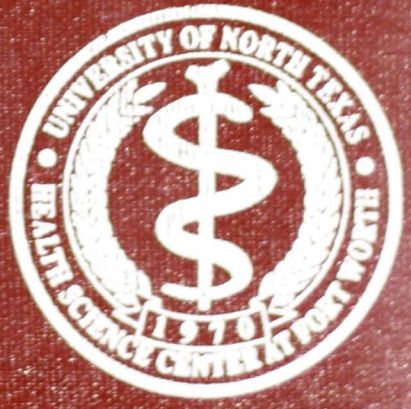
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Photoreceptor cell death mediated by programmed cell death pathways is responsible for many disease states of the retina, which result in vision loss. Examples of this include retinal dystrophies and age-related macular degeneration. Correspondingly, the understanding of programed cell death, or apoptosis, in these cells is important in the formulation of preventative and treatment options. The goals of this dissertation are to characterize a suitable in vitro photoreceptor cell model and explore the molecular mechanisms resulting in apoptotic cell death secondary to an oxidative cell death paradigm. Means of interrupting the cell death process were also investigated.

An immortalized clonal mouse retinal cell line was shown to express photoreceptor-specific genes and proteins by RT-PCR amplification, Western blot analysis, and immunocytochemical localization. Exposing these cultured cells to visible light resulted in oxidative stress, as exhibited by elevated malonyldialdehyde and reduced glutathione levels, as well light exposure-dependent apoptosis. Apoptosis was shown using multiple techniques which identified fragmentation of chromosomal DNA, a key finding in the apoptotic cell death process.

Molecular regulators of apoptotic cell death, including bcl-2 family proto-oncogenes and the nuclear transcription factor NF-κB, were found to be important in oxidative stress-induced pathogenesis of 661W photoreceptor cells. mRNA and protein

levels of the anti-apoptotic proto-oncogene bcl-2 declined following oxidative stress disturbing the balance proto-oncogene regulators and initiating the apoptotic pathway. The nuclear transcription factor NF-κB was found to be constitutionally expressed in the photoreceptor cells with its down-regulation during apoptosis. Permanent transfection of the photoreceptor cells with the bcl-2 gene imparted protection from apoptosis and sustained NF-κB levels.

The results presented in this dissertation help define the molecular mechanisms which occur during apoptosis of photoreceptor cells. Photo-oxidative stress results in programmed cell death mediated through changes in NF-κB binding activity and bcl-2 family genes. The involvement of caspase-1 in the degradation of NF-κB and the execution of apoptosis is also demonstrated. Over-expression of the proto-oncogene bcl-2 interrupts the apoptotic events, protecting against down-modulation of NF-κB binding activity and cell death. Our proposed mechanism for apoptosis in photoreceptor cells provides several points at which targeted gene expression (bcl-2 or NF-κB), or pharmaceuticals (anti-oxidants, caspase inhibitors, or calcium channel blockers) may prevent apoptotic cell death.

MECHANISMS OF PHOTORECEPTOR CELL APOPTOSIS

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CHAPTER I

INTRODUCTION TO THE STUDY

Statement of the Problem

The retina serves the function of converting visible light to electrical signals that are interpreted by visual cortex. This process of photo-electrical conduction is responsible for our ability to see. The retina, an embryologic extension of the brain, is made up of three cell types: neurons, pigmented epithelial cells, and support cells. The outermost neuronal layer of the retina is made up of rod and cone photoreceptor cells responsible for the initial conversion of photic potential to electrical potential. In rod photoreceptors light energy activates rhodopsin via isomerization of 11-cis-retinol, initiating a catalytic pathway resulting in hyperpolarization of the photoreceptor membrane (1). The end result of this process is inhibition of photoreceptor neurotransmitter release at the synapse with bipolar cells, which is the first step in the visual transduction cascade. Any pathologic event altering the function of the photoreceptor cells has the potential to interfere with visual processing.

Retinal degeneration is a general term used to describe pathologic events resulting in altered retinal and photoreceptor function. Included in this diverse group of diseases are hereditary syndromes such as retinitis pigmentosa and cone/rod dystrophy, as well as

age-related changes including macular degeneration (ARMD). In the United States ARMD is the primary cause of blindness in individuals over the age of 45 years, while diabetic retinopathy accounts for most cases of blindness in those under 45 years of age (2). Retinitis pigmentosa is the leading cause of inherited blindness and includes disease of the retinal pigment epithelium and choroid, as well as the photoreceptors.

Photoreceptor Cells

Photoreceptors cells are modified neurons consisting of an inner and outer segment linked by connecting cilium (3). The inner segment is the cell body while the outer segment contains the light absorbing visual pigments. The different pigments are responsible for absorbing light at different wavelengths. There are three types of cone photoreceptors, each with a unique pigment, which reside in the central foveal region and are responsible for color vision. The rod photoreceptor cells contain only one visual pigment, rhodopsin, and are located in the peripheral region of the retina. Rod photoreceptor cells have a low threshold for stimulation and thus are primarily for night and peripheral vision. All visual pigments consist of a retinal and opsin component which act to absorb light and initiate the visual transduction cycle (3). Other proteins important to visual transduction include the G protein transducin, phosphodiesterase, α and β cyclic guanosine monophosphate, and peripherin-RDS. As described later, mutations of any of these proteins may lead to retinal degeneration.

Important to the investigation of photoreceptor involvement in retinal disease is the availability of appropriate cell lines. Animal models have been used extensively in the study of retinal degenerations. A major difficulty in the use of animal models or whole retinal extracts in the study of photoreceptor specific disease is the confounding influence of other retinal cell types. Immortalized cell lines have provided a valuable means of studying both normal and pathologic processes in the retina. Immortalized cell lines currently exist for several retinal cell types including Muller cells (4), corneal endothelial cells (5-8), and retinal pigment epithelium cells (9, 10). At present the Y-79, WERI-Rb, and other related human retinoblastoma cell lines are the only immortalized cell lines available for study of photoreceptors. Initially it was thought that the Y-79 cells were of cone cell origin (11), but more recently these cells were shown to express rod opsin, rod transducin, rod α-phosphodiesterase and recoverin (12, 13). Primary retinal cultures have been created from several mammalian donor retinas, including humans (14, 15). These types of cultures, in addition to being tedious in preparation, are not adequate for some types of experimentation due to their heterogeneity and limited life span. Moreover, some cell types, such as the Y79 cells, are difficult to grow in monolayer. At the time of this dissertation there existed a need for additional photoreceptor cell lines which are homogeneous, passagable, and easily grown as a monolayer using standard tissue culture techniques.

Retinitis Pigmentosa

Retinitis pigmentosa is a syndrome of photoreceptor degeneration with multiple forms. The disease most commonly has a genetic origin with symptoms beginning in late childhood or early adulthood. During childhood there are generally no indications of disease except in the presence of a larger congenital abnormality, such as Usher's syndrome, Bardet-Biedl syndrome, juvenile Leber's amaurosis, or abetalipoproteinemia. One of the earliest manifestations of retinitis pigmentosa is a deficit in night vision. Later in the disease process there is a gradual loss of peripheral vision, resulting in a "tunnel" visual field. Ultimately, there may be involvement of central vision as cone photoreceptors are also affected by the degenerating rods. The endpoint of this progressive disease is often total blindness. Ophthalmologic examination may reveal bone-spicule pigmentation and narrowed arterioles, in addition to altered electroretinographic studies. The two primary forms of retinitis pigmentosa, rod-cone degeneration and cone-rod degeneration, are similar in presentation with the differentiation being made through their unique electroretinographic patterns. A randomized trial of affected individuals taking oral vitamin A supplementation demonstrated the course of retinal degeneration to be slowed in comparison to those who did not take supplements, as evidenced by cone ERG amplitudes (16). A problem with this study is that visual acuity is not a measured outcome and the actual impact of vitamin A in preventing the progression of blindness is not shown. Unfortunately, there are currently no treatments shown to prevent the full progression of retinitis pigmentosa.

The inheritance of retinitis pigmentosa may be in an autosomal dominant, autosomal recessive, or X-linked pattern. Advances in molecular biology have allowed researchers to identify many mutations responsible for the disease. Thus far it appears that mutations in photoreceptor specific proteins are primarily responsible. The phototransduction pigment rhodopsin has been found to have the greatest number of different mutations leading to retinitis pigmentosa, although mutations in α cyclic guanosine monophosphate, β cyclic guanosine monophosphate, and peripherin-RDS have also been found. Table 1 contains a more extensive review of retinal degenerative diseases, their mutations, and proteins affected. The consideration that multiple genotypes lead to a common phenotype, photoreceptor degeneration, suggests a common pathogenesis, a concept first proposed by Alder (17). Since that time it has been discovered that a programmed cell death sequence, apoptosis, is responsible for the photoreceptor cell death. One common finding between the various forms of retinitis pigmentosa is that photoreceptor loss is via apoptosis (18-21).

Necrosis and Apoptosis

Cells are thought to undergo cell death by one of two general mechanisms, necrosis or apoptosis (22). Necrosis is an unregulated cell death process in response to cellular injury or cytotoxic chemical exposure. The cell and mitochondrial contents swell resulting in rupture of cell membranes. Release of cytoplasmic contents into surrounding tissue often results in inflammatory reaction, a hallmark of necrosis. In contrast,

apoptosis, or programmed cell death, refers to a cellular program in which a cell "commits suicide". Apoptosis is regulated by the cell's machinery, enabling removal without excess disruption of surrounding tissue. During normal development apoptosis allows the clean and efficient removal of unwanted cells from surrounding tissue. Examples of this include elimination of cells between developing digits (23) and the creation of a lumen from solid tissue (24). Additionally, apoptosis is important in removing damaged cells (i.e. sloughing of epithelial tissue) as well as immunological functions. Apoptosis also occurs in many disease states including Alzheimer's disease (25), Hodgkin's disease (26), acquired immuno-deficiency syndrome (AIDS) (27), and graft versus host transplant rejection (28). During apoptosis a cell undergoes characteristic morphologic changes including loss of membrane attachment and symmetry, condensation of nucleus, and internucleosomal cleavage of DNA. The fragmentation of the cell into "apoptotic bodies" allows for elimination of cellular remains by phagocytosis.

Apoptosis in the Retina

While apoptosis of photoreceptor cells is an integral component of the pathophysiology of retinal degenerations there is a period during development in which apoptosis in the retina is a normal finding. The role of a controlled cell-death pathway during development, as first described by Glucksmann (29), is for elimination of unneeded cells (phylogenic apoptosis), to control a cell population (histiogenic

apoptosis), and for the molding of structures (morphogenic apoptosis). Earlier Glucksmann saw changes in the number of cells of the developing tadpole (30). Many years would pass before Kerr, Wyllie, and Currie first coined the term apoptosis as a description for the cell death pattern seen in development and disease (31). Since that time apoptosis has been found to be a critically important process in the developing retinal tissue as well as in the pathogenesis of the retina. In the developing retina of vertebrates cell death has been shown in retinal ganglion cells, retinal pigment epithelium, and photoreceptors (32-35). Apoptosis in the retina is not limited to development as evidenced by its involvement in many retinal pathologies. In particular, retinitis pigmentosa in both humans and animal models has been found to occur via apoptosis (18-21). Apoptosis has also been found to occur in age-related macular degeneration (ARMD), retinal detachment, and retinal light damage (36, 37). Essential to the understanding of retinal development and treatment of retinal disease is the knowledge of biochemical pathways and regulation of apoptosis.

Initiation of Apoptosis

Considerable advances have been made in understanding the early events of programmed cell death (PCD). Early theories suspected the nucleus to be the initiation site of the apoptotic cascade by virtue of the structural changes seen during apoptosis (38). Currently there is more knowledge pertaining to the mitochondrial changes which occur prior to chromatin condensation in the nucleus. Among most of the programmed

cell death models, whereby an initial insult consigns a cell to death, there exists the common phenomena of decreased inner mitochondrial transmembrane potential (delta psi) and opening of mitochondrial permeability transition pores (39-44). A shift of cytochrome c from the mitochondria to the cytoplasm has been shown to correlate with decreased delta psi and result in the cytosolic execution phase of apoptosis, including caspase activation (45-47). It is hypothesized that this early PCD event interrupts the mitochondrial electron flow and results in increased levels of reactive oxygen species (ROS). ROS were once recognized only for their involvement in necrotic cell death events; however, recent studies now suggest a role of ROS in the modulation of apoptotic events. Two important nuclear transcription factors responsible for the induction of apoptotic gene expression, AP-1 and NF-κB, are known to be activated by ROS (48). The implication of ROS in programmed cell death pathways suggests that anti-oxidants may attenuate or block the cell death signals. Wong and colleagues demonstrated the ability of mangenous superoxide dismutase, a potent antioxidant, to block tumor necrosis factor alpha (TNF-α) induced apoptosis (49). Superoxide dismutase was also found to delay neuronal apoptosis caused by nerve growth factor withdrawal (50). In the retina, prolonged exposure to visible light results in the production of ROS and apoptosis of photoreceptor cells (51). The importance of retinal light damage models in the study of photoreceptor apoptosis will be discussed later.

Regulation of Apoptosis – Bcl-2 Family Genes

The two most pervasive genes in control of apoptosis are p53 and bcl-2 oncogenes (52). Alterations in the expression of the p53 tumor suppressor gene, whether by mutation or protein inhibition, are by far the largest event in producing human cancers (53). p53 induction following cellular damage allows for growth arrest (for DNA repair) or programmed cell death if the insult is severe enough. When the function of p53 is impaired the cell loses the ability to control growth, thus allowing tumor formation. The Bcl-2 gene has a role opposite to that of p53. Bcl-2 is known as an inhibitor of apoptosis. Over-expression of bcl-2 was found to be responsible for the neoplastic transformation of cells in B-cell lymphoma-2 (hence the name bcl-2) (54, 55). Over-expression of bcl-2 was found to inhibit normal cellular control allowing for tumor growth

The bcl-2 proto-oncogene is a member of a larger family of genes known to regulate apoptotis. Bcl-2 is the prototypical member, and functions as an anti-apoptotic protein with the ability to protect against a variety of physiologic and pathologic insults (56, 57). Other members of the bcl-2 family of apoptotic regulatory molecules include bax, bak, bcl-X_L, bad, and bcl-X_S. Bax, bcl-X_S, bad , and bak are classically described as a pro-apoptotic proteins, while bcl-2 and bcl-X_L have been shown to protect against apoptosis. Interaction between members of the bcl-2 family in the control of apoptosis is complex. It is postulated that bcl-2 and bcl-X_L combine with homodimers of bax, forming heterodimers which interrupt apoptotic signaling (58, 59). A recent in vivo study of synovial cells isolated from rheumatoid arthritis patients found an association

between apoptosis and increased bax/bcl-2 ratio (60). Although this theory is well supported, it has been found that the bcl-2/bax interaction is not the only explanation for regulation of bcl-2 activity. It has been reported that bcl-X_L, acting independently of bax, is able to prevent apoptosis (61).

An established mechanism for bcl-2's anti-apoptotic function is via interaction with antioxidant pathways. Bcl-2 is known to localize to the outer mitochondrial membrane, as well as the endoplasmic reticulum and nuclear membrane (62, 63). The presence of bcl-2 at the sites of free radical generation correlates with its ability to prevent oxidative cellular insults. Hockenbery et al. found that bcl-2 is able to suppress lipid peroxidation and apoptotic cell death induced by hydrogen peroxide treatment (64). Similarly, bcl-2 expression in neuroblastoma cells significantly lowered levels of oxidized lipids following potassium cyanide treatment (65). As discussed earlier, the mitochondrial transmembrane potential is lowered in the early phase of apoptosis due to increased opening of mitochondrial pores. A key finding in explaining bcl-2 family regulation of apoptosis is the ability of these proteins to interact with mitochondrial permeability transition (MPT) pores, either facilitating or inhibiting their opening. The bcl-2 and bcl-X_L proteins are able to block MPT pore opening thus preventing the release of apoptotic proteins (66, 67). In a similar fashion the pro-apoptotic bcl-2 family member bax interacts with MPT pores promoting opening and the resultant decrease in transmembrane potential (68).

Regulation of Apoptosis –Nuclear Factor Kappa B

Nuclear factor-kB (NF-kB) is a transcription factor that has recently been implicated in the regulatory pathways of apoptosis. Subunits of NF-κB, p50 and p65 (RelA), form a heterodimer which exhibits DNA binding potential. NF-κB typically resides in the cytoplasm bound to an inhibitory subunit, IκBα or IκBβ, which prevents its translocation to the nucleus (69-71). The inhibitory constraint upon NF-κB is released by phosphorylation of IκB, which may be induced by cellular exposure to lipopolysaccharides, interleukin-1, tumor necrosis factor-α, or other inducers. NF-κB is constitutively active (i.e. active binding form present in nucleus) in several cell lines including B Cells (72, 73), neurons (74), synovial cells (60), and photoreceptor cells (75). NF-kB has been found to be an integral part of a cell's ability to survive an apoptotic insult (76). Beg and Baltimore demonstrated that mouse fibroblasts deficient in the RelA subunit of NF-κB had reduced viability following TNF-α treatment (76).

Current studies suggest that bcl-2 and NF-κB may be regulatory factors in a common apoptotic pathway. A potential role of NF-κB p50-p50 homodimer in the anti-apoptotic function of bcl-2 has been proposed by Ivanov *et al.* (77). They showed that over-expression of bcl-2 in a T-cell line resulted in increased NF-κB (p50 dimer) binding activity, promoting cell survival. Mandal *et al.* found that over-expression of bcl-2 restored NF-κB activity in HeLa cells undergoing anti-CD95 mAb-induced apoptosis (78). More recently, bcl-2 was shown to degrade the IκBα inhibitory subunit, resulting in NF-κB activation and rescue of neonatal ventricular myocytes from TNF-α induced

apoptosis (79). These results suggest that one mechanism of bcl-2 protection is as an upstream activator of NF-κB. Several reports suggest an alternative interaction between bcl-2 and NF-κB in which bcl-2 related genes act as transcriptional targets for NF-κB. For instance, Dixon *et al.*, observed that the five prime regulatory region of bcl-X contains a consensus sequence for NF-κB which, when bound, results in increased expression of the pro-apoptotic gene bcl- X_S (80). Similarly, NF-κB binding to a regulatory site in the promotor sequence of a pro-survival bcl-2 homolog, Bfl-1/A1, was found to provide protection against apoptosis (81). Currently, there is no consensus on how bcl-2 family genes and NF-κB interplay to alter the apoptotic status of cells. Since NF-κB has been found to effect pro and anti-apoptotic pathways in different cell lines, it is likely that the relationship between bcl-2 and NF-κB may be cell type specific.

Oxidative Stress and Light Damage

Oxidative stress refers to the toxic biological consequences of reactive oxygen species (ROS) formed spontaneously by biological processes (82). ROS formation is dependent upon the reduction of molecular oxygen via the gain of electrons, or realignment of electron spins. Oxygen serves an important biological role as the terminal electron acceptor during cellular respiration, but these reactions also result in the production of reduced oxygen species (83, 84). In addition to normal cellular respiratory

production of ROS, electron leakage from membranes and inadequately coupled reactions are also known to play a role (85).

ROS are often designated as free radicals for the unpaired electrons that allow them to act as powerful oxidizing agents. Formation of ROS follows a stepwise reduction of molecular oxygen via the addition of electrons as demonstrated in the reactions below (86):

$O^2 + e^- + H^+ \rightarrow HO_2°$ (hydroperoxyl radical)

$HO_2° \rightarrow H^+ + O_2^{°-}$ (superoxide anion)

$O_2^{°-} + 2\,H^+ + e^- \rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + e^- \rightarrow OH^- + °OH$ (hydroxyl radical)

Superoxide anion, peroxide anion, and the hydroxyl radical are all highly reactive molecules capable of oxidizing biological membrane components including phospholipids, cerebrosides, short and long chain saturated, unsaturated, and polyunsaturated fatty acids (87). ROS have been found to exert their toxic effects through oxidation of thiols, depletion of tissue glutathione, impairment of ATP, NADH, and NADPH production, inhibition of calcium transport, oxidation of cytochromes, and DNA cleavage (88). Obviously, a paradox exists for aerobic organisms that depend on oxygen for cellular respiration, but are also susceptible to the damaging effects of ROS.

Necessary for cellular survival in an environment which may accumulate pro-oxidant molecules are substances that act to inhibit oxidation. These substances, known as anti-oxidants, are synthesized in the body, or acquired through dietary sources. Specific anti-oxidants produced by cells to offset changes in the redox environment

include catalase, superoxide dismutase, uric acid, selenium dependent glutathione peroxidase, and the transition metal-binding proteins transferrin and ceruloplasmin (89, 90). Dietary anti-oxidants include ascorbic acid (vitamin C), tocopherols (vitamin E), and carotenoids. Ascorbic acids role as an anti-oxidant rests in its ability to react with hydrogen peroxide, hydoxyl radical, peroxyl radical, and superoxide anion to form a semidehydroascorbate radical and dehydroascorbate (91-93). Tocopherols, such as vitamin E, have the potential to prevent lipid peroxidation via a reaction involving donation of hydrogen to peroxyl radicals (94). The use of exogenous anti-oxidants has been implemented to experimentally interrupt oxidative stress in cells. These molecules, which also interact with ROS to inhibit their oxidative potential, include N-acetylcysteine (NAC), dimethylthiourea (DMTU), and mannitol. NAC is de-acetylated in vivo to cysteine, a precursor of the biological anti-oxidant glutathione (95). Mannitol and DMTU exert their anti-oxidant effect through specific reactions involving the inactivation of hydroxyl radicals.

ROS have been shown to play a role of the pathogenesis of a variety of disease, including autoimmune disorders (96), infectious processes (97), cancer (98), AIDS (99). Additionally, oxidative stress and ROS generation are involved in retinal diseases such as, age-related macular degeneration (100), light damage (101), and photoreceptor dystrophies (102).

The use of light to initiate retinal damage has been used for over thirty years. Noel and colleagues provided the initial work showing that light exposure can cause retinal damage in vivo (103). Continued support for light-induced retinal cell death has

been provided by a number of researchers (104-106). Further, the mechanism of light-induced retinal cell death has been shown to be via apoptosis (37, 107-110). Specific cellular changes invoked by light exposure have been shown to include oxidative stress within photoreceptor cells and a subsequent increase in lipid hydroperoxides (111-113). Due to retinal vulnerability to lipid peroxidation, the increased lipid peroxides produced during light exposure lead to retinal damage (114, 115). A rat model for accelerated aging of the retina also found an increase in lipid peroxides, as well as decreased glutathione(GSH) levels and increased superoxide dismutase(SOD) activity (116).

It is clear that light damage results in oxidative changes and damage to the retina, but the exact mechanism by which this occurs is not known. The retina exhibits elevated levels of many anti-oxidants, including vitamin C, vitamin E, GSH, and SOD (117-119). The elevated levels of these anti-oxidants suggests an evolutionary mechanism to protect an organ which is predisposed to oxidative stress. Further support that light damage results in oxidative damage to the retina comes from the finding that intraperitoneal injections of the antioxidant dimethylthiourea prior to light exposure results in partial protection (120, 121). The free radical scavenger WR-77913 provides similar protection from light damage (63). In Chapter 2 we have exhibited protection from light exposure-induced oxidative changes by GSH and Malonyldialdehyde (MDA) levels. Upon light exposure the photoreceptor cells exhibited elevated MDA levels providing evidence of lipid peroxidation of membranes, and decreased GSH levels suggesting a response to oxidative stress. These findings support our use of fluorescent visible light as a means of creating oxidative damage in the 661W cell line.

The proto-oncogene Bcl-2, a 26 kDa integral membrane bound protein, has been of interest to scientists due to its ability to prevent programmed cell death (122, 123). More important to the aims of this dissertation is the mechanism of action for Bcl-2. Since Bcl-2 is found in mitochondrial membranes it has been proposed that it plays a role in oxidative phosphorylation as well as the prevention of formation of reactive oxygen intermediates (ROI) (63, 122, 123). Over expression of Bcl-2 in the retinas of transgenic mice was shown to protect retinas from light damage (124), although one such study postulates that the protection is due to decreased rhodopsin levels (125). Obviously there needs to be additional studies to determine the role Bcl-2 plays in oxidative damage. In the third specific aim I propose that elevated expression of Bcl-2 in photoreceptors will protect them from both oxidative changes, NF-κB degradation, and apoptosis.

Caspases

Caspases are a family of proteases known to play an important role in the regulation of apoptosis. The first caspase to be decribed, interleukin-1β converting enzyme (ICE) or caspase-1, is a novel cysteine protease involved in the conversion of the pro-interleukin-1β to the mature interleukin-1β (126). While caspase-1 is involved in the activation of inflammatory cytokines (along with caspase 4,5, and 11), most caspases are involved in the initiation or activation of apoptosis. Caspases participate in the execution of apoptosis by participating in a cascade of protein activation involving cleavage of protein substrates. Upstream stimuli initiate the caspase signaling, which culminates in

cell death. Like other mediators of apoptosis, caspases appear to be conserved throughout evolution. Caspase-3 is the human homolog of the ced-3 gene of C-elegans that has been shown to be required for apoptosis (127, 128).

