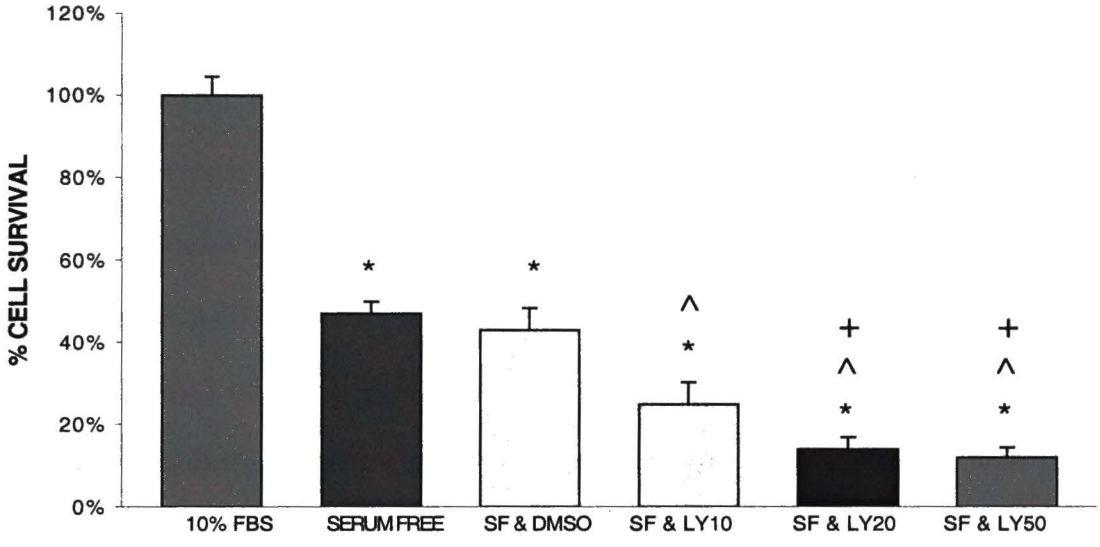
*= statistically significant difference from 10% FBS control ($p<0.05$)
+= statistically significant difference from serum free ($p<0.05$)

Figure 11. Effect of PD 098059 on Serum Deprived RGC-5 Cells.



*= statistically significant difference from 10% FBS control ($p<0.05$)
^= statistically significant difference from serum free ($p<0.05$)

Figure 12. Effect of LY 294002 on Serum Deprived RGC-5 Cells.



*= statistically significant difference from 10% FBS control ($p<0.05$)
^= statistically significant difference from serum free ($p<0.05$)
+= statistically significant difference from SF & LY10 ($p<0.05$)

DISCUSSION

The Phosphatidylinositol 3-Kinase and Mitogen-Activated Protein Kinase pathways are two pathways that are important to neural cells (Meyer-Franke, 1995). To determine if these pathways are important in serum deprived RGC-5 cells, the serum deprived cells were incubated for 48 hours with varying concentrations of inhibitors.

Treatment of the serum deprived RGC-5 cells with JNKII inhibitor resulted in a significant decrease in cell population as compared with the serum deprived cells. On the other hand, the AKT inhibitor had no significant effect on the cell population. When the cells were treated with PD 098059, a MEK-inhibitor, there was no significant decrease between the cells that were serum deprived and the cells that were treated with 10 μM concentrations of MEK inhibitor. There was however a significant decrease when the serum deprived RGC-5 cells were treated with 20 or 50 μM concentrations of MEK inhibitor. Incubation of cells with LY 294002, a PI3K inhibitor, resulted in a significant decrease in cell number between serum deprived cells and cells treated with 10, 20, and 50 mM concentrations.

Taken together these results indicate that the PI3K and MAPK pathways may be important for retinal ganglion cell survival when there is a blockade of neurotrophins. For the PI3K inhibitor LY 294002 there appears to be a dose dependent decrease in cell population. The AKT inhibitor, which blocks the phosphorylation of AKT and occurs late in the pathway, has no significant effect on cell number in the serum deprived cells. This may indicate that the early phosphorylation of the pathway may be more important due to the fact that blocking the later phosphorylation does not affect cell survival. The inhibition of the activation of the MEK 1/2 protein does significantly decreases the cell

number at higher concentrations while the JKNII inhibitor, that inhibits phosphorylation of JNK that is activated during cellular stress, results in a significant cell decrease. This decrease may result from the fact that during this experiment a concentration greater than the IC_{50} was used. From these results we conclude that of the two pathways, the PI3K and MAPK pathways, may be important for retinal ganglion cell survival when there is serum deprivation based on the fact that treatment with PD 098059 resulted in significant decrease at higher concentrations and LY 294002 resulted in significant decreases of cell population not just between serum deprived cells but also between the serum deprived cells treated with inhibitor. The AKT inhibitor really had no effect on the cells. This may indicate that the PI3K pathway may work in an AKT independent pathway due to the fact that the phosphorylation of AKT did not cause a decrease in cell survival. The MAPK pathway shows a significant decrease when the concentrations near the IC_{50} of MEK2. This shows that MEK2 may be more important to the survival of serum deprived retinal ganglion cells. The cells treated with JNK inhibitor did have a significant decrease as shown by the experiments but this may be due to the fact that a concentration larger than the IC_{50} was used.