Throughout the apoptotic pathway caspases serve to activate the effectors of cell death as well as participating in the disassembly of the cell directly. Two caspases, caspase 8 and 9, are known to activate the effectors of apoptosis in response to an upstream signal. It is suggested that caspase-9 is activated following cytotoxic stimuli (129, 130), while caspase-8 is cleaved following receptor-mediated apoptotic signaling (131). Other members of the caspase family, including caspase-3, caspase-6, and caspase-7, do not appear to respond to initiating signals, but instead respond as effectors of apoptosis (132, 133). Regulation of caspase activity is important in preventing inadvertent damage to the cell. Inhibition of caspase activity is one means by which uncontrolled cell death is prevented. A specific inhibitor of capase-8 exists. FADD-like ICE inhibitory protein (FLIP) shares homology with caspase-8, but has no proteolytic activity and prevents its activation (134). Other inhibitors of caspases are known to exist, including the apoptosis repressor with caspase recruitment domain (ARC) (135). Bcl-2 is also thought to interfere with caspase activation primarily by altering cytochrome c, which is necessary for caspase-9 activity (136). Inhibition of ICE activity using cytokine response modifier A (CrmA) has a similar effect in preventing ICE induced apoptosis (137). Figure 1 illustrates our proposed molecular mechanisms of photoreceptor cell apoptosis following light-induced oxidative stress.

Specific Aims

Although the primary mutations that result in a variety of retinal degenerations, including both human forms and animal models of retinitis pigmentosa, are known they fail to explain the common end phenotype, apoptosis of photoreceptor cells. This suggests the existence of a common pathway for photoreceptor cell death in retinal degenerations. The long term goal of my research is to explain the molecular mechanisms of photoreceptor cell death in retinal dystrophies. The objective of this proposal is to determine the role of oxidative damage in light induced photoreceptor cell death paradigm. **We will test the hypothesis that light exposure results in photo-oxidative stress and apoptosis of 661W photoreceptor cells.**

The following specific aims are proposed to test the hypothesis:

Specific Aim 1. The SV 40 immortalized cell line 661W expresses a photoreceptor phenotype. The 661W cell line, cloned from the retinal tumor of a transgenic mouse expressing SV 40, will be used as a tissue culture model for photoreceptor cells in the proposed research. In order to establish the 661W cell's photoreceptor origin, we will characterize them by immunocytochemistry and western immunoblotting, using photoreceptor specific antibodies, and by RT-PCR, using photoreceptor specific primers. Protein and total RNA from BALB/c mouse retina will be used for positive controls, while Madin Derby canine kidney (MDCK) and baboon lung

will be used as negative controls. The analysis of Muller and retinal ganglion cell type specific markers, including GFAP and Thy-1, will further demonstrate the homogeneity of the cell line. Additionally, morphologic and growth characteristics will be evaluated.

Specific Aim 2. Visible light treatment of photoreceptor cells results in oxidative stress, decreased expression of NF-kB, and cell death via apoptosis. The ability of visible fluorescent light to cause oxidative damage in 661W cells will be demonstrated. The analysis of malonyldialdehyde (MDA) levels and GSH/GSSH ratios will provide indices of cell membrane lipid peroxidation and cellular reducing environment changes due to exposure to visible light. Having established light induced oxidative changes within the 661W cells, we will examine if there is a concurrent loss of viability and/or apoptotic cell death using Formazon viability assay, DNA fragmentation, and TUNEL assay for apoptosis. We also propose that the oxidative changes occurring within light exposed 661W cells results in the decreased cellular levels and binding of NF-κB, adversely affecting the cells ability to survive these changes. NF-κB protein levels and binding will be assessed using immunoblot analysis (for the NF-κB proteins, p65, p50, and IκBα) and electrophoretic mobility shift assays (EMSA) (with the consensus binding sequence for NF-κB), respectively. To further establish the oxidative stress paradigm as the cause of the decreased viability, NF-κB cellular changes, and apoptosis in the 661W cells, light exposure experiments will be repeated using a battery of anti-oxidants. To begin to determine the mechanisms by which oxidative stresses result in apoptosis of 661W cells, we will analyze the expression of proto-oncogenes

(bcl-2, bcl-X_S, bcl-X_L, bax, bak, and bad) known to control cell survival and death. The proto-oncogene mRNA levels will be quantitated by ribonuclease protection assay (RPA) using specific primers for each.

Specific Aim 3. Over-expression of the bcl-2 proto-oncogene results in protection from oxidative damage. Since bcl-2 has been implicated in protecting cells from oxidative stress induced apoptosis, an important goal of this proposal is also to explain the role that bcl-2 plays in the photoreceptor cellular response to oxidative stress. Levels of bcl-2 in both dark and light exposed 661W cells will be quantitated by SDS-PAGE western blotting, RT-PCR, and immunocytochemistry. To evaluate a possible protective role of Bcl-2 in cellular response to oxidative changes, the 661W photoreceptor cells will be permanently transfected with the pSFFVneo-Bcl-2 plasmid containing the full length human bcl-2 cDNA. Bcl-2 over-expression will be quantitated by SDS-PAGE western blotting, RPA, and immunocytochemistry. Morphologic analysis and growth characteristic assessment will also be performed on the permanently transfected cells. EMSAs, formazon viability assays, and the TUNEL test for apoptosis will be used to assess the role Bcl-2 over-expression in protecting against oxidative damage.

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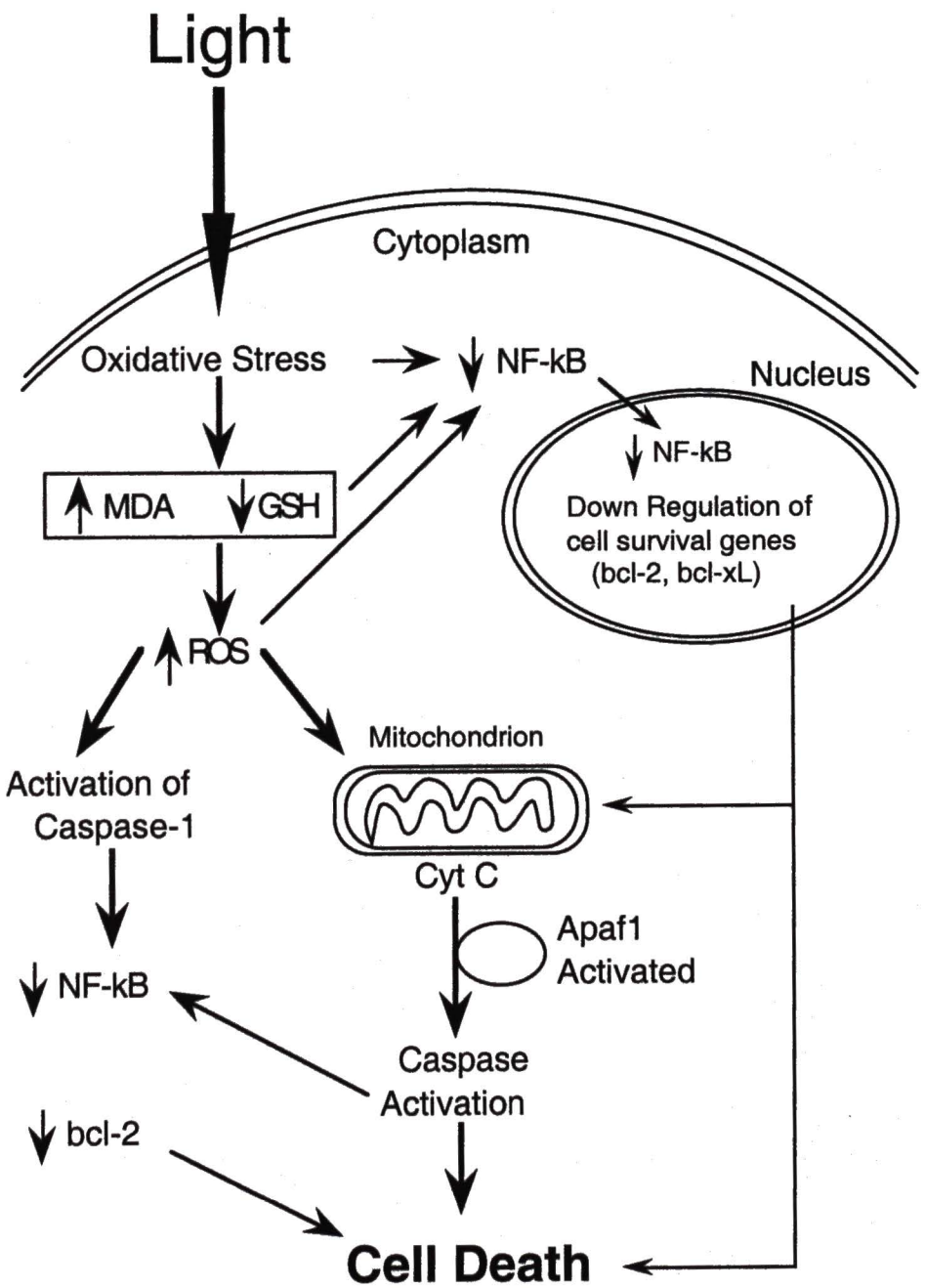
Table 1. Human hereditary retinal degenerations and associated proteins and genes.

Disease	Protein	Location
Usher syndrome type 2a	usherin	1g41
recessive achromatopsia	cone photoreceptor cGMP-gated cation channel	2q11
Oguchi disease	arrestin	2q37.1
congenital stationary night blindness	rod transducin	3p22
dominant and recessive retinitis pigmentosa	rhodopsin	3q21-q24
recessive retinitis pigmentosa	rod cGMP phosphodiesterase	4p16.3
Recessive retinitis pigmentosa	rod cGMP-gated channel	4p12-cen
Recessive abetalipoproteinemia	microsomal triglyceride transfer protein	4q24
recessive retinitis pigmentosa	cGMP phosphodiesterase alpha subunit	5q31.2-q34
dominant retinitis pigmentosa	peripherin/RDS	6p21.2-cen
dominant cone dystrophy	guanylate cyclase activating protein 1A	6p21.1
refsum disease	peroxisome biogenesis factor 1	7q21-q22
dominant tritanopia	blue cone opsin	7q31.3-32
dominant retinitis pigmentosa	RP1 protein	8q11-q13
recessive retinitis pigmentosa	RPE-retinal G protein-coupled receptor	10
recessive RPE degeneration	retinol-binding protein 4	10q24

Table 1. (continued)

Disease	Protein	Location
dominant retinitis pigmentosa	retinal outer segment membrane protein 1	11q13
dominant macular degeneration	bestrophin	11q13
Usher syndrome, type 1	myosin VIIA	11q13.5
fundus albipunctatus	11-*cis* retinol dehydrogenase 5	12q13-q14
recessive congenital stationary night blindness	rhodopsin kinase	13q34
dominant retinitis pigmentosa	neural retina lucine zipper	14q11.2
recessive enhanced S-cone syndrome	nuclear receptor subfamily 2 group E3	15q23
Batten disease	Batten disease protein	16p12.1
Leber congenital amaurosis	arylhydrocarbon-interacting receptor protein-like 1	17p13.1
dominant cone-rod dystrophy	retinal-specific guanylate cyclase	17p13.1
mouse recessive retinal degeneration	cGMP phosphodiesterase gamma subunit	17q21.1
dominant cone-rod dystrophy	cone-rod otx-like PR homeobox transcription factor (CRX)	19q13.3
retinoschisis	X-linked retinoschisis 1 protein	Xp22.2
Oregon eye disease	dystrophin	Xp21.2
X-linked Retinitis pigmentosa	retinitis pigmentosa GTPase regulator	Xp21.1
protanopia and macular dystrophy	red cone opsin	Xq28

Figure 1. Proposed mechanisms of oxidative stress induced apoptosis in 661W photoreceptor cells.



CHAPTER II

A SIMIAN VIRUS 40 IMMORTALIZED MOUSE RETINAL CELL LINE EXPRESSES PHOTORECEPTOR PHENOTYPE

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ABSTRACT

Purpose. The authors sought to examine an immortalized mouse retinal cell line (661W) for markers characteristic of photoreceptor cells, to validate their photoreceptor origin, and to establish these cells as a tool for retina research.

Methods. The 661W cells were cloned from retinal tumors of a transgenic mouse line expressing the Simian Virus 40 (SV-40) T-antigen under control of the human interphotoreceptor retinol binding protein (IRBP) promoter. Morphologic analysis, cell growth patterns, reverse transcription-polymerase chain reaction (RT-PCR), immunocytochemistry, and immunoblot analyses were performed to characterize these cells. Total cellular RNA and protein from mouse retinas were used as positive controls for RT-PCR and immunoblot analysis of various photoreceptor specific genes and proteins, respectively. The formation of isorhodopsin in 661W cell extracts was measured spectrophotometrically after incubating 9-cis retinal with cell membranes.

Results. Morphologic analysis of cultured 661W cells revealed cells growing as a monolayer and exhibiting processes characteristic of neuronal cells. The doubling time for the cultured cells was approximately 24 hours in medium containing 9% fetal bovine serum and 1% penicillin/streptomycin. Expression of photoreceptor specific messages for opsin, arrestin, β-phosphodiesterase (PDE), β-transducin, and rds/peripherin was shown by RT-PCR analysis. Thy-1 and glial fibrillary acidic protein (GFAP) were used as a negative controls, since Thy-1 is a marker for ganglion cells and GFAP, a marker for Muller cells. As expected, 661W cells were negative for Thy-1 as well as GFAP mRNA, shown by RT-PCR analysis. Immunocytochemistry demonstrated the expression of

arrestin, IRBP, opsin, rds/peripherin, phosducin, and β-rod transducin. The immunocytochemistry results for opsin, rds/peripherin, and phosducin were further confirmed by immunoblot analysis. Most significantly, a characteristic isorhodopsin peak was observed at 485 nm upon light bleaching of 661W cell membranes incubated with 9-cis retinal.

Conclusion. The 661W cells demonstrated cellular and biochemical characteristics consistent with photoreceptor cells. These cells also resembled neuronal cells with their spindle-like processes and should prove useful as an alternative in vitro model to study photoreceptor cell biology and disease.

INTRODUCTION

Photoreceptor cells occupy the outermost layer of the neural retina and are responsible for converting transmitted light into electrical impulses that travel to the brain via the optic nerve. There are a number of diseases which directly affect photoreceptors, such as retinitis pigmentosa (RP) and related retinal degenerations. A stable homogeneous photoreceptor cell line with an unlimited life span would be valuable for studying pathophysiologic mechanisms of various photoreceptor related diseases. A homogeneous photoreceptor cell system would also prevent the confounding influence of other retinal cell types such as glial cells or endothelial cells that are encountered while studying extracts of whole retinas.

Photoreceptor cells are terminally differentiated, specialized neuronal cells with a limited capacity for cell division. Therefore, to have an established line of photoreceptor cells it is essential to transform them, possibly with a virus. Immortalized cell lines currently exist for several retinal cell types including Muller cells[1], corneal endothelial cells[2, 3, 4, 5], and retinal pigment epithelium cells[6, 7, 8]. A cell line expressing retina specific genes, including the photoreceptor proteins IRBP and cone transducin, has also been isolated from a mouse ocular tumor[9]. In addition, Y-79[10] and WERI-Rb[11] are other immortalized human retinoblastoma cell lines available for the study of photoreceptors. Initially it was thought that the Y-79 cells were of cone cell origin[12], but more recently these cells were shown to express rod opsin, rod transducin, rod β-phosphodiesterase, and recoverin[13, 14]. Primary retinal cultures have been created from several mammalian

donor retinas, including humans[15, 16]. These types of cultures, in addition to being tedious in preparation, are not adequate for some types of experimentation due to their heterogeneity, limited cell division, and special conditions for their growth as monolayers. Thus, there exists a need for additional photoreceptor cell models which are homogeneous, passageable, and easily grown as a monolayer using standard tissue culture techniques.

Herein, we describe a mouse photoreceptor-like cell line (661W) immortalized by the expression of SV40- T antigen under control of the human IRBP promoter [17]. Cellular, molecular, and biochemical analyses show that these cells resemble mouse photoreceptors cells. They should, therefore, aid in the study of photoreceptor cell function and of diseases affecting retinal photoreceptor cells, including apoptotic mechanisms of photoreceptor cell death in various retinal dystrophies.

METHODS

Immortalization of the Mouse Photoreceptor Cells. The 661W cell line was established by expressing the viral oncoprotein simian virus 40 large tumor antigen (SV40 T-antigen) under control of the human IRBP promoter in a transgenic mouse, as described previously[17]. Briefly, a construct was prepared which contained the sequence for SV40 T-antigen driven by a 1.3 kb fragment containing the human IRBP promoter. This construct, termed HIT1, was used to create a transgenic mouse line. Expression of T-antigen under the IRBP promoter resulted in bilateral retinal tumors. A clonal cell line was isolated from these tumors and given the designation 661W. Early passages of 3-15 were used in these studies. Immunocytochemical localization was performed using a SV40-T antigen specific antibody to ensure expression of the viral oncogene in retinal tumors.

Culture conditions, morphology, and growth rates of 661W cells. The 661W cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 9% fetal bovine serum, penicillin (90 units/ml), streptomycin(0.09 mg/ml), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells grown in culture were photographed using Nomarski optics with a Nikon Microphot-FXA photomicroscope (Nikon, Melville, NY).

To calculate growth rates, $1x10^4$ cells were seeded in triplicate in 35 mm tissue culture dishes for each time point. The growth medium was changed every third day thereafter. The cells were counted at six time points over a nine day period

using a Coulter Counter ZM (Coulter Electronics, Hialeah, FL). At each time point, the cells from three dishes were trypsinized, counted in triplicate, and averaged. These values were plotted as number of cells versus time of culture in days.

Reverse transcriptase polymerase chain reaction analysis. RT-PCR was performed on 661W cells, and BALB/c mouse retinas (positive control), and baboon lungs (negative control) using the specific primers for β-actin, arrestin, β-transducin, β-phosphodiesterase (PDE), rds/peripherin, opsin, Thy-1, and GFAP as described in Table 1. The primers were designed using Entrez Nucleotide QUERY (NIH, Bethesda, MD) and Oligo 4 (National Biosciences, Inc., Plymouth, MN), a primer analysis software program. Total RNA from 661W cells, and BALB/c mouse retinas was isolated using the RNAzol B RNA isolation kit (Biotecx, Houston, TX). cDNA synthesis was performed as described[18]. Initially, to reduce the secondary structure, the RNA (20 μg) and random primers (0.75 μg, Promega Corp, Madison, WI) were added together and incubated at 85^0C for 3 min, followed by addition of 80 units of RNasin (Promega), 40 units avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 0.625 mM each of four deoxyribonucleotides, 50 mM Tris HCl, 0.75 mM potassium chloride, 10 mM dithiothreitol and 3 mM magnesium chloride. The reaction tubes were incubated at 42°C for 30 minutes followed by an incubation at 94°C for 2 minutes. The cDNA were stored at -20°C.

PCR reactions were performed in an automated DNA thermacycler (Perkin Elmer, Norwalk, CT) using optimum annealing temperatures (Table 1) as previously

described[18] using hot start PCR protocol with Taq antibody (Clontech, CA) and Taq polymerase (Promega, WI) as per the following: one initial denaturation cycle for 5 min at 94°C and 5 min at 60°C followed by 40 cycles for 2 min at 72°C, 1 min at 94°C, and 1 min at 60°C, with a final extension phase consisting of one cycle of 10 min at 72°C. Control reactions without template were included with each amplification for each pair of primers. All the test samples were amplified simultaneously with a particular primer pair, using a master mix containing all of the components in the PCR reaction. The control samples included RNA (to rule out genomic DNA contamination) or water, instead of the target cDNA. PCR products (20 µl) were run on a 1% agarose gel stained with ethidium bromide. A 100 bp ladder (Promega, Madison, WI) was included in a separate lane on each gel to determine the size of the PCR product. To establish specificity of primer amplification in the PCR reaction, products were subjected to Southern blot hybridization analysis using specific oligonucleotide or cDNA probes.

Immunocytochemical studies. 661W cells were processed for fluorescent immunocytochemical analysis of photoreceptor cell specific protein expression using the following antibodies: monoclonal H_3B_5 against IRBP (1:25 of cultured supernatant), monoclonal A_9C_{10} against arrestin (1:50 of cultured supernatant) (A kind gift of Dr. Larry Donoso), monoclonal $1D_4$ against opsin (1:100, of cultured supernatant) (Dr. Robert Molday), rabbit polyclonal anti- rds/peripherin at 1: 100 (Dr. Gabriel Travis), rabbit polyclonal anti- phosducin at 1:100 (Dr. Rehwa Lee), and rabbit polyclonal anti-β-rod transducin at 1:100 (Dr. James Hurley). 661W cells were seeded onto 12 mm circular

coverslips (Fisher Scientific, Pittsburgh, PA) and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature.

Prior to immunocytochemistry the cultured cells were permeabilized with 0.3% Triton X-100 in PBS for 2 minutes. The cultured cells were blocked with a 1:10 dilution of serum from the species of the secondary antibody in PBS for 60 minutes. Primary and secondary antibodies were diluted in 1% bovine serum albumin (fraction V, Boehringer Mannheim, Indianapolis, IN). Anti-mouse or anti-rabbit fluorescein isothiocyanate labeled (FITC) secondary antibodies at 1:100 (Sigma, St. Louis, MO) were applied following primary antibody incubations. Between primary and secondary incubations the cells were washed three times for five minutes each with 1% Triton X-100 in PBS. Following a final series of washes in PBS, the cultured cells were mounted with DABCO containing mounting medium for fluorescent secondary antibodies. The cells were photographed using a FITC filter on a Nikon Microphot-FXA photomicroscope.

Immunoblot analysis. Protein extracts from 661W cultured cells were subjected to immunoblot analysis[19, 20] using specific antibodies for opsin (1:5000), phosducin, kindly given by Dr. Rehwa Lee (1:5000), and rds/peripherin (1:5000), as described for immunocytochemistry. Total 661W cellular protein was prepared using a lysis buffer (10 mM Tris-HCl pH 7.4, 0.5% NP-40, 150 mM NaCl, 2 mM PMSF). BALB/c mouse retinas, included as positive controls, were homogenized in buffer C (10 mM HEPES pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 0.5 mM PMSF, 1 mM DTT) using a tissue homogenizer (Omni International, Waterbury, CT). Following

isolation, proteins were quantitated using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) modified from the Lowry assay. Five micrograms of total retinal protein, and 40 μg of total 661W cell protein were separated on 12% SDS polyacrylamide gels. Following transfer to nitrocellulose membranes, the binding of primary antibodies to various proteins was detected by using the appropriate peroxidase labeled secondary antibodies at 1:20,000, which in turn were detected by an enhanced chemiluminiscence technique as per the instructions of the supplier (NEN, Boston, MA).

Measurements of rhodopsin generation by association of opsin to 9-cis retinal. To study the generation of rhodopsin by 661W cells, the cells were homogenized using a polytron in 2 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 8.5% sucrose for 30 sec on ice. The homogenates were centrifuged at 35,000 rpm for 1 hour at 4°C. Supernatants were decanted in a dark room under dim red light illumination. The cell pellets were transferred to glass centrifuge tubes, washed twice with 0.25 ml of 50 mM phosphate buffer, and vortexed. To the resultant pellet, approximately 8 nmol 9-cis retinal in ethanol was added and the tubes were vortexed, then incubated at room temperature for 90 min in darkness. The contents of the tube were centrifuged at 10,500 rpm for 10 min. The supernatant was collected and 1 ml of 1% β-octyl glucopyranoside in phosphate buffer was added, followed by vortexing, and incubation in the dark at 4°C for 90 min. The contents of the tubes were centrifuged at 11,500 rpm for 10 min and 20 μl of 1M hydroxylamine (pH 7.0) was added to the supernatant and mixed. Difference spectra were obtained on a SLM Aminco DW-2C spectrophotometer from samples

before and after bleaching with white light. Rat rod outer segments were prepared as desribed[21] . Bleached rod outer segments were used as a positive control in parallel experiments.

RESULTS

Growth characteristics and morphology of 661W cells. These cells have been maintained in culture for over 60 passages with no apparent slowing of mitotic activity or loss of photoreceptor specific markers. Morphological characterization showed the cells growing as a monolayer and exhibiting processes (Figure 1). Upon attachment to growth surface, the 661 cells appear elongated with a unit ratio of nuclear to cytoplasm (white arrow head, Figure 1A). Shortly after, they begin to project elongated extensions and once established on growth surface, cytoplasm increases in size and cells flatten out (black arrow, Figure 1A). However, frequently multinucleated cells are observed (white arrow, Figure 1A). At this stage cells also start to touch each other through the projected extensions and often multiple cells are connected to each other (white arrow heads, Figure 1B). These cellular connections are best viewed in a confluent culture undergoing trypsinization with a diluted solution of trypsin (white arrow heads, Figure 1C). The 661W cells grew to confluence in culture and proliferated rapidly. A slight lag phase of about 2 days and a log phase extending from days 2-8 characterized the growth of these cells. Following logarithmic growth, the cells reached a plateau at day 9. Based on the growth curve (Figure 2), 661W cells were shown to have a doubling rate of 1.1 days (Figure 2).

RT-PCR analysis of expression of photoreceptor specific messages. RT-PCR allowed us to analyze 661W cells for the expression of the photoreceptor specific messages using

the specific sense and anti-sense primers for β-actin, arrestin, β-transducin, β-phosphodiesterase(PDE), rds/peripherin, and opsin. The messages for arrestin, β-transducin, phosphodiesterase (PDE), rds/peripherin, and opsin were all expressed by 661W cells and BALB/c mouse retinas (positive control) and they were not observed for baboon lungs used as a negative contol (Figure 3). To further confirm the photoreceptor-like characteristics, these cells were tested for the expression of GFAP and Thy-1, since Thy-1 is a marker for ganglion cells and GFAP is a marker for Muller cells. As expected, 661W cells failed to show any amplification for Thy-1 as well as GFAP mRNA. β-actin mRNA expression was used as a control for confirmation of cDNA synthesis (Figure 3). A faint band for arrestin was observed in baboon lungs (Figure 3, F), which could be due to homology of PCR product with β-arrestin in the lung tissue. The specificity of the PCR amplification products were verified by Southern blotting using either the oligonucleotides containg part of the amplified sequence or the cDNA. Further, it is obvious from the results that the expression of these photoreceptor messages is considerably low, which could be due to the fact that it is not a quantitative PCR. On the other hand the RT-PCR analysis may reflect the true levels of these messages, because the cells in culture condition may not be synthesizing these proteins to the same extent as in vivo since they are not undergoing disc shedding and renewal. Further differentiation may be necessary to induce the expression of these messages.

Immunological expression of photoreceptor proteins. 661W cells were immunoreactive for known markers of photoreceptors such as arrestin, opsin, IRBP, β-

rod transducin, phosducin, and rds/peripherin (Figure 4). Immunocytochemistry controls (non-immune immunoglobulins for polyclonal antibodies or cultured supernates for mAbs, and omission of primary antibodies) failed to exhibit labeling for the photoreceptor proteins. The labeling patterns were cytoplasmic for arrestin, transducin, and IRBP, whereas opsin, phosducin, and rds/peripherin labeling pattern was membrane associated as well as in the cytoplasm, probably due to association of these proteins with endoplasmic reticulum. The labeling for rds/peripherin was also observed in the nucleus of 661W cells. The granular appearance of opsin immunostaining in the cytoplasm may be due to the presence of vesicles involved in vesicular opsin transport. Nevertheless, the aberrant pattern of labeling by immunocytochemistry is not readily explainable in these cells. It could be due to the fact that these cells are transformed and are in culture, lacking adjoining retinal pigment epithelium or Muller cells. To assess further, it may be necessary to study these proteins by electron microscopy immunocytochemistry.

To further ascertain the photoreceptor nature of 661W cells, expression of some photoreceptor proteins, including opsin, phosducin, and rds/peripherin, was studied by immunoblot analysis. The results of immunoblot analysis showed that the 661W cells expressed opsin, phosducin, and rds/peripherin as did BALB/c retina used as a positive control (Figure 5). The band intensities of opsin, phosducin, and rds/peripherin from immunoblot analysis suggest that there are major quantitative differences in the levels of expression of these photoreceptor proteins in 661W cells, compared to BALB/c mice retinas (Figure 5). The higher background could be due to differences in the amount of protein loaded in the lane of 661W cells, as compared to BALB/c retinas.

Photobleaching difference absorption spectrum of rhodopsin. To ascertain the generation of isorhodopsin, exogenous 9-cis retinal was incubated with 661W membranes followed by the addition of hydroxylamine. This reaction is a sensitive measure of the quantity and conformational integrity of apoprotein. The absorption spectra were recorded prior to and after photobleaching for 661W and rat rod outer segments (Figure 6 A and B).

On photobleaching a characteristic difference in absorption was seen at 485 nm, characterstic of isorhodopsin (Figure 6 C). Isolated rat rod outer segments, used as a positive control, also showed a characteristic isorhodopsin absorption peak on incubation with 9-cis retinal (Figure 6C).

DISCUSSION

Our study of the expression of photoreceptor specific genes and proteins by the 661W cell line supports their purported photoreceptor phenotype. Therefore, this cell line provides another opportunity for investigating normal and pathologic processes involving photoreceptors. These cells were found to be readily transfected with plasmid constructs using calcium phosphate precipitation and liposome mediated techniques (Krishnamoorthy et al., submitted to J Biol Chem; Crawford, unpublished results), thereby offering an in vitro system to study the effect of expression of genes of interest on photoreceptor function.

Previously described homogenous photoreceptor cell lines include the Y-7910 and WERI-Rb-111 human retinoblastoma cell lines, as well as a mouse retinoblastoma cell line[9]. The Y-79 cells have been shown to express cone and rod specific gene transcripts providing valuable insights into the molecular mechanisms of retinal disease[12, 13], while the WERI-Rb-1 cells have not been characterized for photoreceptor specific markers. The mouse retinoblastoma line was found to express the RNA message and protein for IRBP and cone transducin, but failed to express opsin and arrestin. Similarities between the Y-79 retinoblastoma line and the 661W cells include their tumor origin, doubling times (33 hr vs. 26.4 hr, respectively) and expression of photoreceptor specific genes. One difference between 661W cells and Y-79 cells is that 661W cells readily attach and grow as monolayer, while the Y-79 cells preferably grow in suspension cultures. Y-79 cells can only be coaxed into attachment and monolayer

growth by treating flasks with poly-ornithine or other attachment substrates. Morphological differences also exist between the cell types, as Y-79 cells in monolayer appear rounded and form rosettes, while the 661W cells show a flattened orientation with spindle-like processes. A critical finding in support of the 661W ís professed photoreceptor origin is the ability of the 661W cells to show characteristic absorption spectrum for isorhodopsin at 485 nm in the presence of 9-cis retinal. This suggests that quantities of opsin produced in these cells is sufficient for detection of spectral changes in opsin upon photobleaching of 661W cell membranes which indicates the cells ability to generate functional rhodopsin molecules. This is a major difference between previously described human and mouse retinoblastoma cell lines and the 661W cells. Although, compared to BALB/c retinas, there are major quantitative differences in photoreceptor specific protein expression, 661W cells do express many photoreceptor proteins. Further studies will be needed to ascertain its usefulness to study phototransduction process in vitro. These results strongly suggest that 661W cells have the necessary proteins for forming outer segments. Based on these observations, 661W cells seem to be true representatives of photoreceptor cells. It remains to be determined if 661W cells are capable of transducing light, since many of the proteins involved in transduction are expressed in these cells. Currently, we are in the process of determining if 661W cells can be stimulated to differentiate, i.e., to form outer segments, by co-culturing 661W cells with retinal pigment epithelium.

Primary cultures of retinal cells are a means to study retinal and photoreceptor function. Recent reports indicate that primary cultures are not limited to immature,

developing retinal cells since adult human retinal cells, including photoreceptor cells, have been cultured and exhibited ~1% survival of original cells after 2 months in culture[16]. Although both immature and mature retinal cells have been shown to exhibit plasticity in primary culture, there exists a lack of homogeneity for a particular cell type. For those interested in the cell processes occuring exclusively within photoreceptors, the use of primary cultures introduces the confounding effect of other cell types. For this experimentation, photoreceptor cell types such as 661W, offer a valuable tool. For example, these cells undergo apoptosis upon exposure to photo-oxidative stress, thereby providing an in vitro model for studying mechanisms of photoreceptor cell death[23, 24]. Further, these cells appear to be of rod cell origin, as several of the rod specific genes are expressed by these cells.

Thus, in conclusion, the 661W cell line reported in this paper appears to be photoreceptor-like. These cells should be useful for investigating photoreceptor cell biology and function in physiologic and diseased states.

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Table 1. Primer sequences used for PCR amplification of photoreceptor specific genes by 661W cells and their product size. The optimum annealing temperature was 55°C except for β-actin, which was 60°C.

Gene	Primer sequence	Size(bp)	cycles
Arrestin	(S) GCGGGAAGACCAATAAAT (A) CTGAGGGAGACTGAGAGG	622	40
β-transducin	(S) ATGCTCGGAAGGCGTGTG (A) GAATGTGGTCGTCTGCTG	482	30
β-phosphodiesterase	(S) CGTTTTCCCATCAATACT (A) TACACTCTGCCACATCCT	423	40
rds/peripherin	(S) GGGCTCTGGCTTATGAAC (A) GGTCTCGGGCACGCTCTT	891	40
Opsin	(S) TGTTCCTGCTCATCGTGCTG (A) ACTTCCTTCTCTGCCTTCTG	619	30
Thy-1	(S) TGCCTGGTGACCAGAACCTT (A) TCACAGAGAAATGAAGTCCGTGGC	415	40
GFAP	(S) GCTGCTCGCCGCTCCTAC (A) GCCATTGCCTCATACTGC	713	40
β-actin	(S) TGTGATGGTGGGAATGGGTCAG (A) TTTGATGTCACGCACGATTTC	535	40

Figure 1. Cultured 661W cells appeared flattened with a prominent nucleus (long arrow), exhibiting processes (short arrow). Shortly after they begin to project neuronal processes and once established on growth surface, the cells flatten and the cytoplasm increases. 661W cells were passaged every 3-4 days up to 60 passages.

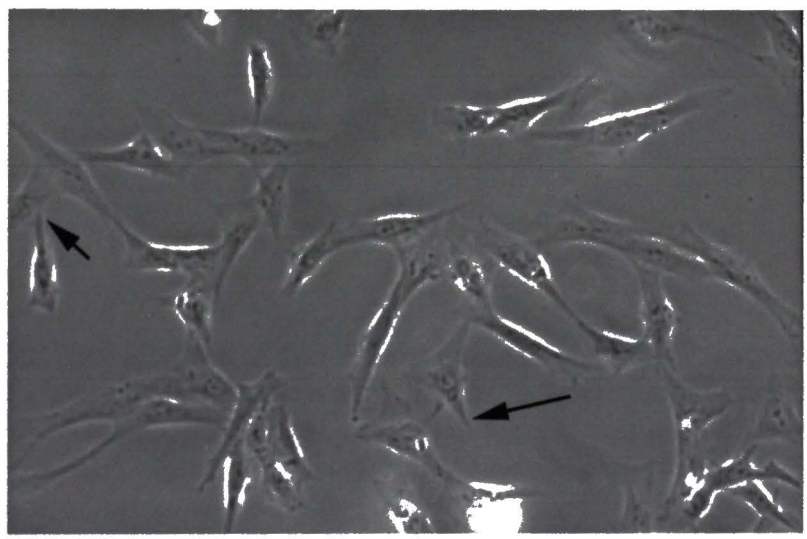


Figure 2. Logarithmic growth curve of 661W cells cultured in DMEM containing 9% fetal calf serum and 1% penicillin/streptomycin. The cells showed a lag phase that lasted for 1-2 days and then divided logarithmically for up to 8 days. For each time point three culture dishes were counted. Although the cell monolayer appeared to be confluent by 3-4 days, the cells continued to grow for another 4-5 days. Doubling time for 661W cells was calculated to be 1.1 days.

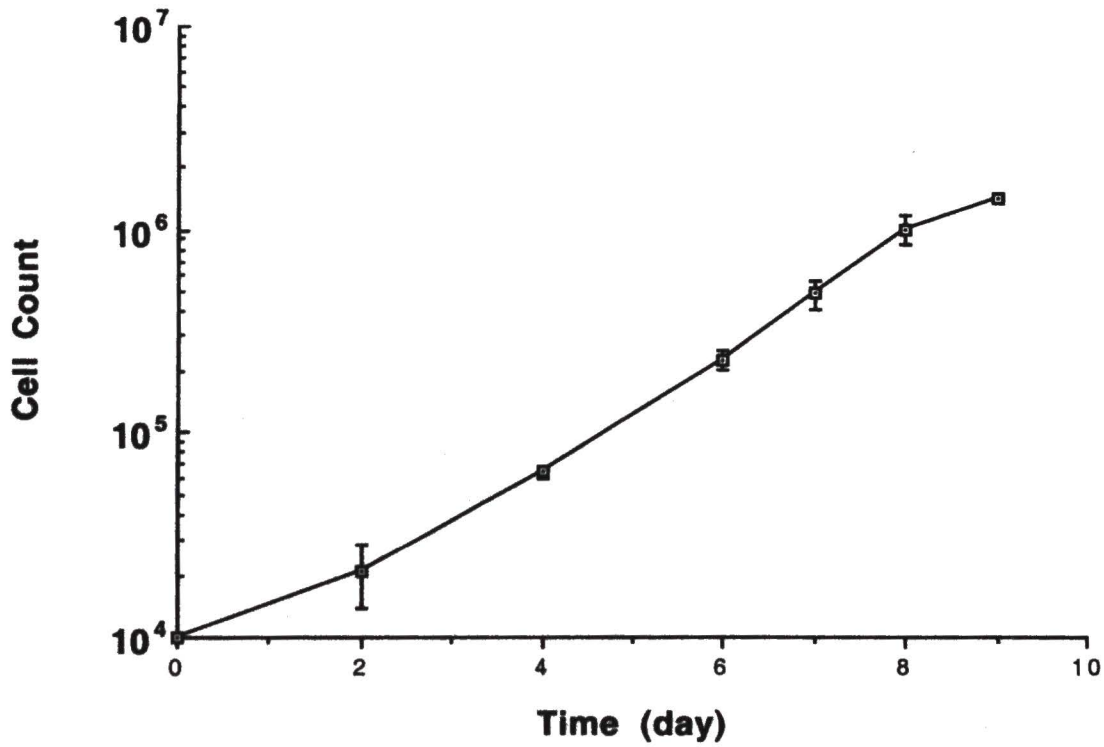

Cell Count
10^7
10^6
10^5
10^4
0
2
4
6
8
10
Time (day)

Figure 3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of β-actin, β-transducin, opsin, β-phosphodiesterase, and arrestin. Total RNA was extracted from 661W cells, BALB/c mouse retinas which were included as a positive control and baboon lungs, included as negative control. The RT-PCR analysis was performed after synthesizing the first strand cDNA, and using sense and antisense primers as described in the methods and Table 1. Experiments were carried out in triplicate. The results of the PCR products from 661W cells, and mouse retina showed 661W cells express the messages for arrestin, opsin, β-transducin, β-PDE, arrestin, and β-actin. The 661W cells did not show expression of Thy-1 or GFAP used as markers for ganglion cells and muller cells, respectively (results not shown).

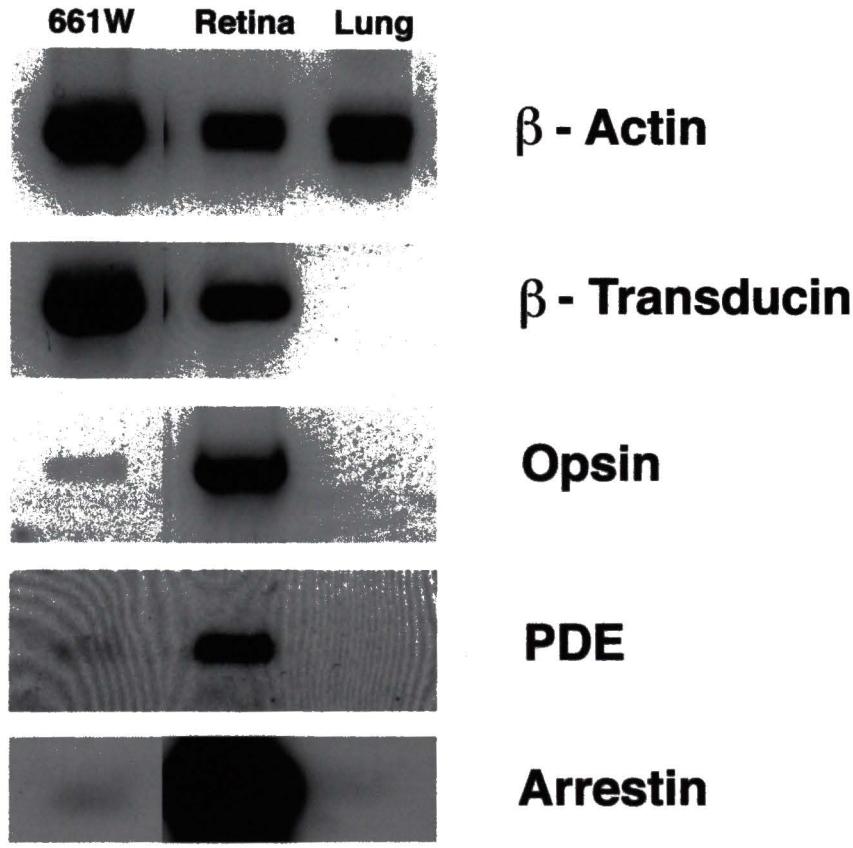

661W
Retina
Lung
β - Actin
β - Transducin
Opsin
PDE
Arrestin

Figure 4. Immunocytochemical labeling of various photoreceptor proteins in 661W cells. 661W cells were grown on glass cover slips, fixed with 4% paraformaldehyde, and immunolabeled using primary antibodies for β-transducin, IRBP, arrestin, opsin, rds/peripherin, and phosducin. Following primary antibody incubation, appropriate FITC-labeled second antibodies were applied. 661W cells were positive for expression of arrestin, opsin, IRBP, β-transducin, phosducin, and rds/peripherin. Controls samples were not treated with primary antibodies and either included pre-immune serum or cultured supernates and did not show any positive labeling (results not shown).

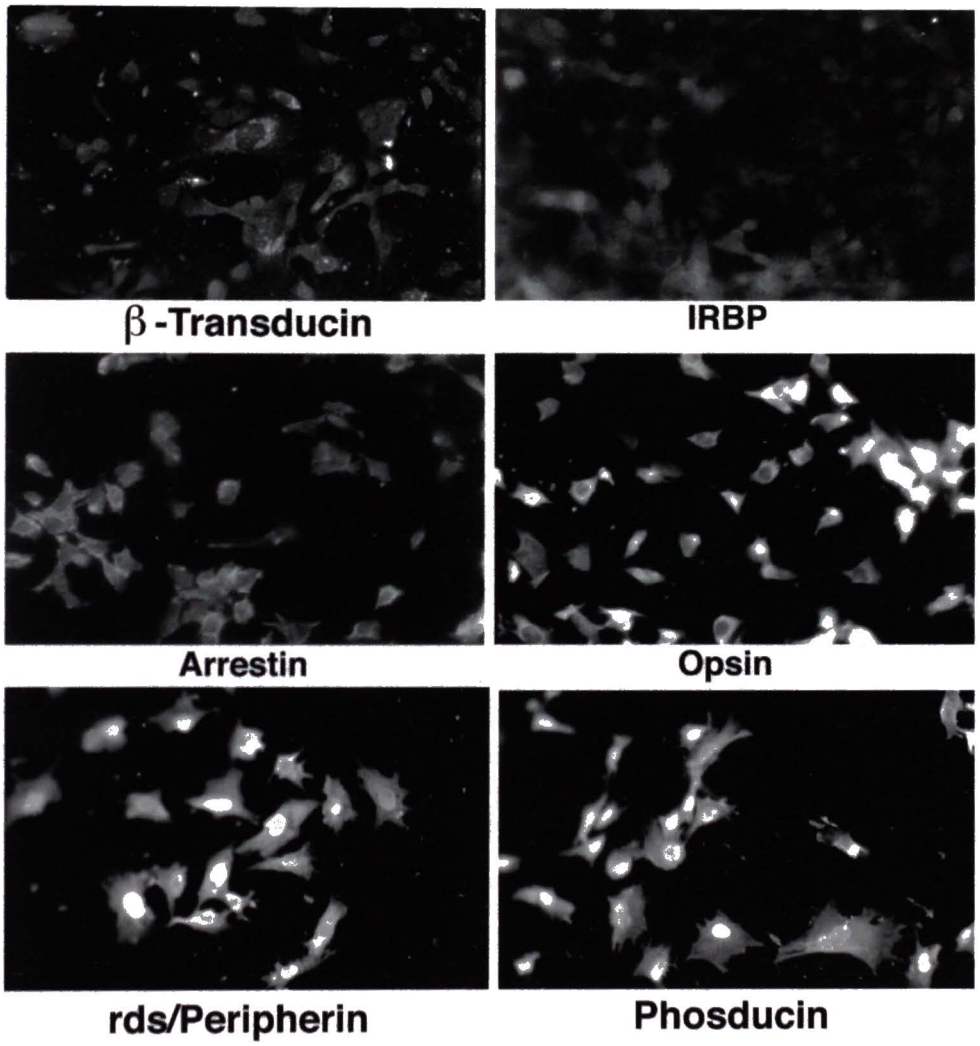

β-Transducin
IRBP
Arrestin
Opsin
rds/Peripherin
Phosducin

Figure 5. Immunoblot analysis of various photoreceptor markers in 661W cells. 661W cell and BALB/c retinal lysates were separated on 12-15% SDS-polyacrylamide gels and blotted on to nitrocellulose membrane (MSI) and immunoreacted with opsin, phosducin, and rds/peripherin, primary antibodies. Following incubation with peroxidase labeled secondary antibodies the immunoblots were developed with ECL reagent (NEN). The results shown were typical findings of western blot experiments carried out in douplicate or triplicate. Positive immune reaction for is seen for opsin, phosducin, and rds/peripherin in 661W cells (arrows). An opsin dimer was also visible in BALB/c retinas (arrowhead), which was not seen in 661W cells.