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CHAPTER 5

Conclusions

Glaucoma is characterized by an increase in intra-ocular pressure. This leads to a loss of retinal ganglion cells which are responsible for transmitting visual information to the brain along the optic nerve. One hypothesis of retinal ganglion cell death is that a disruption occurs to the axonal transport along the optic nerve. This disruption consequently blocks the retrograde transport of neurotrophins leading to death of the retinal ganglion cells. Based on this, we decided to create a model that mimics the blockage of axonal transport. To do this, we deprived the retinal ganglion cells of serum to imitate the withdrawal of neurotrophins. Using this model, we attempted to determine the pathways that retinal ganglion cells utilize when they undergo apoptosis.

For this study, we used an established permanently transformed rat retinal cell line of retinal ganglion cells, RGC-5. To determine the effect of serum deprivation on RGC-5 viability, a neutral red dye uptake assay was performed which showed a 50% cell decrease with 48 hours of deprivation. To further show that serum deprivation results in apoptosis, genomic DNA laddering was performed which showed nucleosomal fragments of genomic DNA. The nuclei of the serum deprived RGC-5 cells were condensed as shown by the propidium iodide staining. These results suggest that serum deprivation results in apoptosis of RGC-5 cells.

Based on these findings we attempted to determine the exact pathway that cells use when they undergo apoptosis. When cells were deprived of serum for 48-72 hours there was an increase in bax protein levels with a decrease in bcl-2 protein levels as shown by immunoblot analysis. As expected, there was also an up-regulation of bax mRNA with a simultaneous down-regulation of bcl-2 mRNA. A densitometry of the RT-PCR products showed a significant decrease in the ratio of bcl-2/bax in serum deprived RGC-5 cells.

Serum deprivation also resulted in a time dependent increase in caspases 3, 8, and 9, protein levels as shown by immunoblot analysis. In addition to the caspases, death receptors 3 and 4 were studied to determine if an alternate apoptotic signaling pathway could play a role in RGC-5 apoptosis. With immunoblot analysis, there was an increase in the DR-3 and DR-4 levels present in the serum deprived cells. In addition to caspase and death receptor up-regulation there was also considerable release of cytochrome-c from the mitochondria of serum deprived cells into the cytoplasm. The serum deprived cells also showed signs of disruption of the mitochondrial membrane, which was expected since mitochondrial disruption was one of the main steps of apoptosis.

During this study of apoptosis, we began looking at survival pathways that may help to protect retinal ganglion cells. Since phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways were known to be important to neurons we looked at their role in RGC-5 survival. Treatment of RGC-5 cells with PD 098059, a MEK 1/2 inhibitor, didn't show a significant cell loss at concentrations that blocked MEK1, but there was a significant decrease when concentrations that inhibited MEK2 were used. When the cells were treated with LY 294002, a PI3K inhibitor, there

was a dose dependent decrease that resulted. Treatment with an AKT inhibitor, which is activated later in the pathway didn't show any significant cell loss. The decrease of cell number with LY 294002 suggests that the PI3K pathway is important but that it may be AKT independent as shown by the fact that AKT inhibitors have no effect on the cell population.

Based on these results, we believe that the mitochondrial pathway is utilized during retinal ganglion cell apoptosis, but that there also is some involvement of the extrinsic death receptor mediated pathway as evidenced by the upregulation of caspase 8 and death receptor 3 and 4. In addition to these pathways, the PI3K pathway may be important to retinal ganglion cell survival as evidenced by blocking of PI3K which causes a significant decrease in cell number. We believe that the absence of trophic factors leads to the cells not activating the PI3K pathway. This can lead to bad, a bcl-2 family proapototic protein, remaining unphosphorylated which allows it to bind with bax. These two proteins can translocate to the mitochondria causing the disruption of the mitochondrial membrane and activating the mitochondrial apoptotic pathway. With this increase in mitochondrial membrane permeability, cytochrome-c is released which binds procaspase-9 activating the apoptotic cascade. The extrinsic pathway is activated by death signals which act through caspase 8. Caspase 8 then acts on caspase 3 and this commits the cell to death. In addition to this, the mitochondrial pathway can also act on the extrinsic pathway by activation caspase 3, which then causes activation of caspase 6 which can upregulate the expression of caspase 8. This leads to the conclusion that there is some amount of crosstalk that may be occurring between the pathways.

In conclusion, this study suggests that mitochondrial and extrinsic pathways are important during apoptosis of retinal ganglion cells deprived of trophic support. There is also evidence that the PI3K pathway may be important to the survival of retinal ganglion cells as shown by the preliminary data.