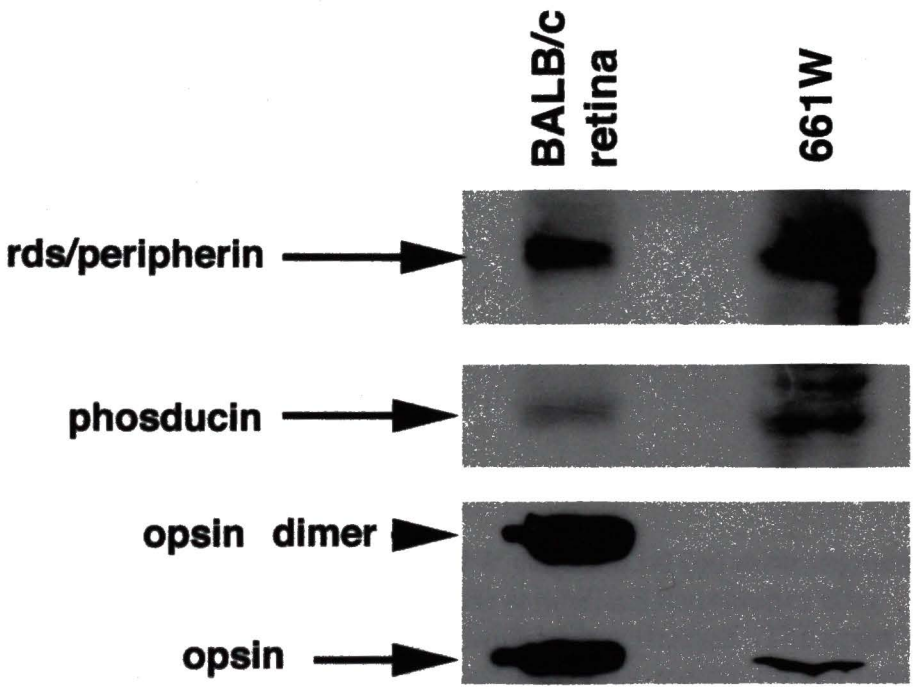
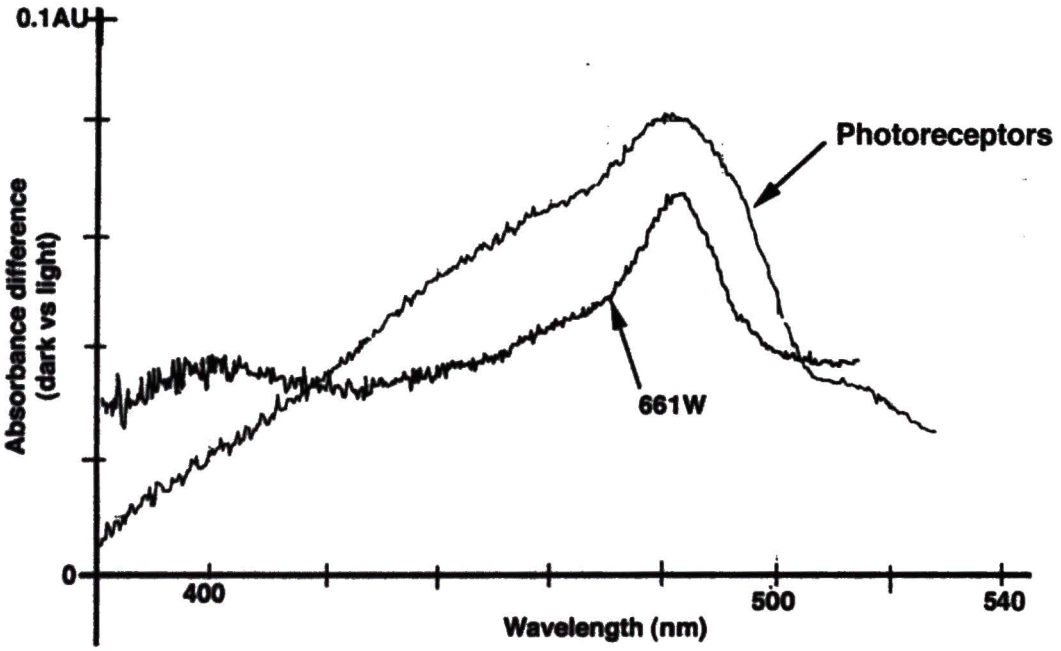
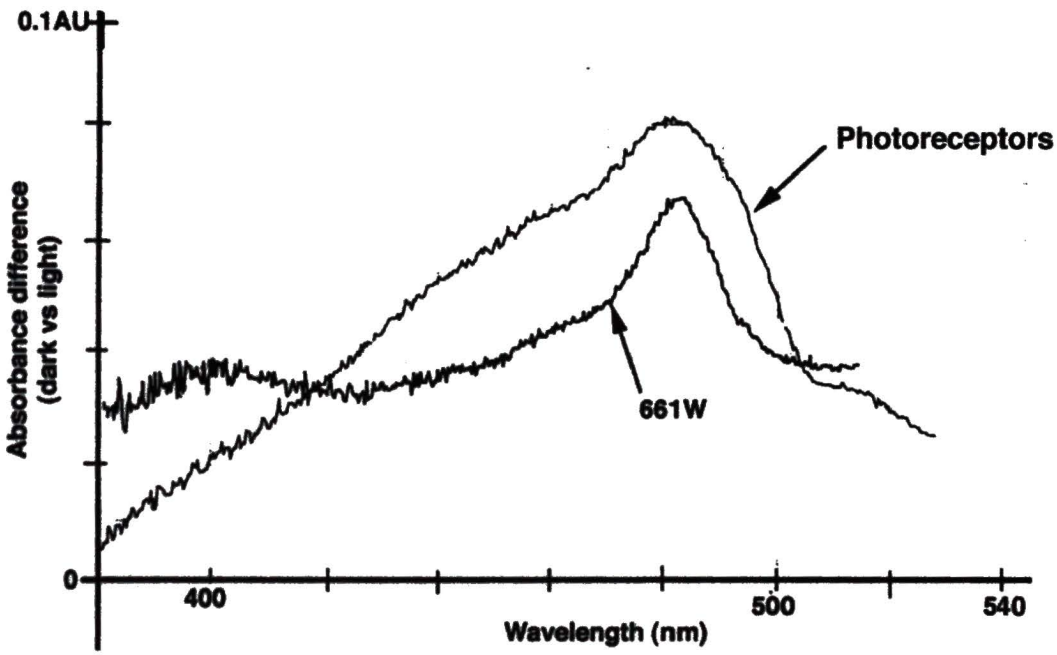

BALB/c retina
661W
rds/peripherin
phosducin
opsin dimer
opsin

Figure 6. Absorbance spectrum of rhodopsin in 661W cells and rat rod outer segments. The difference spectrum is shown for glucopyranoside solubilized membranes after incubation with 9-cis retinal followed by incubation with hydroxylamine. The upper curve shows the difference between pre- and post-photobleaching spectra for rod outer segments, while the lower curve is for 661W cells. Both rod outer segments and 661W cells showed a 485 nm peak on bleaching, a characteristic absorption of isorhodopsin.


0.1AU
Absorbance difference
(dark vs light)
Photoreceptors
661W
0
400
500
540
Wavelength (nm)


0.1AU
Absorbance difference
(dark vs light)
Photoreceptors
661W
0
400
500
540
Wavelength (nm)

PREFACE TO CHAPTER III

The 661W clonal cells express proteins and mRNA message specific to photoreceptor cells, including arrestin, opsin, IRBP, β-transducin, phosducin, and rds/peripherin. Additionally, solubilized membranes of 661W cells incubated with 9-cis retinal exhibited a 485 nm absorbance peak on bleaching, a characteristic absorption of isorhodopsin. Having established the photoreceptor origin of the 661W cells these studies focus on demonstrating oxidative changes and apoptosis following visible light exposure. The redox state of the cells was studied by measuring malonyldialdehyde and glutathione levels following exposure to visible light. The effect of oxidative stress on the viability of the photoreceptor cells was evaluated using several apoptotis assays. Furthermore, the effects of photo-oxidative stress on the expression and binding activity of nuclear factor kappa B was studied using electrophoretic mobility shift assays, immunocytochemistry, and protein immunoblots.

CHAPTER III

PHOTO-OXIDATIVE STRESS DOWN-MODULATES THE ACTIVITY OF NF-κB VIA INVOLVEMENT OF CASPASE-1, LEADING TO APOPTOSIS OF PHOTORECEPTOR CELLS

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ABSTRACT

The mechanisms of photoreceptor cell death via apoptosis, in retinal dystrophies, are largely not understood. In the present report we show that visible light exposure of mouse cultured 661W photoreceptor cells at 4.5 mwatt/cm^2 caused a significant increase in oxidative damage of 661W cells leading to apoptosis of these cells. These cells show constitutive expression of NF-κB and light exposure of photoreceptor cells results in lowering of NF-κB levels in both the nuclear and cytosolic fractions in a time dependent manner. Immunoblot analysis of IκBα and p50, and p65 (RelA) subunits of NF-κB, suggested that photo-oxidative stress results in their depletion. Immunocytochemical studies using antibody to RelA subunit of NF-κB, further revealed the presence of this subunit constitutively both in the nucleus and cytoplasm of the 661W cells. Upon exposure to photo-oxidative stress, a depletion of the cytoplasmic and nuclear RelA subunit was observed. The depletion of NF-κB appears to be mediated through involvement of caspase-1. Further, transfection of these cells with a dominant negative mutant IκBα greatly enhanced the kinetics of down modulation of NF-κB resulting in a faster photo-oxidative stress induced apoptosis. Taken together, these studies show that the presence of NF-κB RelA subunit in the nucleus is essential for protection of photoreceptor cells against apoptosis mediated by an oxidative pathway.

KEY WORDS: photoreceptor cell, apoptosis, nuclear factor-kappa B (NF-κB), oxidative damage, retinal dystrophies.

ABBREVIATIONS: EMSA, Electrophoretic mobility shift assay; NF-κB, nuclear factor-kappa B; PFA, paraformaldehyde; TUNEL, terminal deoxynucleotidyl transferase mediated fluorescinated dUTP nick end labeling; GAPDH, Glyceraldehyde phosphate dehydrogenase; MDA, Malonyl dialdehyde; GSH, Glutathione reduced; TBA, Thiobarbituric acid adduct; NAC, N-acetyl cysteine; ALLN, N-acetylleucylleucylnorleucinal; IκBα, Inhibitory subunit of NF-κB; IκBΔN, Superrepressor of IκBα; FITC, Fluorescine isothiocyanate.

INTRODUCTION

Nuclear factor kappa B (NF-κB) is a widely distributed transcription factor that plays a role in the regulation of a number of cellular and viral genes involved in early defense reactions in higher organisms[1]. NF-κB exists in an inactive form bound to the inhibitory protein IκBα or IκBβ[2-4]. Treatment of cells with inducers such as lipopolysaccharide, IL-1 and tumor necrosis factor-α (TNF-α), generally result in degradation of IκB proteins. This releases NF-κB of its inhibitory constraint, facilitating its translocation to the nucleus resulting in regulation of expression of genes encoding cytokines, hematopoietic growth factors and cellular adhesion molecules. NF-κB exhibits its DNA binding activity in its dimeric form and the most commonly occurring dimer is that of the p50 and the p65 (RelA) subunits. NF-κB has been shown to be constitutively active in several cell types including B cells[5], thymocytes[6] and neurons[7].

Most of the earlier studies on NF-κB focussed on its role in immunological and inflammation responses[1, 8, 9]. Recent reports suggest that NF-κB is also activated by oxidative signaling[10-13]. It has been suggested in many of these studies that reactive oxygen intermediates (ROI) may be involved in the activation of NF-κB. Another area of research where NF-κB involvement is gaining momentum, is the regulation of apoptosis. One of the earliest significant observations in this direction was made by Beg et al.[14], 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)[15], 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, Wang et al., (1996)[16], Van Antwerp et al., (1996)[17], and Liu et al., (1996)[18] showed a role of NF-κB in suppression of TNF-α induced apoptosis. 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[19].

In several experimental models of retinal dystrophies including certain forms of retinitis pigmentosa, photoreceptor cells of the retina have been shown to undergo apoptosis[20-23]. A number of studies have identified the primary mutations involved in the etiology of these diseases[24]. These studies however, do not completely explain the ultimate phenotypic manifestation of the disease namely apoptosis of photoreceptor cells. The difficulty in studying the disease process of retinal dystrophies is further compounded by non-availability of a homogenous permanent photoreceptor cell line, since retina is a complex tissue of multiple cell types. In the current studies we have used a transformed mouse photoreceptor cell line 661W (Crawford et. al., 1998, submitted to Investigative Ophthalmology and Visual Sciences).

In vivo studies have shown that exposure of rats to constant light results in apoptosis of photoreceptor cells[25-29]. Even moderate intensities of light exposure have been shown to damage the retinas of rats[30]. Since then, light has been extensively studied as an initiator of retinal cell death in a number of in vivo[31-33] and in vitro experimental conditions.

In the current study, we assessed the contribution of an oxidative stress paradigm to the propensity of photoreceptor cells to proceed to cell death via apoptosis, using cultured photoreceptor cells. We provide evidence in this paper that visible light exposure to photoreceptor cells results in oxidative damage leading to apoptosis via down-modulation of NF-κB. NF-κB which was constitutively expressed in the 661W cells, was found to be progressively down-regulated upon exposure of the cells to light. By immunocytochemistry using NF-κB RelA antibody, the NF-κB activity appeared to be localized both in the nucleus and cytoplasm of dark exposed 661W cells. Upon exposure to light the nuclear and cytoplasmic NF-κB RelA immuno-labeling was largely diminished in these cells. Pretreatment of the cells with various antioxidants prevented to a great extent the down-modulation of NF-κB and also protected the cells from apoptosis. Further, transient transfection of the 661W cells with a dominant negative IκBαΔN (super-repressor), caused a rapid decline in NF-κB binding activity in the cells, leading to a faster kinetics of photo-oxidative stress-induced apoptosis. Down-modulation of NF-κB in these cells appears to be mediated by caspase-1. Our results suggest that NF-κB which is constitutively expressed in 661W photoreceptor cells undergoes degradation when subjected to oxidative stress leading to apoptosis of the photoreceptor cells. Thus, the presence of NF-κB in the nucleus is essential for photoreceptor cell survival and protection against oxidative stress induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials- Following materials were purchased from: Fetal bovine serum (FBS) from JRH Biosciences, Lenexa, KS; PFA and H_2O_2 from EM Sciences, Gibbstown, NJ; HEPES, PMSF, ALLN, and DTT from Sigma, St. Louis, MO; Poly (dI-dC) Poly (dI-dC) from Amersham Pharmacia Biotech, Piscataway, NJ; and Polynucleotide kinase from New England Biolabs, Beverly, MA;

Antibodies- p50 subunit of NF-κB, a goat polyclonal IgG; p65, of NF-κB , a rabbit polyclonal IgG; IκBα rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA. GAPDH, chicken anti-rabbit GAPDH immunoaffinity-purified monospecific antibody (34). β-Tubulin, a mouse monoclonal antibody from Sigma, St Louis, MO. Peroxidase labeled secondary antibodies either anti-rabbit IgG or anti-mouse IgG were from Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD. Anti-cyclin D1 antibody was against amino acids 1-295, which represents full-length cyclin D1 of human origin (Santa Cruz Biotechnology, Santa Cruz, CA). FITC labeled anti-rabbit IgG from Vector Laboratories, Burlingame, CA.

Cell Culture - The 661W cells were originally isolated from a transgenic mouse line expressing the construct HIT1 comprising of SV40 T-antigen driven by the human interphotoreceptor retinol binding protein (IRBP) promoter[35]. The construct HIT1 resulted in SV40 T-antigen expression and retinal and brain tumors. 661W cells are

routinely grown in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Light Exposure of the Cells - The 661W cells were seeded either in 35/60/100 mm tissue culture dishes or on round coverslips kept in 35 mm dishes and exposed to fluorescent visible light at 4.5 mwatt/cm^2 for varying durations up to 4 hrs at 37°C in tissue culture. The accompanying control cells were shielded from light for similar intervals and left in similar conditions as the cells in light exposed paradigm.

3' End Labeling of Fragmented DNA by Terminal Deoxynucleotidyl Transferase Mediated Fluorescinated dUTP Nick End Labeling (TUNEL) - The TUNEL procedure as described by Gavrieli et al. (1992)[36] was employed to study apoptosis, using a commercially available apoptosis kit (In situ cell death detection kit, Boehringer Mannheim) as per the supplier's instructions.

Measurements of Membrane Lipid Peroxidation and Reduced Glutathione (GSH) Levels- The membrane lipid peroxidation of light exposed cultured cells was studied by measuring the malonyldialdehyde (MDA) levels by a colorimetric method involving thiobarbituric acid (TBA) adduct formation[37]. The GSH levels in light exposed cells was studied by using the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reagent[38].

Immunoblot Analysis - Protein extracts from 661W cultured cells exposed to light, were subjected to immunoblot analysis (39, 40) using specific antibodies for IκBα, p50, and RelA subunit of NF-κB using commercially available antibodies (Santa Cruz Biotechnologies, CA). Cytoplasmic extracts were used for IκBα analysis whereas nuclear extracts were used to study RelA and p50 subunits of NF-κB. Control blots were run using total cellular extracts and an antibody to GAPDH. The binding of primary antibodies was detected by using peroxidase labeled appropriate secondary antibodies, which were detected by using diamino benzidine as substrate. Immunoblots were performed at least two times to verify results.

Immunolocalization Studies- The 661W cells were exposed to light and fixed in 4% paraformaldehyde. The immunofluorescence for p65 subunit of NF-κB was done, by using a specific antibody against p65 and a FITC labeled goat anti-rabbit secondary antibody[20]. The immunofluorescent cells were photographed using a Nikon Microphot-FXA photomicroscope.

Preparation of Cytoplasmic and Nuclear Extracts - The 661W cells were exposed to light for the desired amount of time and the nuclear and cytoplasmic extracts were prepared[41]. Briefly, the cells were suspended in 100 µl of buffer C (10 mM N-2 hydroxyethylpiperizine-N'-2-ethanesulfonic acid (HEPES), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 10% glycerol 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 15 min. 3 µl of 10% NP-40 was added to the suspension and briefly vortexed. Following this, the

nuclei were pelleted by centrifugation at low speed. The supernatant (cytoplasmic extract) was collected and stored at -80°C. The nuclear pellet was resuspended in 70 µl of buffer D (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF). The suspension was incubated for 20 min at 4°C followed by a centrifugation at 8000 g for 5 min. The supernatant containing the nuclear protein extract were transferred to a fresh microfuge tube and stored at -80°C. Protein concentrations of the cytoplasmic and the nuclear extracts were measured with a DC Protein Assay Kit (Bio-Rad), using BSA as a standard.

Electrophoretic Mobility Shift Assays (EMSA) - 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, CA) were used to study the DNA binding activity of NF-κB by EMSA as described[42]. For super shift assay, 4 µg of nuclear extract was incubated with 1 µg of antibodies for 30 min at room temperature and analyzed by EMSA. EMSA studies were performed either two or three times to verify consistency of results.

Transfection of 661W with the dominant negative IκBα - The dominant negative IκBα (super-repressor) constuct IκBα was obtained from Dr. Dean Ballard, Vanderbilt University, Nashville TN (43). IκBαΔN is a deletion mutant in which N-terminal 36 amino acids are deleted from the IκBα protein. The 661W cells were transiently

transfected with the construct using the lipofectamine reagent (Life Technologies, Gaithersburg, MD), as per the manufacturer's instructions using 8μl lipofectamine and 5μg of the IκBα superrepressor plasmid DNA for 1 ml of transfection mix. The untransfected control cells were treated in a similar manner except for the exclusion of the plasmid DNA. The transfected cells and their controls were used 48 h post-transfection for making either total cellular extract for immunoblot analysis or cytoplasmic and nuclear extracts for EMSAs and for TUNEL assay as described above.

RESULTS

Membrane lipid peroxidation and depletion of glutathione are observed in cultured photoreceptor cells exposed to light- The 661W cells were exposed to light for up to 4 h and membrane lipid peroxide formation and GSH levels were measured. There was almost a 2 fold increase in MDA formation following light exposure, as compared to controls (Table 1) and inclusion of NAC in the medium before light exposure of cultured cells, prevented the increase in MDA levels (Table 1). These results indicate that photic injury to photoreceptor cells occurs due to a possible involvement of an oxidative pathway. To explore this possibility further, we used other anti-oxidants such as thiourea and mannitol in our studies. As shown, photo-oxidative stress resulted in significant lowering of GSH levels as compared to control cells maintained in dark. The presence of thiourea (7 mM) in the medium of 661W cells was protective against photo-oxidative damage, as seen by the maintenance of GSH levels close to control values (Table 1).

NF-κB activity is down-modulated upon exposure of photoreceptor cells to light - The photoreceptor cells were exposed to light for various time intervals for 15, 30, and 60 min. There was no change in NF-κB activity up to 30 min of light exposure in both the nucleus (Figure 1a, lanes 2 and 3 for nucleus) and cytoplasm (Figure 1a, lanes 6 and 7) compared to dark exposed control cells (Figure 1a, lanes 1 and 5, for nucleus and cytoplasm, respectively). Upon 60 min of light exposure, there was approximately 80% loss of NF-κB binding activity in both the nucleus and cytoplasm (lanes 4 and 8

respectively). These results indicate that the cultured photoreceptor cells express NF-κB constitutively and that the activity of NF-κB decreases on exposure to photo-oxidative stress.

The specificity of the binding of NF-κB was shown by competition with cold NF-κB consensus oligo (Figure 1b, lanes 2-4). As expected, a lack of competition was observed with cold NF-κB mutant oligo (Figure 1b, lanes 6-8). The DNA protein complex seen in the EMSA appears to be a heterodimer of p50 and p65 subunits of NF-κB, as revealed by a decrease in the binding upon additional incubation with the antibodies to the p65 and p50 (Figure 1c) subunits, in a supershift assay. An unrelated antibody, anti-cyclin D1 used as a negative control, did not inhibit the DNA protein complex formation (Figure 1c). These results confirm the identity of the NF-κB DNA-protein complex seen in the EMSAs.

Pretreatment of photoreceptor cells with antioxidants protects against down-modulation of NF-κB upon exposure to light - In order to establish the involvement of oxidative damage in the lowering of NF-κB activity during conditions of photo-oxidative stress, we studied the effects of antioxidants, namely NAC, mannitol, and thiourea under these conditions (Figure 2a). Lanes 1, 5, and 9 represent NF-κB binding activity in dark exposed cells. Lanes 2, 6, and 10 represent NF-κB binding activity in dark exposed cells in the presence of NAC, mannitol, and thiourea respectively. The presence of these anti-oxidants did not appreciably alter the NF-κB binding activity in dark exposed control cells. As expected, the activity of NF-κB decreased on exposure to photo-oxidative

stress (lanes 3, 7, and 11). The inclusion of NAC, mannitol, and thiourea in the growth medium prior to light exposure partially protected against the down-modulation of NF-κB (Figure 2a, lanes 4, 8, and 12 respectively) in light exposed 661W cells. The differences in the extent of protection of NF-κB levels in these groups may be attributed to differences in efficacy of these anti-oxidants in affording protection against photo-oxidative damage. These results indicate that oxidative damage plays a major role in decreasing the NF-κB activity in cultured photoreceptor cells exposed to light. It remains to be seen if these antioxidants offer an additive protection of NF-κB levels, if used simultaneously.

Treatment with H_2O_2 does not significantly alter the NF-κB binding activity and apoptosis of the 661W cells - Our results so far suggest that light may down-modulate NF-κB through generation of ROIs. To further confirm the role of ROIs in this process we treated the cells with H_2O_2 for various times up to 120 min and measured NF-κB activation and apoptosis. The EMSA revealed no significant change in the NF-κB binding activity in these cells treated with 300μM H_2O_2 for 30 and 60 min (Figure 2b, Lanes 2 & 3 and Lanes 6 & 7 for nuclear and cytoplasmic extracts respectively), compared to untreated control cells (Lanes 1 and 5 for nucleus and cytoplasm respectively). However, treatment of H_2O_2 for 120 min, resulted in a modest increase in the NF-κB binding activity both in the nucleus (lane 4) and the cytoplasm (lane 8). The TUNEL assay revealed no significant increase in the number of apoptotic cells on treatment with H_2O_2 compared to untreated controls for all the durations of H_2O_2

treatment (data not shown). Therefore, this data indicate that ROIs alone are not sufficient for light induced down regulation of NF-κB and activation of apoptosis in these cells.

Effect of photo-oxidative stress on NF-κB levels in Madin-Derby Canine Kidney (MDCK) Cells - To assess the specificity of response of 661W cells to photo-oxidative stress, we studied the effect of light exposure on MDCK cells, using them as an unrelated control. The light exposure of MDCK cells did not cause a decrease in NF-κB binding activity, in both the nucleus and cytoplasm (data not shown). These results indicate that the cell-specific response of 661W cells to light is different from that of MDCK cells.

Immunoblot analysis of IκBα, p50, and RelA subunit of NF-κB in 661W cells exposed to light - To further confirm the down-modulation of NF-κB, the protein levels of IκBα, p50, and RelA subunits of NF-κB were studied in 661W cells exposed to light, with or without pre-treatment with NAC and thiourea, by immunoblot analysis using specific antibodies. The light exposed cells showed lowering of IκBα, p50, and RelA subunit of NF-κB, as compared to dark controls. Pretreatment of the cells with both anti-oxidants protected the levels of NF-κB p50, RelA, and IκBα subunits, albeit partially, upon exposure to light (Figure 3). To ensure that light exposure does not result in a generalized protein degradation, a control protein GAPDH was included, which was not greatly altered in all samples under these experimental conditions. On quantitation, there was ~50% decrease of IκBα protein levels on 2 h light exposure. On the other hand,

there was ~90% decrease in p50 and RelA subunit with no change in GAPDH levels under similar conditions. Inclusion of anti-oxidants protected to a large extent against degradation of these proteins. IκBα was protected 100% whereas p50 and p65 were protected to 40-45% of control values. Based on the results of GAPDH protein levels, these data suggest that down-modulation of IκBα and p50 and p65 subunit of NF-κB is not due to random protein degradation.

Cultured photoreceptor cells undergo cell death via apoptosis upon light exposure - To establish that oxidative damage along with a down-modulation of NF-κB results in apoptosis of these cells, we studied the effect of photo-oxidative stress on apoptosis of these cells in presence or absence of the anti-oxidant, NAC. We found that exposure of 661W cells to light up to 1 hr did not result in any significant apoptosis of cultured photoreceptor cells (Figure 4A). However, after 2 and 4 hrs of light exposure, many cells underwent apoptosis (Figure 4C, and E respectively), compared to dark exposed control cells as seen by incorporation of fluorescinated dUTP in the nuclei of apoptotic cells containing fragmented DNA. Approximately, 80% of the cells were seen to undergo apoptosis in 661W cells exposed to light for 4 hrs (Figure 4 E). Inclusion of NAC protected the cells from apoptosis at the 1, 2, and 4 hr intervals of light exposure respectively (Figure 4 B, D, and F). As expected, dark exposed control cells did not show any TUNEL positive apoptotic cells (data not shown).

Photo-oxidative stress and immunocytochemical localization of NF-κB RelA subunit in 661W cells - To study the effect of photo-oxidative stress on the localization of RelA subunit of NF-κB, immunocytochemistry was performed in these cells in presence and absence of NAC, using RelA specific antibody. It was seen that RelA was present in the nucleus and also in the cytoplasm of dark exposed control cells (Figure 5 A). Upon exposure to light, the nuclear and cytoplasmic labeling of NF-κB diminished to a large extent (figure 5 B). But, in the presence of NAC, a number of cells exposed to light, retained positive immunoreactivity of RelA, in the nucleus as well as cytoplasm (figure 5 C). There were still some cells, which had diminished RelA immunoreactivity in their nuclei and cytoplasm (figure 5C). These data indicate that light exposure caused the degradation of RelA subunit from the nucleus as well as cytoplasm by an oxidative pathway.

Pretreatment with the proteasome inhibitor N- acetylleucylleucylnorleucinal (ALLN) does not protect 661W cells against photo-oxidative stress induced apoptosis - Since our results suggested that preservation of NF-κB levels in the cells protects against apoptosis, we investigated the down-regulation of constitutive NF-κB activity and its effect on apoptosis, by a proteosome inhibitor, ALLN. ALLN pretreatment of the 661W cells did not protect the NF-κB binding activity, upon exposure to light (Figure 6a, Lanes 4 and 8 for nucleus and cytoplasm respectively) compared to NF-κB binding activity in both the nucleus and the cytoplasm of 661W cells exposed to light without any pretreatment (Figure 6a, Lanes 3 and 7 for nucleus and cytoplasm respectively). ALLN treatment to

cells maintained in the dark, also did not alter their NF-κB activity (Figure 6a, Lanes 2 and 6 for nucleus and cytoplasm respectively).

The TUNEL assay revealed that ALLN pre-treatment also caused some of the cells maintained in the dark, to undergo apoptosis (Figure 6b, B). Further, ALLN treatment did not protect against photo-oxidative stress induced apoptosis (Figure 6b, D) compared to cells exposed to light without any pre-treatment of ALLN (Figure 6b, C). ALLN by virtue of being a proteosome inhibitor, could block IκBα degradation, thereby, NF-κB activation could be inhibited thus leading to apoptosis. This provides further confirmation of our hypothesis that NF-κB is involved in blocking apoptosis.

Transient transfection of 661W cells with a dominant negative IκBα accelerates photo-oxidative stress-induced apoptosis by down-modulating NF-κB binding activity - To further delineate the role of NF-κB in apoptosis we also transfected the cells with IκBα super-repressor and examined its effects on NF-κB binding activity and apoptosis upon exposure to photo-oxidative stress. To determine this, 661W cells were transiently transfected with IκBα super-repressor and evaluated for their IκBα and IκBαΔN levels, NF-κB binding activity, and DNA fragmentation by TUNEL. Immunoblot analysis of the transfected cells revealed higher protein levels of IκBα in these cells, compared to the mock transfected controls (Figure 7a, compare lane 2 with lane 1). A faster moving band corresponding to the super-repressor was also detected in the transfected cells representing a truncated form of IκBα, the IκBαΔN, but not in the mock-transfected cells

(Figure 7a, compare lane 2 with lane 1). A control protein, β-tubulin, did not appear to change between the two samples (Figure 7a, bottom panel).

The NF-κB binding activity in the untransfected cells was higher than that of the superrepressor transfected cells (Figure 7b, Lanes 1 & 2). Upon exposure to light, there was a rapid decline in NF-κB activity in the transfected cells compared to that in the untransfected cells, both in the nucleus and cytoplasm (Figure 7b, Lanes 4, 6, and 8 compared to Lanes 3, 5, and 7 for nucleus; Lanes 12, 14, and 16 compared to lanes 11, 13, and 15 for cytoplasm, for 15, 30, and 60 min of light exposure, respectively). Quantitation of a typical EMSA autoradiogram showed practically no change in NF-κB binding activity of control cells exposed to light up to 30 min, with a 75 to 85% decrease in binding activity upon 60 min of light exposure in nucleus as well as cytoplasm. In contrast, in IκBα super-repressor transfected cells, there was a rapid loss of NF-κB binding activity, which decreased by 40% and 95% at 30 and 60 min in the nucleus and by 50%, 80%, and 90% at 15, 30, and 60 min of light exposure in the cytoplasm. There was no decrease in the NF-κB binding activity of nucleus at 15 min of light exposure in the transfected cells.

The TUNEL assay revealed that mere transfection of the IκBαΔN super-repressor caused a number of cells to undergo apoptosis, even without being exposed to light (Figure 7c, E). There was a rapid increase in the number of TUNEL positive cells in the transfected cells by as early as 15 min of exposure to light (Figure 7c, F), when compared to untransfected 661W cells (Figure 7c, B). It reached a maximum by 30 min of light exposure in the IκBα super-repressor transfected group (Figure 7c, G), in contrast with

just a few apoptotic cells in the untransfected 661W cells exposed to light for 60 min (Figure 7c, D).

Thus, transfection with the super-repressor hastens the kinetics of down-modulation of NF-κB binding activity as well as apoptotic cell death of 661W cells upon exposure to light. Pretreatment with the caspase 1 inhibitor (YVAD-CMK) protects against down-modulation of NF-κB and apoptosis of 661W cells upon exposure to light. In order to identify proteases involved in the down-regulation of NF-κB in the 661W cells, we studied the effect of caspase inhibitors on NF-κB binding activity and apoptosis of 661W cells exposed to photo-oxidative stress. Pretreatment of 661W cells with the caspase 1 inhibitor, YVAD-CMK (100μM) could protect against down-regulation of NF-κB upon exposure of the cells to light in both nucleus (figure 8a, compare lane 4 to lane 3) and cytoplasm (figure 8a compare lane 8 to lane 7). On the other hand, inclusion of caspase-3 inhibitor, DEVD-CHO did not protect the levels of NF-κB in the light exposed cells (data not shown).

To further study if the caspase-1 inhibitor could protect against light-induced apoptosis, we performed TUNEL assay of these cells exposed to light in presence of the inhibitor. As seen in figure 8b, pretreatment with the caspase 1 inhibitor could protect against photo-oxidative stress induced apoptosis (panel C) as compared to 661W cells exposed to light in absence of caspase-1 inhibitor (panel B), and dark-exposed controls (panel A). Similar studies with caspase-3 inhibitor showed that it did not render protection of 661W cells against photo-oxidative stress induced apoptosis (data not

shown). These studies indicate that down-modulation of NF-κB may be due to an activation of a specific caspase, namely, caspase-1.

DISCUSSION

Apoptosis of photoreceptor cells is a common phenotype in retinal dystrophies, shared by patients with age related macular degeneration and autosomal dominant retinitis pigmentosa (ADRP), a family of disorders characterized by photoreceptor cell degeneration and a corresponding loss of vision. Point mutations of several genes involved in visual transduction have been identified in retinitis pigmentosa and other forms of retinal dystrophies (24). These studies while pointing to the primary cause of the disease, do not provide clues to the downstream effectors that play a crucial role in the progression of the disease, ultimately leading to photoreceptor cell death by apoptosis.

Light has been extensively used as a initiator of photoreceptor cell death in a number of in vivo and in vitro experimental conditions (31-33). In vivo studies have also shown that exposure of rats to constant light results in apoptosis of photoreceptor cells (25-30). Production of lipid hydroperoxides has been observed in light exposed retinas (44). Retina has been shown to be susceptible to lipid peroxidation (45, 46) despite having high levels of antioxidants (47-49).

In the current study, we have assessed the involvement of oxidative damage in the apoptotic cell death of 661W photoreceptor cells in an in vitro model. The cell line expresses several markers of differentiated photoreceptors including opsin, arrestin, rds/peripherin, phosducin, and IRBP (Crawford et al., 1998, submitted to Investigative Ophthalmology and Visual Sciences). The 661W cell line therefore, is a valuable cell

line to study photoreceptor cell death. When the cells exposed to light for 4 hrs were subjected to an apoptotic TUNEL assay, it was seen that 80% of the cells were labelled with fluorescinated dUTP, suggestive of apoptosis. In a time course study, the extent of apoptosis of 661W cells had a direct correlation to duration of light exposure, and inclusion of NAC was protective of this effect. This experimental system thus, afforded a convenient model to analyze, the molecular events leading to apoptosis of photoreceptors by an oxidative pathway.

The important role of oxidative events has been further emphasized for a variety of biological processes, such as signal transduction and gene expression (19). A few studies have demonstrated that in particular, activation of NF-κB requires oxidative signaling i.e., its expression is dependent on the redox state of the cell (1, 10). Treatment of several cell lines with H_2O_2 resulted in activation of NF-κB (50, 51) and this can be blocked by inclusion of antioxidants (52-54).

By electrophoretic mobility shift assay, we found constitutive NF-κB activity, both in the nucleus and the cytoplasm of 661W photoreceptor cells. Visible light exposure to photoreceptor cells creates conditions of photoxidative stress leading to oxidative damage. This causes the cells to proceed to cell death via apoptosis. The NF-κB activity in 661W cells, was found to be progressively down-regulated upon exposure of the cells to light. Pretreatment of the cells with antioxidants namely NAC, thiourea, and mannitol partially prevented the down-modulation of NF-κB and NAC also protected the cells from apoptosis, indicating involvement of hydroxyl radicals and superoxide anions in the pathway leading to cell death via apoptosis. The NF-κB activity profile in

the photoreceptor cells exposed to light appear to be radically different from that seen in the unrelated MDCK cells. While the light exposure of 661W cells leads to decrease in NF-κB binding activity and apoptosis, the same stimulus does not greatly alter the nuclear NF-κB activity and does not lead to cell death in the MDCK cells. This points to a cell type specificity of 661W cells' response to light.

In the cell, NF-κB is stored in the cytoplasm in its inactive state by interaction with IκBα. On activation, IκBα undergoes degradation through an ubiquitin-dependent pathway (55, 56), allowing translocation of NF-κB to nucleus (57, 58) and subsequently binding to DNA regulatory elements within NF-κB target genes. Under our experimental conditions we find degradation not only of IκBα, but also the NF-κB p50 and RelA subunits. How the p50 and p65 subunits of NF-κB are degraded in this oxidative stress paradigm, is not fully understood. It is plausible that exposure of photoreceptor cells to light causes activation of one or more proteases which leads to degradation of not only IκBα, but also the p50 and p65 subunits. One of the protease responsible for degradation of NF-κB proteins in the 661W cells, appears to be the Interleukin 1β-converting enzyme (ICE) also called caspase-1 according to the new nomenclature. Recently, it was shown that caspase-3, which activates apoptosis, can also induce proteolytic cleavage of IκBα (59). In addition, the ubiquitin- conjugating enzymes that control IκBα degradation are known to be induced by ROI (60). Thus in our system, it appears that exposure of photoreceptor cells to light generates ROI, which activates caspase-1 resulting in proteolytic cleavage of NF-κB proteins leading to apoptosis. This is consistent with studies which show that NF-κB activation is essential for cell survival (61).

The role of NF-κB in apoptosis is not very clear with reports of both pro- and anti-apoptotic aspects appearing in literature. It was recently demonstrated that NF-κB is needed for TNF-α mediated induction of IAP-2, a protein belonging to the inhibitors of apoptosis (IAP) protein family. When over-expressed in mammalian cells, cIAP-2 activates NF-κB and suppresses TNF cytotoxicity (62). Exposure of human alveolar epithelial (A 549) cells to hyperoxia resulted in activation of NF-κB leading to necrotic cell death (63). These authors speculated that apoptosis occurs in the absence of NF-κB activation but protection from cell death by NF-κB is limited to apoptosis. It was further shown that TNF-α induces cell death in RelA -/- mouse fibroblast cells, whereas RelA+/+ cells were unaffected, demonstrating the role of RelA in protection of the cells from TNF-α induced apoptosis (15). A similar anti-apoptotic role of the RelA subunit was also demonstrated by Wang et al., 1996 (16), and Van Antwerp et al., 1996 (17). These studies indicate that either the inhibition of NF-κB RelA subunit or the prevention of its translocation to nucleus, is essential to induce apoptosis (15). However, several groups have suggested that NF-κB may function pro-apoptotically under some conditions and in certain cell lines (19, 64, 65).

In the 661W cells the NF-κB protein does not seem to follow the prototype pathway of its activation, upon exposure to an oxidative stimuli. For instance we do not observe an increase in NF-κB binding activity in these cells treated with an oxidizing agent like H_2O_2. Similarly, exposure of 661W cells to photo-oxidative stress which causes a degradation of IκBα, does not activate NF-κB, but infact degrades the p50 and p65 subunits. It therefore appears that in these cells, oxidative damage activates other

cellular mechanisms that trigger selective protein degradation, culminating in apoptosis. It needs to be emphasized that NF-κB signaling is indeed essential for survival of these cells, as seen by the induction of apoptosis in the 661W cells upon transfection with the IκB superrepressor. But the overwhelming stimulus for cell death appears to result from the oxidative degradation of NF-κB, as seen by the greatly accelerated kinetics of apoptosis in the super-repressor transfected 661W cells. A similar response was observed when the cells were pretreated with the proteasome inhibitor, ALLN which also inhibits calpain I, and II, cathepsin B, cathepsin L, and neutral cysteine proteases. Treatment with ALLN would slow down the degradation of IκBα, thereby perturb the NF-κB signaling in the cell and exacerbate cell death via apoptosis. The results of ALLN treatment further suggest that the down-modulation of RelA subunit is not due to generalized protein degradation, but may indeed require an activation of a specific caspase involved in apoptosis (66). Evidence is accumulating to attest to the fact that a constitutive expression/activation of NF-kB is essential for a cell to survive an apoptotic insult (67-69). All these observations underscore the importance of the protective role of NF-κB against photo-oxidative stress induced apoptosis in the 661W cells. A schemetic representation of the molecular events occurring in the course of photo-oxidative stress induced apoptosis of photoreceptor cell death are shown in figure 9.

Taken together, these results suggest that the RelA subunit of NF-κB constitutively expressed in 661W photoreceptor cells, may be important for photoreceptor cell survival. Exposure of the cells to visible light creates conditions of photo-oxidative stress, causing the production of reactive oxygen intermediates leading to

oxidative damage as evidenced by depletion of glutathione and increase in malonyldialdehyde formation. These oxidative events further result in the down-modulation of NF-κB (predominantly the RelA subunit), thereby exacerbating the oxidative damage and channeling the cells along a pathway of cell death, via apoptosis.

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Table 1. **Effect of photo-oxidative stress on malonyldialdehyde formation and glutathione levels in cultured photoreceptor cells.**

Treatment	MDA (nmol/ mg protein)	GSH Levels (μmol/mg protein)
Controls	0.306 ± 0.109 (n=4)	0.0258 ± 0.005 (n=3)
Light exposed	0.598 ± 0.040* (n=4)	0.010 ± 0.003* (n=3)
N-acetyl cysteine (2 mM) pre-treated and light exposed (n=4)	0.197 ± 0.067*	ND
Thiourea (7 mM) pre-treated and light exposed	ND	0.037 ± N.D.

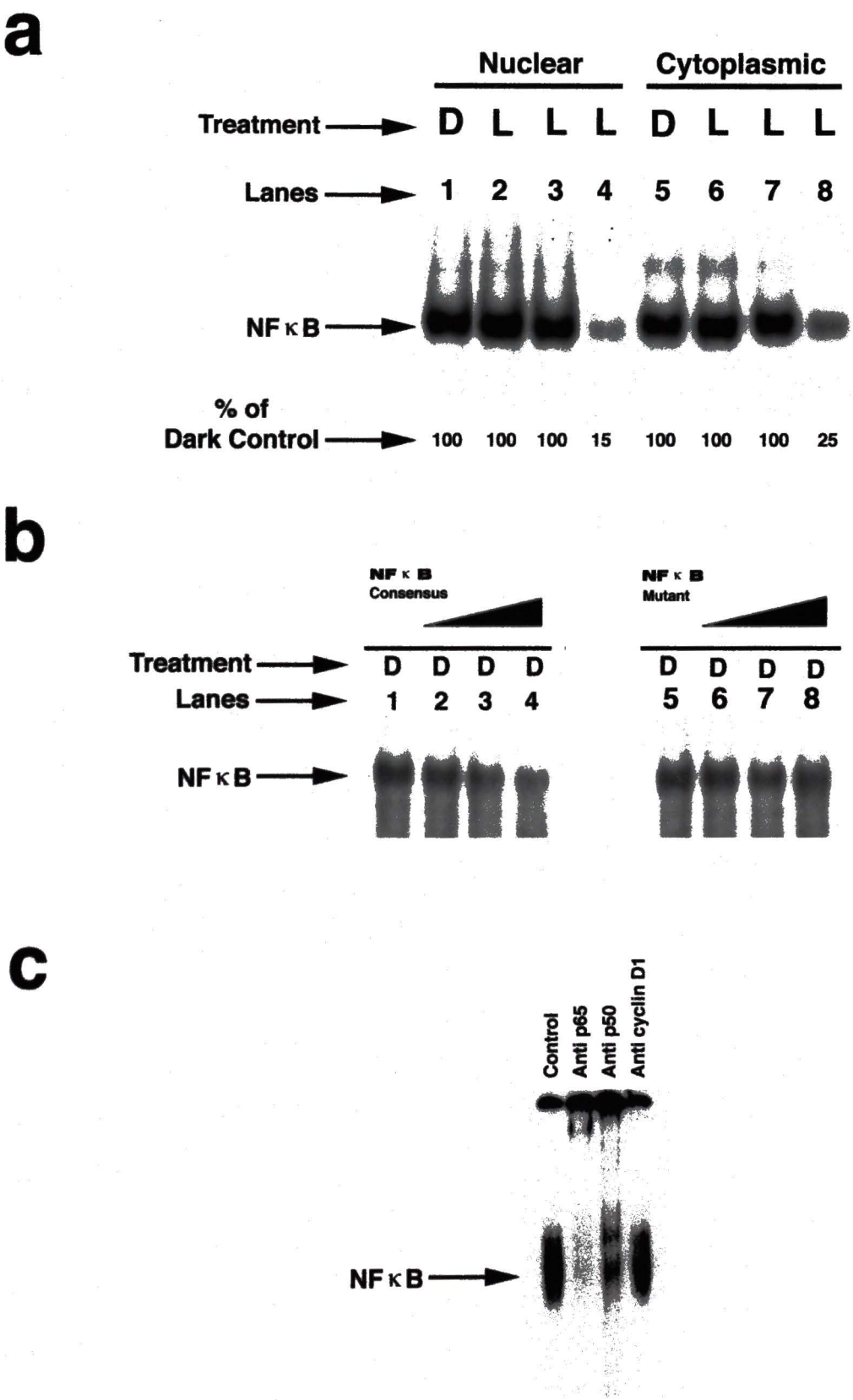
The values are expressed as mean ± standard error of mean.

Statistical analysis was done by Student's t test.

* significant at $p<0.05$.

N.D. = not determined.

Figure 1. **Effect of a time course of light exposure on NF-κB levels in 661W cells.** (a) 661W cells express NF-κB constitutively (lanes 1 and 5, for nuclear and cytoplasmic fractions respectively). Lanes 2 and 6; 3 and 7; and 4 and 8 represent NF-κB binding activity in nucleus and cytoplasm respectively after 15, 30 and 60 min of light exposure to the cells. For quantitation of the bands of the autoradiogram, a value of 100% was taken for dark exposed controls and the values for other samples were calculated as % of control and are shown below each lane. "D" and "L" represent dark exposed controls and light exposed cells respectively. (b) The identity of the shifted band seen in the EMSA was confirmed to be that of NF-κB by competition EMSA using molar excesses of cold consensus and mutant NF-κB oligos. Lanes 1 and 5 show NF-κB levels in dark exposed 661W cells in the absence of any competitor oligo. Competition EMSA using 50, 100, and 200 fold molar excess of cold consensus NF-κB oligo resulted in reduction of the NF-κB binding to the consensus sequence (Figure 1b, lanes 2-4). On the other hand competition with cold mutant NF-κB oligo using 50, 100, and 200 fold molar excess, did not result in a reduction of the NF-κB binding to consensus sequence (Figure 1b, lanes 6-8). (c) Further, the specificity of NF-κB binding was established by super shift assays using p50 and p65 antibodies . Anticyclin D1 was used as an unrelated antibody serving as a negative control. The results show that both p65 and p50 antibodies resulted in decrease in NF-κB band intensity, whereas anticyclin D1 did not have any effect on the binding reaction (Figure 1c).


a
Nuclear
Cytoplasmic
Treatment
D L L L D L L L
Lanes
1 2 3 4 5 6 7 8
NFκB
% of
Dark Control
100 100 100 15 100 100 100 25
b
NFκB
Consensus
NFκB
Mutant
Treatment
D D D D D D D D
Lanes
1 2 3 4 5 6 7 8
NFκB
c
Control
Anti p65
Anti p50
Anti cyclin D1
NFκB

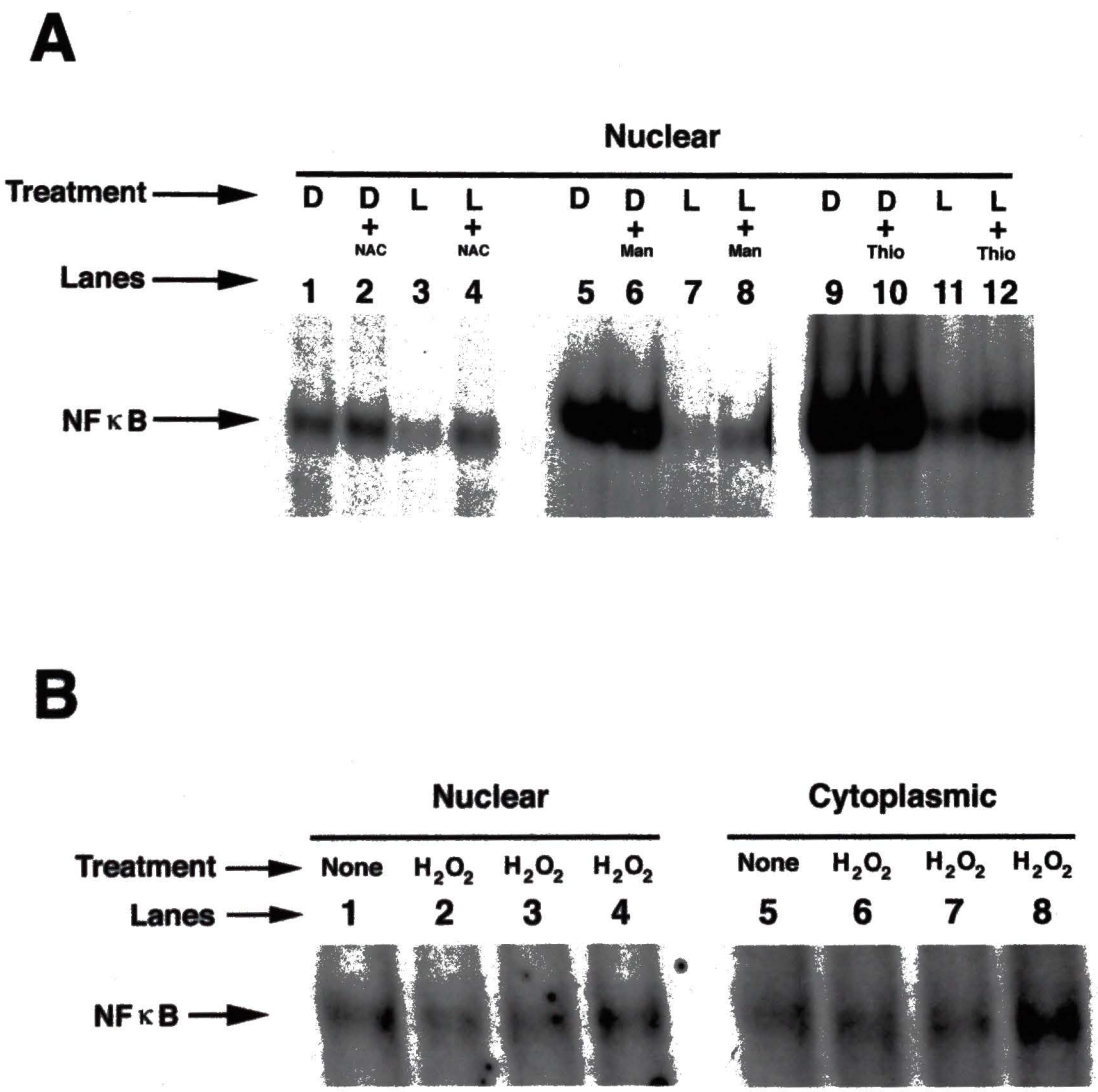

A
Nuclear
Treatment
D
D + NAC
L
L + NAC
D
D + Man
L
L + Man
D
D + Thio
L
L + Thio
Lanes
1 2 3 4 5 6 7 8 9 10 11 12
NFκB
B
Nuclear
Cytoplasmic
Treatment
None
H2O2
H2O2
H2O2
None
H2O2
H2O2
H2O2
Lanes
1 2 3 4 5 6 7 8
NFκB

Figure 3. **Immunoblot analysis of NF-κB p50, RelA, IκBα subunit in 661W cells exposed to photo-oxidative stress.** The cells were exposed to light for 2 hrs in presence or absence of NAC (2 mM), and thiourea (7 mM) and the levels of NF-κB subunits p50, RelA, and IκBα subunit were studied by immunoblot analysis. There was a decrease in the levels of IκBα, p50, and RelA subunit of NF-κB upon light exposure (lane 2) and these were protected to varying extents by pre-treatment of the cells with NAC and thiourea (lanes 3, and 4) respectively. The GAPDH levels were not altered between the experimental groups studied. "D" and "L", represent dark and light exposed 661W cells, respectively. "NAC", and "Thio" indicate N-acetyl cysteine, and thiourea respectively.

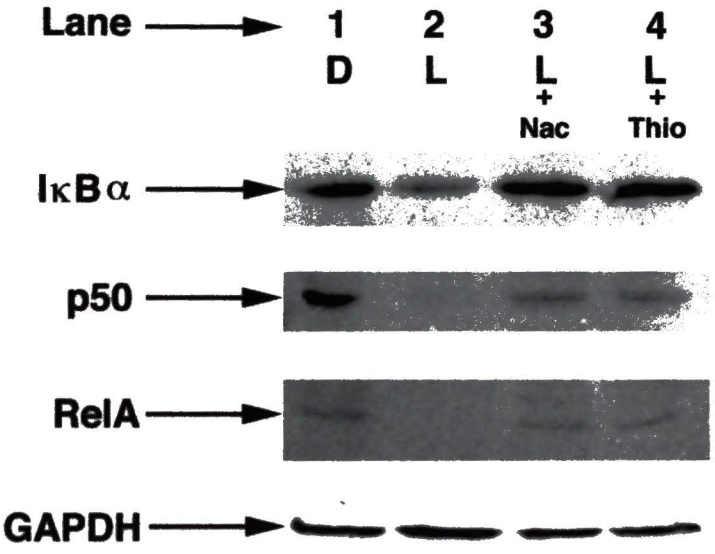

Lane
1
D
2
L
3
L
+
Nac
4
L
+
Thio
IκBα
p50
RelA
GAPDH

Figure 4. **Fluorescent TUNEL labeling of 661W cells after exposure to light for various time durations in presence or absence of NAC.** 661W cells with or without pre-treatment with NAC were exposed to light for various durations, fixed with 4% paraformaldehyde and processed for TUNEL labeling. There was a time dependent increase in the number of the cells labeled with the fluorescinated dUTP suggestive of apoptosis (white arrows) in 661W cells, with increasing duration of light exposure for 1 hour (A), 2 h (C) and 4 h (E). Not all the cells were undergoing apoptosis in 2 h light exposed group (C, arrowheads). Inclusion of NAC in the culture medium before light exposure, protected the cells from apoptosis (B, D, and F) for 1, 2, and 4 h of light exposure respectively. There were also a few apoptotic cells in the NAC pre-treated cells exposed to light for 4 h (F, arrow). Non-apoptotic cells were devoid of fluorescence.

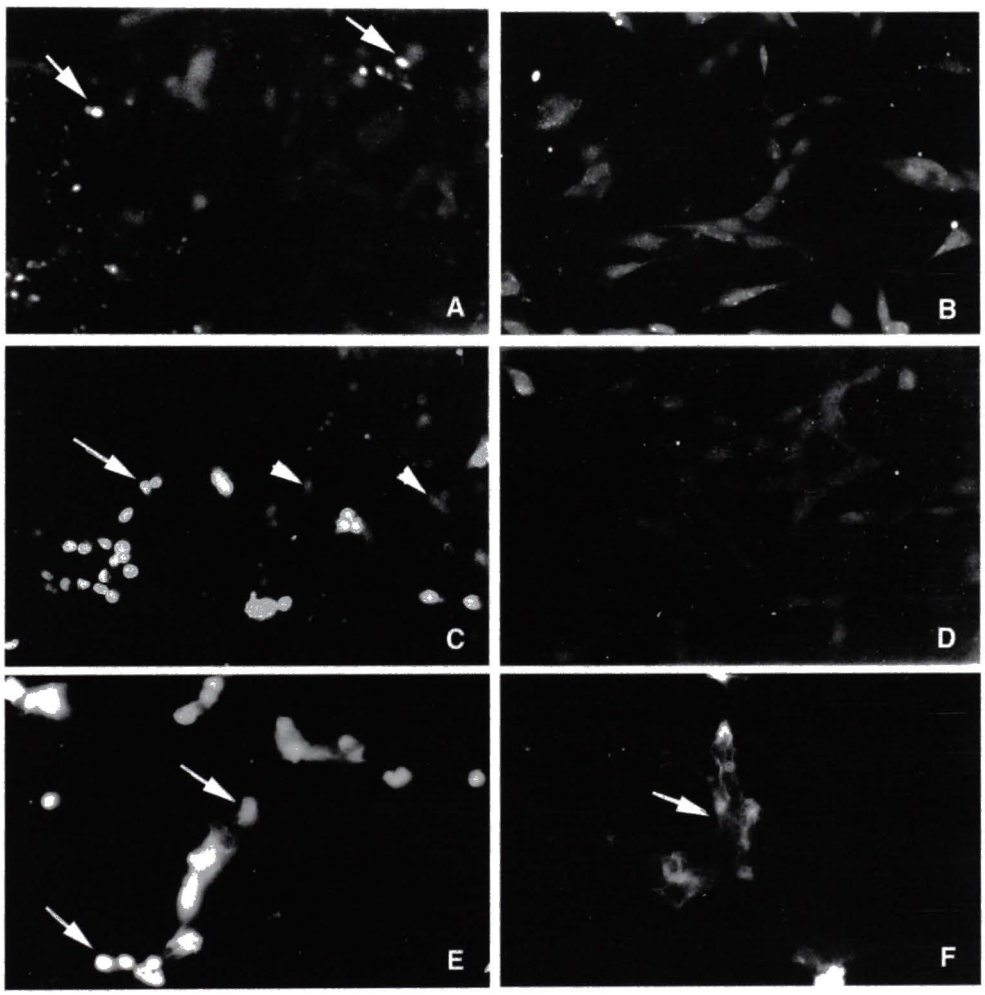

A
B
C
D
E
F

Figure 5. **Photo-oxidative stress and immunocytochemical localization of NF-κB RelA subunit in 661W cells.** The RelA subunit of NF-κB was predominantly present in the nucleus and in the cytoplasm of dark exposed control cells (A, arrows). Upon exposure to light, the nuclear and cytoplasmic labeling diminished considerably (B, arrows). In presence of NAC, a number of cells showed positive immunoreactivity in the nucleus as well as cytoplasm (C, arrows). Some nuclei devoid of RelA were also seen in NAC pre-treated cells exposed to light (arrowheads, C). These data indicate that light exposure brought about lowering of NF-κB levels in the nucleus as well as cytoplasm by an oxidative pathway.

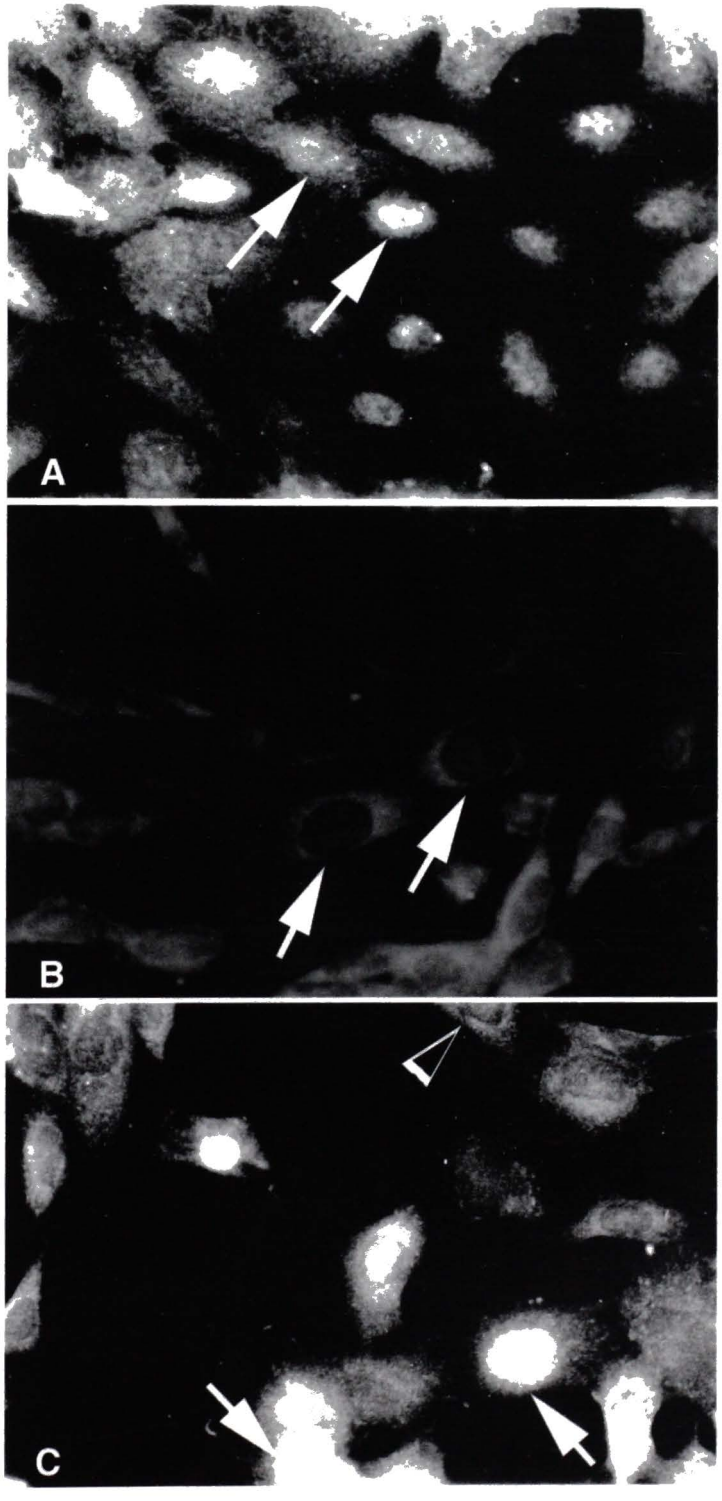
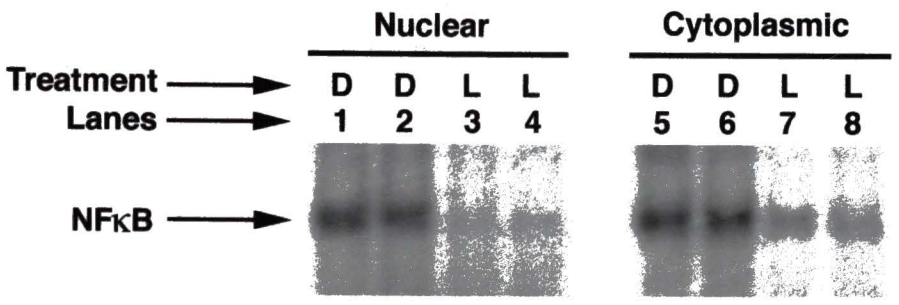

A
B
C

Figure 6. **NF-κB binding activity and apoptosis of ALLN-pretreated, dark and light exposed 661W cells.** (a) 661W cells were pretreated with ALLN (100 μM) and exposed to light for 1 hr. Pretreatment with ALLN did not protect against the down-regulation of NF-κB binding activity upon light exposure (lanes 4 and 8 for nucleus and cytoplasm respectively). Lanes 1 and 5 represent NF-κB binding activity in nucleus and cytoplasm of dark-exposed control cells. Lanes 2 and 6 show the NF-κB binding activity in ALLN pretreated, dark-exposed control cells. The activity was decreased upon exposure to light (lanes 3 and 7 for nucleus and cytoplasm respectively). (b) TUNEL assay of ALLN pretreated, dark- and light-exposed 661W cells. Pretreatment with ALLN caused a few cells to undergo apoptosis even in cells maintained in dark (B, arrows), compared to the untreated controls (A). Upon light exposure, a number of cells were TUNEL positive (C, arrows). Pretreatment with ALLN did not protect against photo-oxidative stress-induced apoptosis (D, arrows).

a


Nuclear
Cytoplasmic
Treatment
D D L L D D L L
Lanes
1 2 3 4 5 6 7 8
NFκB

b

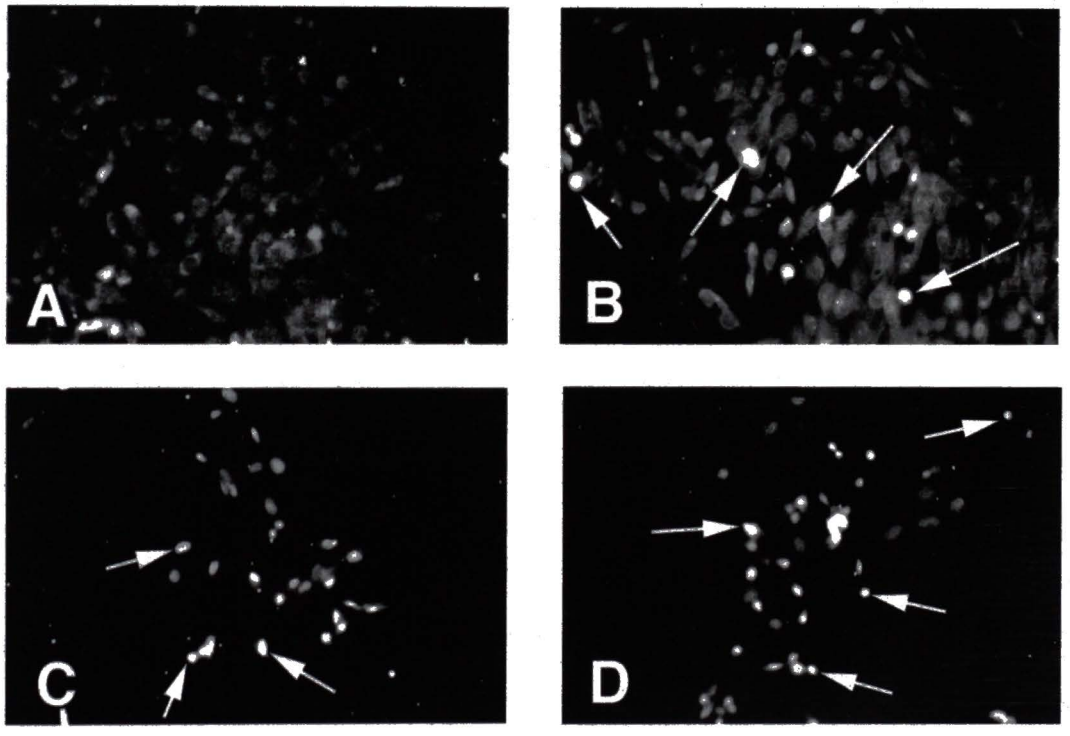

A
B
C
D

Figure 7. **Effect of IκBα super-repressor expression on NF-κB levels and apoptosis of 661W cells.** (a) 661W cells transfected with the super-repressor IκBα (+) and their corresponding control cells (-) were used for an immunoblot analysis of IκBα and IκBαΔN expression. There was a higher amount of IκBα expression in the transfected cells (lane 2) compared to the mock transfected control cells (lane 1). A faster moving band corresponding to the super-repressor IκBαΔN was also detected in the transfected cells, but not in the mock-transfected cells. The lower panel shows a duplicate immunoblot of β-tubulin levels, used as a control to show equal loading of protein in each lane. (b) EMSA of NF-κB binding activity in 661W cells transfected with the IκBα super-repressor, exposed to light for different time durations. The untransfected cells showed higher NFκB binding activity, in comparison to the transfected cells both in the nucleus (compare lane 1 with lane 2) and the cytoplasm (compare lane 9 with lane 10). A short time course of light exposure caused a rapid loss of NF-κB binding activity in the transfected cells in nucleus (lanes 4, 6 and 8 for 15, 30 and 60 min of light exposure) and cytoplasm (lanes12, 14 and 16 for 15, 30 and 60 min of light exposure). The untransfected cells showed much slower kinetics of NF-κB down regulation showing practically no change in the activity up to 30 min of light exposure in both nucleus (lanes 3 and 5) and cytoplasm (lanes 11 and 13). A significant decrease in the NF-κB binding activity in the untransfected cells was seen only upon 60 min of light exposure in both nucleus (lane 7) and cytoplasm (lane 15). For quantitation of the bands of the autoradiogram, a value of 100% was taken for dark treated controls for both the transfected and untransfected cells and the values for other samples were calculated as %

of control for each group and are shown below each lane. "D" and "L" represents dark and light repectively. (c) TUNEL assay of super-repressor IκBαΔN transfected 661W cells exposed to light for various time durations. There were no TUNEL positive cells in the untranfected cells maintained in the dark (A) and in those exposed to light for 15 min (B) and 30 min (C). There was induction of apoptosis only after 60 min of light exposure (D, arrows), in the untransfected cells. In contrast, transfection with the IκBα superrepressor caused a few cells to undergo apoptosis even in the cells maintained in the dark (E, arrows). There was a rapid increase in the number of TUNEL positive cells, in the transfected cells exposed to light for 15 (F), 30 (G) and 60 min (H) of light exposure (arrows).

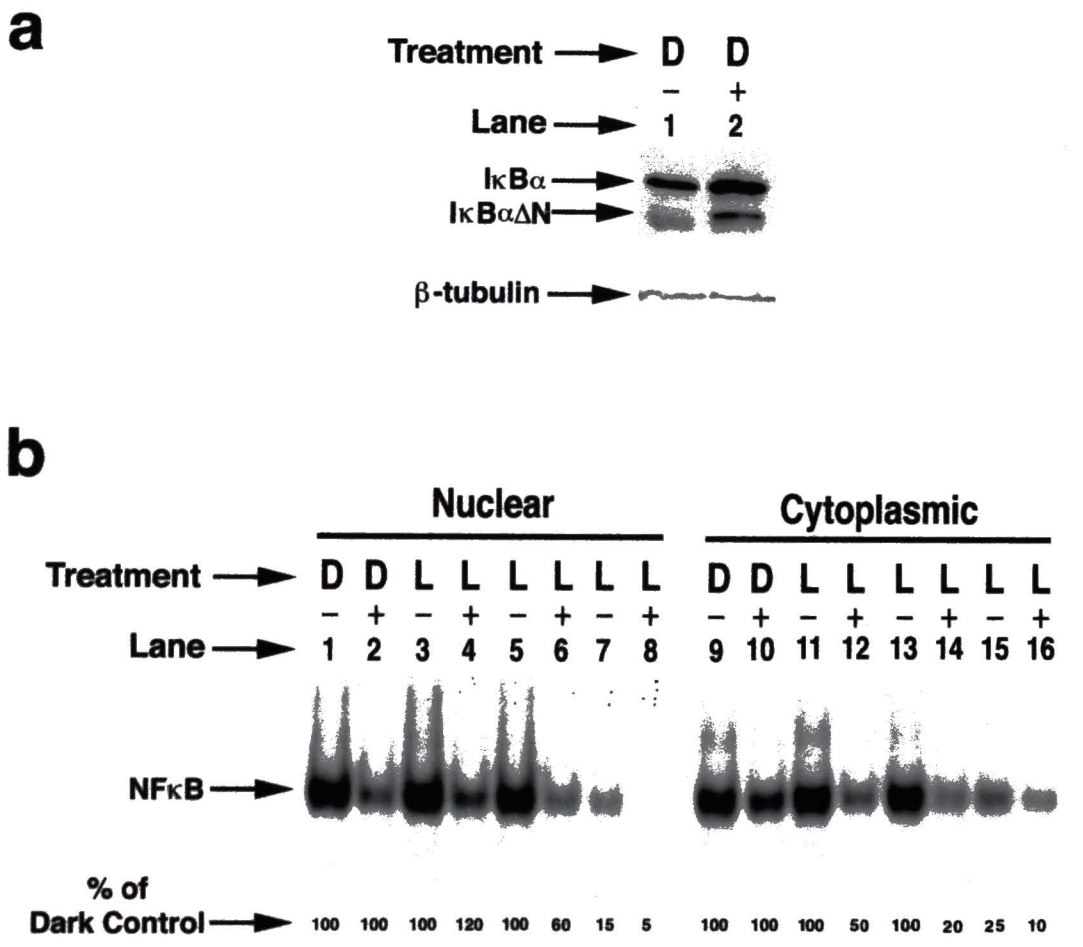

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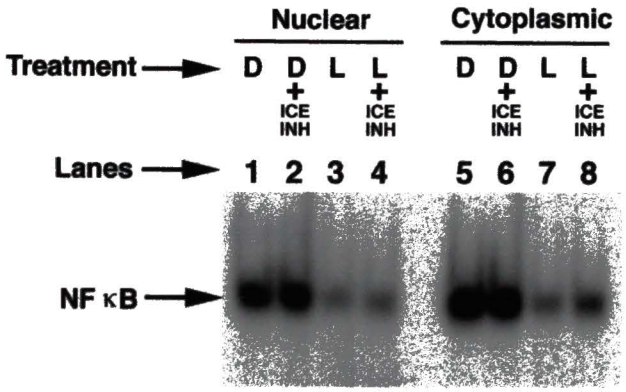
Figure 8. **Effect of pretreatment with caspase-1 inhibitor on NF-κB levels and apoptosis of 661W cells, upon exposure to light:** (a) 661W cells were pretreated with caspase-1 inhibitor (YVAD-CMK) (100μM) and NF-κB levels were studied in nuclear and cytoplasmic fraction, after exposure of the cells to light for 2 h. The inclusion of YVAD-CMK resulted in protection of NF-κB levels in light exposed 661W cells in both the nuclear (compare lane 4 with lane 3) and cytoplasmic fractions (compare lane 8 with lane 7). Inclusion of the caspase-1 inhibitor in dark exposed control cells did not alter NF-κB levels in both nuclear (lane 2 compared to lane 1) and cytoplasmic fractions (lane 6 compared to lane 5) respectively. "D" and "L" represent Dark- and Light-exposed 661W cells. (b) Fluorescent TUNEL labeling of caspase-1 inhibitor pre-treated 661W cells exposed to light. The cells maintained in the dark were devoid of apoptotic cells (A, arrowheads). A number of TUNEL positive cells were seen in the light exposed group (B, arrows). Pretreatment with the caspase-1 inhibitor was protective against apoptosis as seen by decreased TUNEL labelling in these cells (C, arrowheads). A few cells undergoing apoptosis were also seen in this group (C arrows).

a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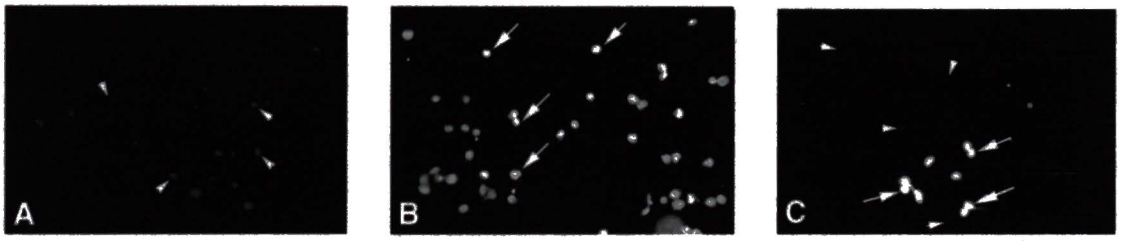
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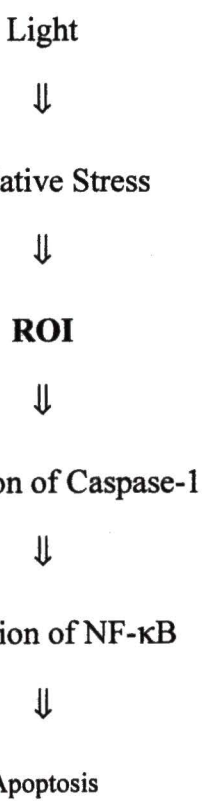
Figure 9. **Summary of the proposed mechanism of photo-oxidative stress induced apoptosis of photoreceptor cell death.**



PREFACE TO CHAPTER IV

The previous chapters have described the characterization of a mammalian clonal photoreceptor cell line and established that photo-oxidative stress results in cell death via apoptosis. The importance of constitutive expression of nuclear factor kappa B in the photoreceptors response to oxidative insults was also shown. This last chapter will further investigate the molecular changes in specific regulators of apoptosis, including bcl-2 and bax. The ability of bcl-2 gene over-expression to prevent light induced apoptosis and enhance survival will also be explored. Lastly, the effect of bcl-2 over-expression on the binding activity of nuclear factor kappa B will be addressed.

CHAPTER IV

Bcl-2 Over-Expression Protects Photo-Oxidative Stress Induced Apoptosis of Photoreceptor Cells via NF-κB Preservation

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Abbreviations Used: NF-κB, nuclear factor -κB; EMSA, electrophoretic mobility shift assay; TUNEL, terminal deoxynucleotidyl transferase mediated fluoresceinated dUTP nick end labeling, IκBα, Inhibitory subunit of NF-κB; MPT, mitochondrial permeability transition; AMD, age related macular degeneration

We recently showed that photo-oxidative stress to cultured photoreceptor cells results in down-modulation of the NF-κB activity which then leads to apoptosis of cultured 661W photoreceptor cells (1). In an effort to further delineate the mechanism of photo-oxidative stress induced apoptosis of photoreceptors, we sought to determine the effects of Bcl-2 over-expression on cell survivability. Wild-type 661W cells were transfected with the plasmid construct pSFFV-neo-Bcl-2 and several clones were isolated. All clones demonstrated increased Bcl-2 mRNA and protein levels with the B4 clone exhibiting the greatest enhancement. On exposure to photo-oxidative stress the B4 cells showed protection from photo-oxidative stress as compared with the untransfected cells, as seen by TUNEL apoptosis assay and formazan based estimation of their metabolic activity. The Bcl-2 over-expressing cells also maintained a higher Bcl-2/Bax ratio following photo-oxidative stress, as compared with the untransfected cells, suggesting that this ratio is important in protection from photo-oxidative stress. Electrophoretic mobility shift assays for NF-κB demonstrated higher activity in both nuclear and cytosolic fractions of the B4 photoreceptors as compared with the 661W cells at all light exposure time points. Furthermore, following photo-oxidative stress, immunocytochemistry for NF-κB revealed that protein levels of the RelA subunit of NF-κB were protected in the nucleus as well as in the cytoplasm of Bcl-2 over-expressing B4 cells, as compared to the corresponding wild type 661W photoreceptors subjected to similar light exposure. These results suggest that Bcl-2 over-expression protects NF-κB protein levels and activity in the nucleus. Thus, preservation of NF-κB binding activity

in the nucleus may be essential for photoreceptor cells to survive photo-oxidative damage induced apoptosis.

INTRODUCTION

Apoptosis has been described as the common pathway to photoreceptor cell death in many eye diseases such as hereditary retinal degenerations (2, 3). It has also been found to occur in age related macular degeneration (AMD), retinal detachment, and retinal light damage (3, 4). However, it is not obvious how the initial insult to the retina, which could be genetic, environmental, or both, drives the photoreceptor cell towards cell death. Therefore, an understanding of mechanisms that control apoptosis, is integral to the treatment and prevention of retinal diseases.

The Bcl-2 proto-oncogene, a member of the Bcl-2 gene family, is known to be a key regulator of apoptosis, functioning as an anti-apoptotic protein with the ability to protect against a variety of physiologic and pathologic insults (5, 6). Other members of the Bcl-2 family of apoptotic regulatory molecules include Bax, Bak, Bcl-X_L, Bad, and Bcl-X_S. Bax, Bcl-X_S, bad , and bak are classically described as a pro-apoptotic proteins, while Bcl-2 and Bcl-X_L have been shown to protect against apoptosis. Interaction between members of the Bcl-2 family in the control of apoptosis is complex. It is postulated that Bcl-2 and Bcl-X_L combine with homodimers of Bax, forming heterodimers which interrupt apoptotic signaling (7, 8). A recent in vivo study of synovial cells isolated from rheumatiod arthritis patients found an association between apoptotis and increased Bax/Bcl-2 ratio (9). Although Bcl-2/Bax interaction is well documented, it does not provide the sole explanation of how apoptotic events may be

regulated. For example, Bcl-X_L, acting independently of Bax, is also able to prevent apoptosis (10).

A proposed mechanism for Bcl-2's anti-apoptotic function is Bcl-2's interaction with members of certain anti-oxidant pathways. Bcl-2 is known to localize to the outer mitochondrial membrane, as well as to the endoplasmic reticulum and nuclear membranes (11, 12). The presence of Bcl-2 at the sites of free radical generation correlates with its ability to prevent oxidative cellular insults. Hockenbery et al. found that Bcl-2 is able to suppress lipid peroxidation and apoptotic cell death induced by hydrogen peroxide treatment (13). Similarly, Bcl-2 expression in neuroblastoma cells significantly lowered levels of oxidized lipids following potassium cyanide treatment in the absence of glucose (14).

Nuclear factor-κB (NF-κB) is a transcription factor which has recently been implicated with the cellular apoptosis. Subunits of NF-κB, p50 and p65 (RelA), form a heterodimer which exhibits DNA binding potential. NF-κB typically resides in the cytoplasm bound to an inhibitory subunit, IκBα or IκBβ, which prevents its translocation to the nucleus (28-30). The inhibitory constraint upon NF-κB is released by phosphorylation of IκB, which may be induced by cellular exposure to lipopolysaccharides, interleukin-1, tumor necrosis factor-α, or other inducers. NF-κB is constitutively active (i.e. the DNA- binding form is present in nucleus) in several cell lines including B Cells (31), thymocytes (32), neurons (33), synovial cells (9), and photoreceptor cells (1). NF-κB has been found to be an integral part of a cell's ability to survive apoptotic insult (34). Beg and Baltimore demonstrated that mouse fibroblasts

deficient in the RelA subunit of NF-κB had reduced viability following tumor necrosis factor-α treatment (34).

Recent studies suggest that Bcl-2 and NF-κB may be regulatory factors of a common apoptotic pathway in photoreceptor cells. A potential role of NF-kB p50 homodimer in the anti-apoptotic function of Bcl-2 has been proposed by Ivanov et al. (35). They showed that over-expression of Bcl-2 in a T-cell line resulted in increased NF-κB (p50 dimer) DNA binding activity, promoting cell survival. Furthermore, Mandal et al. found that over-expression of Bcl-2 restored NF-κB activity in HeLa cells undergoing anti-CD95 mAb-induced apoptosis (36). More recently, Bcl-2 was shown to degrade IκBα inhibitory subunit, resulting in NF-κB activation and rescue of neonatal ventricular myocytes from TNF-α induced apoptosis (37). These results suggest that one mechanism to explain Bcl-2 anti-apoptosis protection is that Bcl-2 acts as an upstream activator of NF-κB. However, other reports suggest an alternative interaction between Bcl-2 and NF-κB in which Bcl-2 related genes act as transcriptional targets for NF-κB. For instance, Dixon et al., observed that the five prime regulatory region of Bcl-X contains a consensus sequence for NF-κB which, when bound, results in increased expression of the pro-apoptotic gene Bcl- X_S (38). Similarly, NF-κB binding to a regulatory site in the promotor sequence of a pro-survival Bcl-2 homolog, Bfl-1/A1, was found to provide protection against apoptosis (39). Currently, there is no consensus on how Bcl-2 family genes and NF-κB interplay to alter the apoptotic status of cells. Since NF-κB has been found to effect pro and anti-apoptotic pathways in different cell lines, it is likely that the relationship between Bcl-2 and NF-κB may be cell type specific.

Earlier using 661W photoreceptor cells, we showed that photo-oxidative stress induced apoptosis was due to down-modulation of NF-κB activity via involvement of caspase-1 (1). Furthermore, photo-oxidative stress induced apoptosis could be prevented by the inhibition of caspase-1 and the inclusion of anti-oxidants in the cell culture media (1). Since Bcl-2 activation in other neural cells blocks activation of caspase proteases (40), it stands to reason that over-expression of Bcl-2 in our system should protect against caspase mediated down modulation of NF-κB, thus rescuing photoreceptors from an oxidative stress induced apoptosis. This report demonstrates the potential of Bcl-2 to rescue photoreceptors from apoptosis and implicates the involvement of NF-κB in the protection mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture - 661W and B4 cells are routinely grown in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Depending upon the experiment, cells were grown in either 35 mm or 100 mm cell culture dishes, 96 well culture plates, or multiwell culture plates with 12 mm coverslips. Cell counts were made using a model ZM Coulter Counter (Cereltu Electronics, Hialeah, FL).

Permanent transfection of 661W photoreceptor cells with the Bcl-2 gene - The 661W photoreceptor cells were originally isolated from a transgenic mouse line expressing the construct HIT1 comprised of SV40 T-antigen driven by the human interphotoreceptor retinol binding protein (IRBP) promoter (41). The 661W cells were permanently transfected with the Bcl-2 construct, pSFFV-neo-Bcl-2 (a kind gift from Dr. Korsmeyer), using the lipofectamine reagent (Life Technologies, Gaithersburg, MD), as per the manufacturer's instructions.

Briefly, 661W cells were grown to about 40% confluence in 100 mm cell culture dishes. The cells were washed thrice with 3 ml EBSS. In a 1.6 ml tube, 8µl lipofectamine was added to 92 µl serum free DMEM. In a second tube, 5 µg of the pSFFV-neo-Bcl-2 plasmid DNA was added and the volume bought to 100 µl with serum free DMEM. The contents of the two tubes were mixed together and allowed to incubate at room temperature for 30 min. Serum free DMEM (800 µl) was then added to the transfection

mix before placing it on the washed cells. After 5 hr incubation, after which 1 ml of DMEM containing 18% fetal bovine serum was added to the cells. They were then incubated for an additional 16 hr. The transfection mix was aspirated out of the plates and replaced with complete medium. Cells were subjected to selection pressure using Geneticin (300 μg/ml). Several Geneticin resistant clones were isolated by dilution cloning growth in 300 μg/ml antibiotic. One of the clones (B4) showed the highest amount of Bcl-2 expression as determined by immunoblot analysis. The B4 transfected cells and the mock transfected 661W wild type control cells were used for further studies.

Light Exposure of the Cells - The 661W and B4 cells were seeded either onto 35 or 100 mm cell culture dishes or onto 35 mm dishes containing12 mm diameter coverslips prior to exposure to fluorescent visible light at 4.5 mwatt/cm^2 for 1 or 2 hr. The UV light was blocked by using a plexi-glass filter. The control cells were shielded from light for similar time intervals.

3' end labeling of fragmented DNA by terminal deoxynucleotidyl transferase mediated fluorescinated dUTP nick end labeling (TUNEL) - The TUNEL procedure, as described by Gavrieli et al., was employed using a commercially available apoptosis kit (In situ cell death detection kit, Boehringer Mannheim, Indianapolis, IN) as per the supplier's instructions (42). 661W and B4 cells were seeded onto 12mm coverslips as described above and exposed to 1 and 2 hour light treatment periods.

Cell proliferation assay - A non-radioactive colorimetric assay for determining cell viability was employed to assess cell proliferation (CellTiter 96 AQueous Assay, Promega, Madison, WI). The assay is based on the bioreduction of Owen's reagent (MTS) to a formazon in the presence of an electron coupling agent (phenazine methosulfate). Aliquots containing 5 x 10^3 661W and B4 cells, respectively, were seeded in alternate wells of 96 well plates. Following 2 hr of light exposure, the cells were processed according to the supplier's instructions. The absorbance of the formazan at 490nm, as measured by an ELISA plate reader, estimates the number of viable cells. Measurements of the formazan in 661W and B4 controls (cells not exposed to light) served as a baseline for calculating the proliferative change following light exposure.

Immunoblot analysis - The cell lysates from 661W and B4 cells were subjected to immunoblot analysis (43) using Bcl-2 specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The binding of primary antibodies was detected using peroxidase labeled secondary antibodies and developed with a diamino benzidine substrate.

Immunolocalization studies - The 661W and B4 cells were exposed to light for 1 or 2 hr and fixed in 4% paraformaldehyde. Immunolocalization of the p65 subunit of NF-κB was performed using NF-κB (rel A) specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (44). Following secondary labeling with FITC

conjugated antibody, the immunofluorescent cells were photographed using a Nikon Microphot-FXA photomicroscope (Tokyo, Japan).

Preparation of Cytoplasmic and Nuclear Extracts - The 661W cells were exposed to light for the desired amount of time and the nuclear and cytoplasmic extracts were prepared (45). Briefly, the cells were suspended in 100 µl of buffer C (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 10% glycerol 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 15 min. Three ml of 10% NP-40 was added to the suspension which was then briefly vortexed. Following this, the nuclei were pelleted by centrifugation at low speed. The supernatant (cytoplasmic extract) was collected and stored at –80°C. The nuclear pellet was resuspended in 70 µl of buffer D (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF) and incubated for 20 min at 4°C before being centrifuged at 8000 g for 5 min. The resulting supernatant containing extracted nuclear proteins was transferred to a fresh microfuge tube and stored at -80°C. Protein concentrations of the cytoplasmic and the nuclear extracts were measured with the DC Protein Assay Kit (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assays (EMSA) - 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, CA) were used to study the DNA binding activity of NF-κB by EMSA as described (46). Briefly, the

double-stranded NF-κB oligos (50 ng) were end labeled with (γ-32P)-ATP (NEN) using T4 polynucleotide kinase. This labeled probe was then purified by ethanol precipitation. A DNA-binding reaction mixture 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 fmole of ^{32}P-labeled oligonucleotide (approximately 20,000 cpm) 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 min at 37°C. Subsequently, the samples were subjected to electrophoresis on a 4% native polyacrylamide gel using 0.25 X TBE. The gel was dried and autoradiographed.

RESULTS

Stable transfection of 661W photoreceptor cells with a plasmid construct encoding the Bcl-2 gene results in a new cell line which over-expresses the Bcl-2 protein - 661W photoreceptor cells were transfected with the plasmid construct pSFFV-neo-Bcl-2, containing the full length mouse Bcl-2 gene. The cells were subjected to selection pressure using a neomycin analog allowing only transfectants producing the neomycin transferase protein to survive. From the surviving cells, multiple clonal cell lines (B1, B2, B3, B4, B5) were isolated from single cell clones and maintained under continuous selection pressure. Analysis of Bcl-2 mRNA over-expression by RT-PCR analysis using a Bcl-2 specific primer set revealed a qualitative increase in Bcl-2 mRNA expression in the all the clones as compared to the 661W wild type (data not shown). One of the clones, B4, was shown to have the greatest Bcl-2 mRNA expression and was henceforth used as the experimental cell line (Figure 1, compare lanes 1 and 4). The results of RT-PCR analysis were further confirmed with immunoblot analysis using a polyclonal Bcl-2 antibody. As expected, the B4 clone exhibited higher levels of Bcl-2 expression (approximately 5 times of normal) as compared with the parent 661W cells (Figure 2). These results indicate that the B4 cells have both elevated levels of Bcl-2 mRNA and protein.

Over-expression of the Bcl-2 gene in 661W photoreceptor cells does not alter their inherent growth characteristics - Differences in growth characteristics between the 661W and B4 cells were compared to evaluate the influence of Bcl-2 over-expression on

growth rate of B4 cells. Six sets of either 661W or B4 cells were seeded at 1 x 10^4 cells/dish in triplicate and cell numbers determined at 3, 5, 7, and 8 days. There was no difference between growth characteristics of wild type and B4 cell lines as shown by doubling times derived from regression lines through the growth curve data (Figure 3). Both cell types divided approximately every 20-24 hr. These data indicate that over-expression of Bcl-2 did not alter the inherent growth characteristics found in the wild-type 661W photoreceptor cell.

Photo-oxidative stress results in a decrease in the Bcl-2/Bax ratio in photoreceptor cells due to down-regulation of Bcl-2 gene expression - As pointed out earlier, the balance between the pro-apoptotic and anti-apoptotic Bcl-2 family genes, Bax and Bcl-2, seems to play an integral role in determining whether or not cells will enter into the apoptotic pathway. The ratio of Bcl-2 to Bax in 661W photoreceptor cells was examined after zero, one hour, and two hours of light exposure. RT-PCR using Bax and Bcl-2 specific primers showed a progressive decline in the ratio following light exposure (Figure 1). The decreased ratio is accounted for by down regulation of the Bcl-2 mRNA in the light exposed cells, with no concomittant changes seen in Bax mRNA. As discussed previously, both B4 and 661W photoreceptor cells exposed to light, exhibited decreased Bcl-2 levels, but the Bcl-2/Bax ratio remained elevated in B4 cells compared to the wild-type cells.

To further corroborate the Bcl-2 mRNA results, we studied the effect of photo-oxidative damage on Bcl-2 protein levels. A decrease in Bcl-2 protein levels following

2 hours of light exposure was seen by immunoblot analysis of light exposed B4 cells (Figure 4). It is important to remember that the Bcl-2 protein level in the Bcl-2 transfected cells remained higher than wild-type 661W cells under similar light exposure.

Over-expression of the Bcl-2 gene protects photoreceptor cells from photo-oxidative stress induced metabolic and apoptotic changes - To establish the role of the Bcl-2 in preventing oxidative damage to photoreceptor cells, 661W and B4 cell lines were subjected to 1h or 2h of light treatment. TUNEL labeling revealed a decreased incorporation of fluoresceinated dUTP in the nuclei of B4 cells indicating a relative protection from apoptosis (Figure 4). The 661W cells showed no such decrease. Approximately all of the wild-type 661W photoreceptors underwent apoptosis versus only 40% of the Bcl-2 over-expressors.

We also sought to determine the effect of oxidative stress on the metabolic activity of 661W photoreceptor cells and potential protection afforded by Bcl-2. To measure metabolic activity, wild-type and B4 photoreceptor cells that had been exposed to 2 hr light were evaluated for their ability to catalyze the reduction of MTS to a formazan. Values for formazan appearance in dark controls served as a baseline for calculating percent decreased proliferation following light exposure. As expected, the wild-type photoreceptors showed a dramatic decrease in survival/proliferation following light exposure, while B4 cells were protected as reflected by a nearly three-fold increase in cell survival (Figure 5).

Over-expression of the Bcl-2 gene results in protection of NF-κB rel A subunit activity - Immunocytochemistry using antibodies specific for the relA subunit of NF-κB was performed on 661W and B4 photoreceptor cells after they had remained in the dark or light for 2 hr (Figure 6). NF-κB relA was distributed in both the cytoplasm and nucleus in the dark exposed 661W and B4 cells. But following 2 hr of light exposure there is an obvious depletion of relA subunit of NF-κB labeling in the nucleus and cytoplasm of the 661W wild-type photoreceptors (Compare A and B) while nuclear levels of NF-κB in the cytoplasm and the nucleus of B4 cells are relatively preserved (Compare C and D).

The protection of photoreceptor cells from light induced apoptotis is associated with increased binding activity of the nuclear transcription factor NF-κB - Electrophoretic mobility shift assays were performed on nuclear and cytoplasmic protein extracts from dark control and light exposed wild type and B4 cells, using a NF-κB consensus oligo. We found that at both durations of light exposure, the levels of NF-κB were found to remain constant in B4 photoreceptors, as compared to the wild-type cells. There was 45% less NF-κB binding following 2 hr of light exposure in 661W cells as compared with the B4 cells, in which only a negligible 10% decrease was observed. Similarly, cytoplasmic NF-κB binding diminished 70-80% in 661W cells after 1 and 2 hr light exposure as compared to the B4 cells, which showed only a 30-40% decrease following the same light exposure (Figure 7).

DISCUSSION

The mechanism by which photoreceptor cells degenerate in many hereditary and environmentally acquired eye diseases is via apoptosis. For nearly all diseases involving photoreceptor cells, treatment options are limited to interrupting the pathophysiologic processes which ultimately result in vision deficits. For example, in age related macular degeneration treatment is limited primarily to laser photocoagulation of affected areas of the retina as a means of interrupting the progressive disease process. In hereditary retinal dystrophies, such as retinitis pigmentosa, there are currently no treatments effective at stopping the progression of vision loss. Vitamin A supplementation has been shown to slow the rate of retinal function decline (as evaluated with cone electroretinogram amplitude) in retinitis pigmentosa, but has no statistically significant effect on visual acuity (47). Without knowledge of the pathogenesis of ocular disease at a molecular level, there is little hope of prevention or adequate intervention. Understanding the biochemical control pathways for apoptosis in photoreceptor cells may provide answers to critical questions involved in the etiology of eye diseases and lead to new treatments/and or prevention.

Our in vitro model for examining the apoptotic pathways in photoreceptor cells is a transformed mouse photoreceptor cell line, 661W. The 661W cell line expresses markers of differentiated photoreceptor cells. Previous work from our lab has shown that light exposed 661W cells undergo oxidative changes including increased malonyldialdehyde and decreased glutathione levels. Additionally, we have shown that light exposure resulted in down modulation of the NF-κB activity and apoptosis of the

661W cells (1). Caspase-1 activity appears to be responsible for the NF-κB degradation, as evidenced by the protection of NF-κB activity provided by the caspase-1 inhibitor, YVAD-CMK (1). Transient transfection with a repressor of NF-κB activity, IκBαΔN, resulted in increased apoptosis of the 661W photoreceptor cells suggesting NF-κB is essential for survival. We have expanded upon these studies in an effort to determine upstream regulation of photo-oxidative stress induced photoreceptor cell death and elucidate the mechanism of protection. With the goal of formulating intervention strategies to rescue photoreceptors from apoptosis, we have transfected the cells with the Bcl-2 gene.

The current study focuses on the involvement of the Bcl-2 family of genes as upstream modulators of NF-κB activity and the ability of Bcl-2 to protect against NF-κB degradation and subsequent photoreceptor cell apoptosis. RT-PCR analysis using Bcl-2 and Bax specific primers, showed an increase in the ratio of Bcl-2 to Bax in the B4 cells compared to the wild type 661W cells both under normal conditions and after photo-oxidative insult. Consistent with the literature (7- 9) we found that the increased levels of Bcl-2 mRNA and the resultant greater amounts of Bcl-2 protein imparted a greater degree of protection from apoptosis. There are no specific studies addressing Bcl-2 and Bax levels in photoreceptor cells following oxidative insult, but human leukocyte HL-60 cells respond similarly to the 661W cells with Bax remaining constant and Bcl-2 declining (48). Since in both the leukocyte study and our own, no changes in Bax mRNA were seen, it is possible that changes in other pro-apoptotic members of the Bcl-2 family (Bak, Bad, Bcl-XS) may occur following oxidative stress.

By TUNEL labeling for apoptotic nuclei, Bcl-2 over-expression was shown to confer protection against apoptosis in the photoreceptor cells. These findings support previous experiments with Bcl-2 over-expressing transgenic mice (49, 50). Both of these articles describe protection from prolonged light exposure, the results differ regarding the ability of Bcl-2 to protect against the ultimate retinal degeneration. In our studies, after two hours of light exposure less than half the B4 cells exhibited apoptotic changes versus nearly 100% of the wild type 661W cells. Our prior research demonstrated the ability of antioxidants to protect 661W cells from photo-oxidative stress induced apoptosis (1). Considering the known oxidative changes occurring in these cells following light exposure, and the well established role of Bcl-2 as an anti-oxidant, it follows that this protection may be mediated by a change in the redox potential of the cells. Assays measuring metabolic activity suggested B4 cells were better able to continue proliferating even after 2 hrs of light exposure. Since increased expression of Bcl-2 is known to have the potential of being tumorogenic (hence the origin of the Bcl-2 name: B cell lymphoma), the inherent growth characteristics of the transfected cells were a concern. But wild-type 661W cell and B4 cells had nearly identical doubling times (Figure 3), indicating no difference in proliferative potential. This finding is important in demonstrating that the B4 cell lines increased metabolic activity is directed to Bcl-2's protective role against oxidative stress rather than towards an increasing rate of proliferation.

Our research has demonstrated an important relationship between the Bcl-2 protein and NF-κB expression in photoreceptors. When the 661W cells are forced to

over produce the Bcl-2 protein, NF-κB rel A labeling in the cytoplasm and nucleus is preserved following light exposure. Additionally, electrophoretic mobility shift assays of NF-κB in B4 photoreceptor cells revealed that there was preservation of NF-κB activity in both cytoplasmic and nuclear fractions compared with the untransfected wild-type 661W cells. Following exposure to light, the NF-κB activity was down-modulated in both the 661W and B4 cells, but concentrating on the 2 hr time, for the B4 cells there was a greater degree of safeguarding of NF-κB levels. In addition, there was preservation of NF-κB protein labeling in the nucleus of B4 cells subjected to oxidative stress which correlated with the maintenance of NF-κB activity seen by EMSA. We assume that this was due to the higher basal activity of the B4 cells prior to light exposure, although, it is possible that Bcl-2 interacts with NF-κB during the actual insult to preserve NF-κB activity. Taken together these findings illustrate that NF-κB protein levels and binding activity are protected in photoreceptor cells as a result of Bcl-2 over-expression.

Bcl-2 interaction with NF-κB in the prevention of apoptosis is a relatively new concept that is well supported by the work of Zong et al., and de Moissac (37, 39). In support of the role of Bcl-2 as an upstream regulator of NF-κB was the revelation that Bcl-2 could degrade the cytoplasmic inhibitor of NF-κB, IκBα. IκBα degradation resulted in an increase in the nuclear activity of NF-κB and guarded ventricular myocytes from tumor necrosis factor alpha (TNF-α) induced apoptosis (37). However, Zong and colleages (39) present an alternative means to explain Bcl-2's protective effect on NF-κB activity. They suggest an interaction between NF-κB and Bcl-2, in which NF-κB binds to the promoter site of the anti-apoptotic Bcl-2 homolog, Bfl-1/A1, thereby increasing its

expression and preventing TNF-α induced apoptosis (39). Thus, the evidence supporting a connection between Bcl-2 and NF-κB in apoptotic pathways is strong, but more information is needed to elucidate the different biochemical interactions which may be occurring.

Our previous research implicates caspase-1 in the degradation of NF-κB following oxidative stress and reactive oxygen intermediate formation (ROI) (1). Bcl-2 has been shown to effect caspase activation through its control of cytochrome c, a mediator of caspase activation and apoptosis (51, 52). A likely explanation for the ability of Bcl-2 to protect against ROI induced down-modulation of NF-κB activity in the 661W photoreceptor cell is through the regulation of caspase activation. Our findings of elevated NF-κB protein labeling and activity in the B4 cells suggest Bcl-2 also positively affects NF-κB homeostasis in the photoreceptor after oxidative insult. In short, our results suggest a positive role for the Bcl-2 protein in the regulation of apoptotic pathways of photoreceptor cells. In addition to the ability of Bcl-2 to protect against photo-oxidative stress induced changes in viability, we suggest that the nuclear transcripton factor NF-κB is involved in the protection mechanism.

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Figure 1. **RT-PCR analysis of Bcl-2 and Bax mRNA levels of 661W and B4 cell lines in dark and light exposed conditions.** RT-PCR analysis of Bcl-2 and Bax mRNA levels for 661W and B4 photoreceptor cells incubated in dark, 1 h of light, and 2 h of light. Dark exposed B4 photoreceptors, exhibited a large elevation of Bcl-2 mRNA expression. Following 1 h and 2 h of light exposure, the amount of Bcl-2 message is down-modulated in both cell lines, but the B4 photoreceptors maintain higher levels of Bcl-2 than 661W cells after all periods of light exposure. Neither 661W nor B4 cells exhibited any difference in Bax mRNA levels following light exposure. D=dark exposed cells; 1 and 2= cells exposed to 1h and 2h of light respectively; and B=blank water control. On quantitation of Bcl-2/Bax ratios using NIH Image program, B4 cells exhibited higher Bcl-2/Bax ratio than did the 661W wild type cells (bar graph). RT-PCR experiments were performed three times to verify consistency of results.

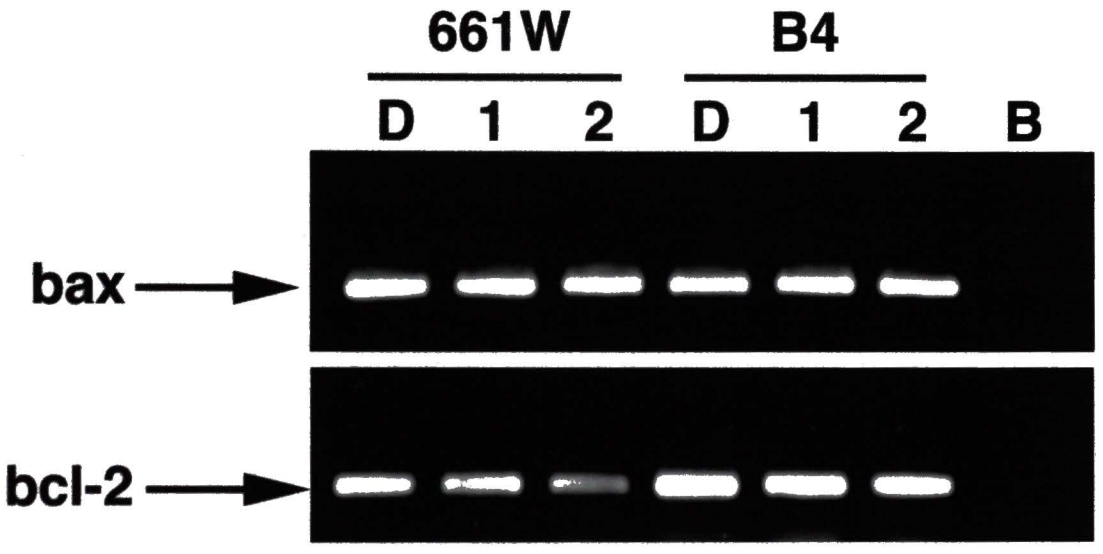

661W
B4
D
1
2
D
1
2
B
bax
bcl-2

Figure 2. **Over-expression of Bcl-2 protein in B4 photoreceptor cell.** Cell lysates from 661W photoreceptor cells transfected with pSFFV-neo-Bcl-2 (B4 cell line) and wild-type 661W cells were used for immunoblot analysis of Bcl-2 protein expression. The B4 cell line exhibited a nearly five-fold increase in Bcl-2 protein expression as compared with the wild-type photoreceptors (compare lanes 2 and 4 for B4 with lanes 1 and 3, for 661W wild type cells respectively).

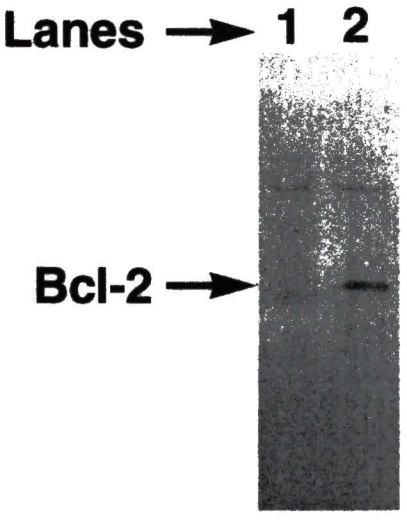

Lanes → 1 2
Bcl-2 →

Figure 3. **Growth characteristics of the 661W and B4 photoreceptors.** Equal numbers of 661W and B4 photoreceptor cells were seeded in triplicate in multiple culture dishes and counted with coulter counter at 3, 5, 7, and 8 days. The cell counts were plotted on a log y-axis. Regression lines showed no significant difference between the log growth curves of the 661W and Bcl-2 over-expressing B4 cell lines.

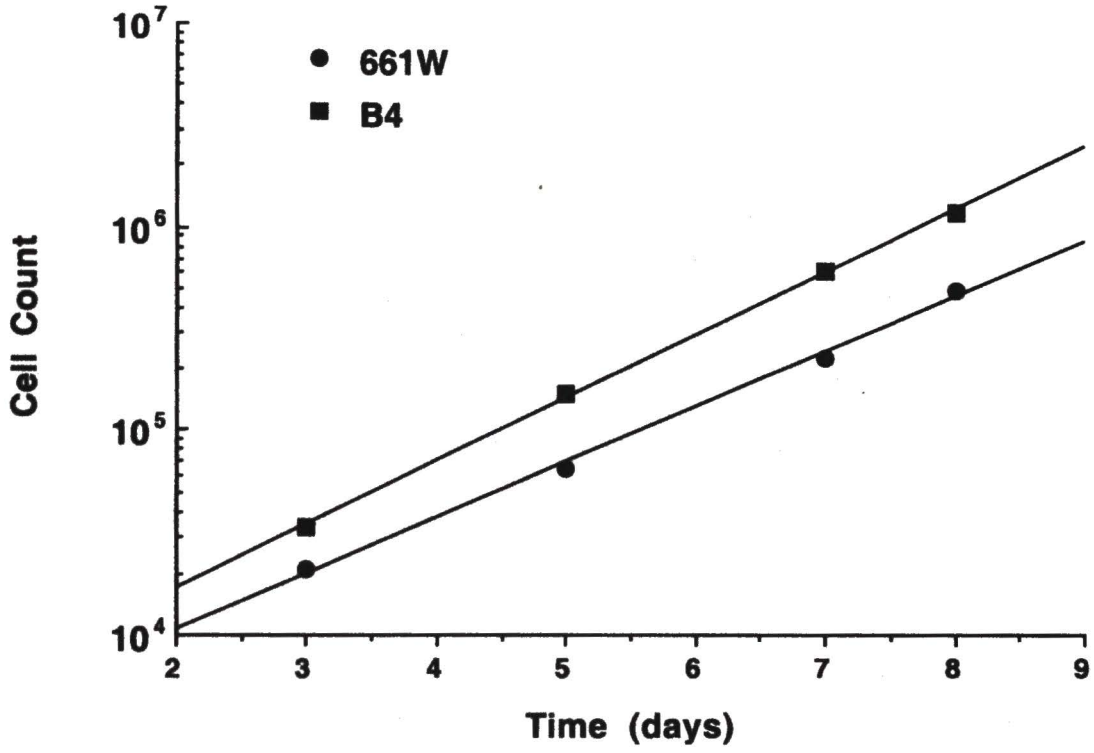

661W
B4
Cell Count
10^7
10^6
10^5
10^4
2
3
4
5
6
7
8
9
Time (days)

Figure 4. **Effect of Bcl-2 over-expression on photo-oxidative stress induced apoptotic cell death.** 661W and B4 photoreceptors were exposed to a dark control environment or 2 h visible light exposure and evaluated for apoptotic changes by TUNEL labeling. Neither the 661W (A) nor B4 (B) dark-exposed controls exhibited any apoptotic cell death. Following the 2 h light exposure, 100% of the 661W photoreceptors (C) were undergoing apoptosis, while less than half of the B4 photoreceptors (D) showed apoptotic changes under similar conditions.

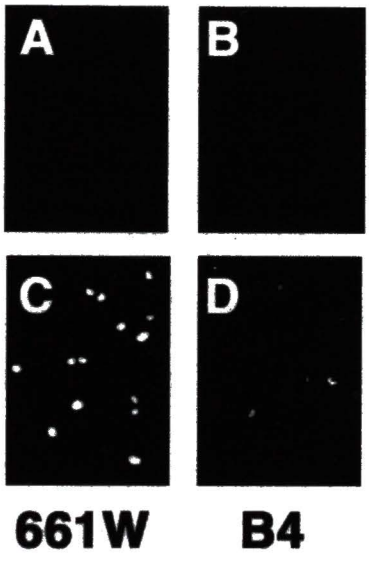

A
B
C
D
661W
B4

Figure 5. **Effect of Bcl-2 over-expression on photoreceptor cellular proliferation following photo-oxidative stress.** 661W and B4 cells were evaluated for light exposure induced viability changes by measuring the reduction of MTS to formazan using dark exposed controls as the baseline activity. The percentage of cellular viability remaining in 661W and B4 cells was calculated following the 2 hr of light exposure. * denotes statistical difference ($p < 0.001$, ANOVA) from the control group of 661W wild type cells. Error bars indicate standard error.

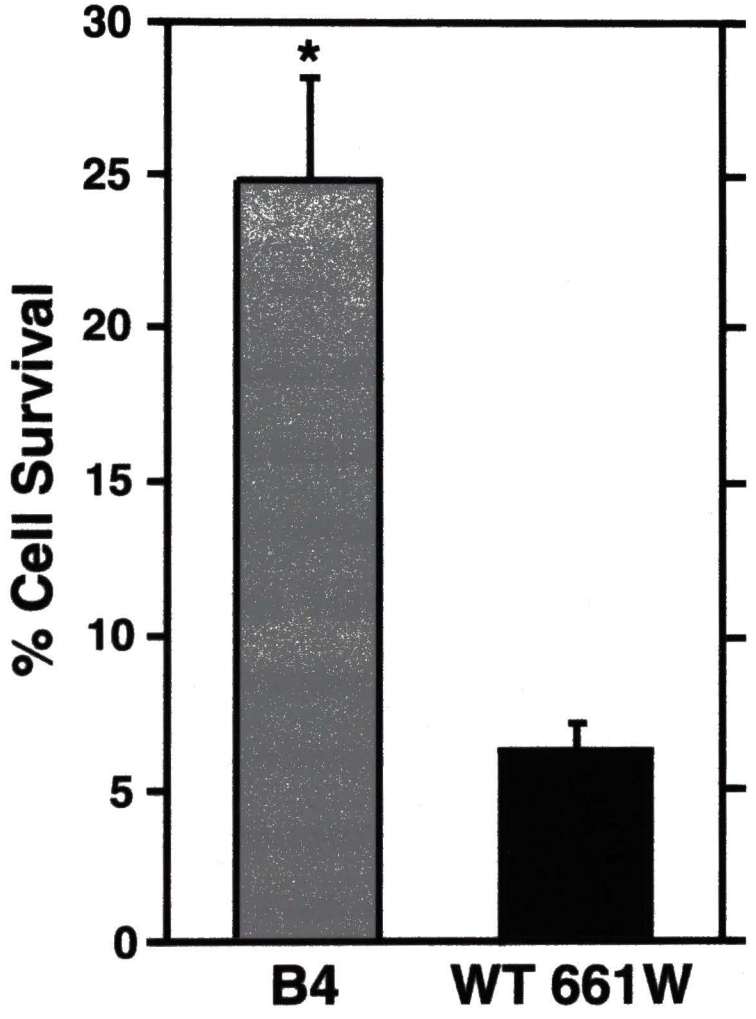
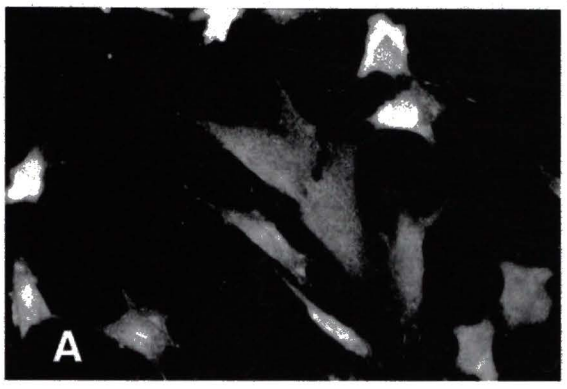
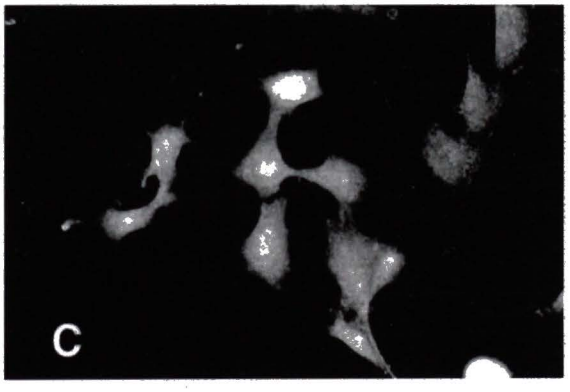

30
25
20
15
10
5
0
*
% Cell Survival
B4
WT 661W

Figure 6. **Immunocytochemical localization of NF-κB (RelA) in 661W and B4 photoreceptors exposed to light.** RelA labeling of the NF-κB subunit in 661W and B4 cells was studied. The results show RelA labeling in both the cytosol and nucleus of 661W and B4 cells (A and C respectively). Following 2 hr light exposure, 661W cells show loss of nuclear and cytosolic RelA labeling, while the Bcl-2 over-expressing B4 cells exhibit relative preservation of RelA labeling in both the cytosol and nucleus (compare B and D for 661W and B4 cells, respectively). Arrowhead in B indicates a cell with depleted RelA fluorescence in 661W cells exposed to photo-oxidative damage.


A


B


C

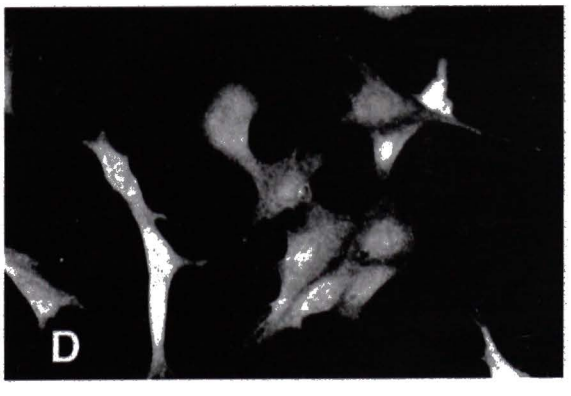
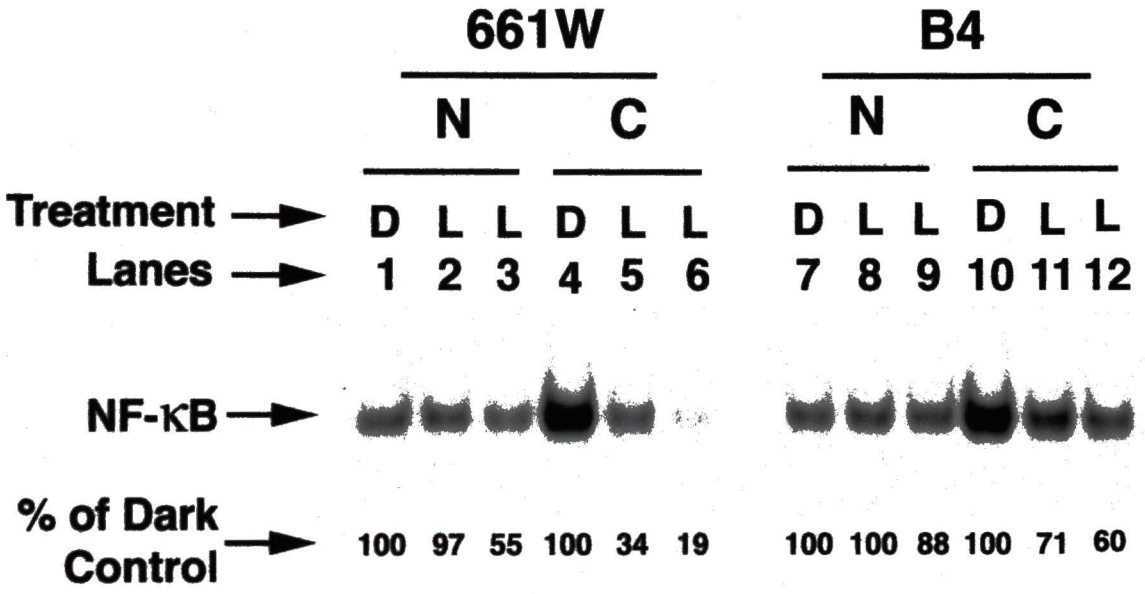

D

Figure 7. **NF-κB electrophoretic mobility shift assays for 661W and B4 photoreceptors exposed to light.** Radiolabled NF-κB consensus binding sequence was used to determine the activity of NF-κB binding in nuclear and cytoplasmic fractions of 1 h and 2 h light exposed 661W and B4 cells. The Bcl-2 over-expressing B4 photoreceptor exhibit preservation of NF-κB binding activity as compared with the wild-type 661W cell line. For quantitation of the NF-κB bands of the autoradiogram using NIH Image program, a value of 100% was taken for dark treated (D) controls for 661W and B4 and are shown below each lane. The lanes 1, and 2 represent 1h and 2h of light exposure (L), respectively. N and C= nuclear and cytosolic fractions respectively.


661W
B4
N
C
N
C
Treatment
D L L D L L D L L D L L
Lanes
1 2 3 4 5 6 7 8 9 10 11 12
NF-κB
% of Dark Control
100 97 55 100 34 19 100 100 88 100 71 60

CHAPTER V

CONCLUSIONS AND FUTURE STUDIES

Apoptosis is the predominant mechanism responsible for photoreceptor cell death in most retinal diseases. The initial insult leading to photoreceptor cell death may be environmental or genetic, but the final pathway of cell death is a common denominator. Without knowledge of the pathogenesis of ocular disease at a molecular level, there is little hope of prevention or adequate intervention. Understanding the biochemical control pathways for apoptosis in photoreceptor cells may provide answers to critical questions involved in the etiology of eye diseases and lead to new treatments or preventative measures. Although multiple regulatory mechanisms play a role in the control apoptosis, the involvement of proto-oncogenes of the bcl-2 family are both predominant and well conserved throughout evolution. While bcl-2 regulation of apoptosis has been studied nearly as long as apoptosis itself, investigation into nuclear transcription factor kappa B involvement is nascent. A key component of this dissertation is the elucidation of how bcl-2 and NF-κB conspire in the control of photoreceptor apoptosis.

A limitation of researchers investigating photoreceptor disease has been the lack of an appropriate cell line. In retinal cultures, there is a confounding influence of multiple cell types which interferes in the study of photoreceptors. Additionally, preparation of retinal cell cultures is time-intensive and the cells have limited passage

potential. We identified the need for a homogeneous cell line which expresses a photoreceptor phenotype and has unlimited ability for passaging. The 661W cell line was established by expressing the viral oncoprotein simian virus 40 large tumor antigen (SV40 T-antigen) under control of the human IRBP promoter in a transgenic mouse. Studies within this dissertation characterize the 661W cell line as having photoreceptor origin. This is based upon the expression of photoreceptor specific genes and proteins including opsin, phosducin, rds/peripherin, arrestin, and IRBP. Also important is the characteristic absorption spectrum for isorhodopsin at 485 nm in the presence of 9-cis retinal. This suggests that quantities of opsin produced in these cells are sufficient for detection of spectral changes in opsin upon photobleaching of 661W cell membranes, indicating the cells ability to generate functional rhodopsin molecules. The 661W cells grow well using basic tissue culture techniques and retain their photoreceptor characteristics beyond 50 passages.

The in vitro study of apoptosis typically requires an induction protocol that initiates the cell death pathway. Our experiments utilize a photo-oxidative stress paradigm that results in apoptotic cell death, allowing observation of molecular changes. Oxidative stress in these cells was evidenced by elevated malonyl dialdehyde and decreased glutathione levels. Additionally, we demonstrated the ability of anti-oxidants to prevent the light-induced ROS production and apoptosis. The constitutive expression of NF-κB in the 661W cell line and its apparent role in the prevention of apoptosis are important revelations of our research. Following photo-oxidative stress, nuclear NF-κB protein levels and binding activity are diminished, suggesting a role for this nuclear

transcription factor in protection against oxidative apoptotic stresses. Inclusion of the anti-oxidants N-acetylcysteine, dimethylthiourea, and mannitol prior to light exposure resulted in protection against down-modulation of NF-κB binding activity. These findings suggest a role for ROS in the regulation of NF-κB activity. To test the hypothesis of caspase involvement in oxidative stress down-regulation of NF-κB activity, we evaluated the effect of protease inhibitors specific to caspase-1 and caspase-3 on NF-κB activity. Caspase-1 inhibitor, but not caspase-3 inhibitor, was found to protect against light induced apoptosis and NF-κB activity down-regulation. Together, these experiments demonstrate a role for ROS and caspases in modulating photoreceptor cell response to oxidative stress via NF-κB activity. To the best of our knowledge, these are the first studies demonstrating NF-κB's role in the prevention of photoreceptor cell death.

Bcl-2 message and protein was shown to decrease following our light exposure protocol, suggesting transcriptional regulation of proto-oncogenes as a means of executing apoptosis in photoreceptor cells. As a prospective method of interrupting oxidatively mediated photoreceptor cell death we transfected the 661W cells with the bcl-2 gene. Bcl-2 over-expression was able to protect against light-induced apoptotic cell death, without interfering with the inherent growth characteristics of the 661W cell. Having established the role of bcl-2 in protecting 661W cells from apoptosis we sought to determine how elevated bcl-2 levels affected NF-κB activity within the photoreceptor cells. We previously described the importance of active binding NF-κB in the 661W photoreceptor cells ability to overcome an oxidative insult. We found bcl-2 over-expression in photoreceptor cells resulted in increased NF-κB binding activity before and

after light exposure, as compared to non-transfected cells, demonstrating a role for bcl-2 in the regulation of NF-κB. Identification of this relationship is an important step understanding PCD pathways in photoreceptor cells. Whether bcl-2 provides protection via increasing nuclear translocation of NF-κB or by preventing its degradation is not known. It is possible that bcl-2 acts to block caspase activity, either directly, or through modulation of ROS. Further studies are proposed to elucidate the precise mechanism by which bcl-2 protects NF-κB function.

Studies proposed as extensions of this dissertation research focus on further describing the mechanisms of apoptotic photoreceptor cell death. Our research suggest that NF-κB, which is constitutively expressed in the 661W cell, undergoes degradation in response to photo-oxidative stress. Moreover, it appears that caspase-1 is involved in this process. Repeating the light exposure protocol with the 661W and B4 cells and processing with ICE/Caspase-1 enzyme activity assays would provide definite evidence of the role of caspase-1, as well as providing insight into interaction between bcl-2 and caspases.

We are currently testing the hypothesis that the relA/p65 subunit of NF-κB is mandatory for photoreceptor survival of oxidative insult. Results presented in this dissertation illustrate an important role for NF-κB in preventing apoptosis. We have developed a clonal 661W photoreceptor cell that over-expresses relA and plan to subject these cells to oxidative damage to evaluate for any conferred protection. We will also evaluate the potential role of relA as a transcriptional activator of bcl-2 family genes by

RT-PCR evaluation of bcl-2, bax, bcl-x_L, and bcl-x_S mRNA message. Promoter studies could also be used to investigate -κB regulatory sites for bcl-2 family genes.

Following further identification of NF-κB, bcl-2, and caspase interaction in the regulation of photo-oxidative stress induced PCD in the 661W photoreceptor cell line, experiments will focus on in vivo studies and animal models. Appropriate animal models for our research would include, but not be limited to, retinal degeneration mouse models (retinal dystrophy and retinal dystrophy slow mice), aging models (Fisher rat), and light damage models for mice or rats. Using the current understanding of photoreceptor apoptotic mechanisms outlined in this dissertation several key regulatory points could be targeted in attempts to block apoptosis in vivo. The most feasible approach is with pharmaceuticals, such as inhibitors of caspases or anti-oxidants. An additional pharmaceutical approach would be to target calcium homeostasis at the mitochondria, which is often lost during apoptosis, using currently available calcium channel blocking drugs. We have identified NF-κB and bcl-2 as being important in photoreceptor survival following oxidative stress. Targeted gene expression for either of these proteins has the potential to interfere with the apoptotic cascade. While the clinical applications of bcl-2, NF-κB, or caspase inhibitor gene therapy are on the distant horizon, there is hope that future advances in gene targeting will make these approaches a reality.