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Glaucoma is a leading cause of blindness worldwide. A major risk factor for glaucoma is increased intraocular pressure that leads to pathological changes in the optic nerve head (ONH). Astrocytes within the ONH become activated in glaucoma and may create an environment detrimental to retinal ganglion cell axons. The factors that cause activation of the ONH astrocytes (ONA) are unknown, although there is evidence that CNTF, FGF-9, and IL-1α activate glial cells within the CNS. The purpose of this research was to determine if exogenous CNTF, FGF-9, and/or IL-1α activate human ONH astroctyes.

IN VITRO EFFECT OF CNTF, FGF-9, AND IL-1α

ON HUMAN OPTIC NERVE HEAD

ASTROCYTES

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IN VITRO EFFECT OF CNTF, FGF-9, AND IL-1α ON HUMAN OPTIC NERVE HEAD ASTROCYTES

THESIS

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LIST OF ABBREVIATIONS

CNTF- Ciliary Neurotrophic Factor

CNTFRα- Ciliary Neurotrophic Factor Receptor alpha

Gp130- Glycoprotein 130

LIFR- Leukemia Inhibitory Factor

FGF-9- Fibroblast Growth Factor-9

FGFR1- Fibroblast Growth Factor Receptor 1

FGFR2- Fibroblast Growth Factor Receptor 2

FGFR3- Fibroblast Growth Factor Receptor 3

FGFR4- Fibroblast Growth Factor Receptor 4

IL-1α- Interleukin-1 alpha

IL-1RI- Interleukin-1 Receptor I

NCAM 180- Neural Cell Adhesion Molecule 180

GFAP- Glial Fibrillary Acidic Protein

LC- Lamina Cribrosa

NO- Nitric Oxide

ONA- Optic Nerve Head Astrocytes

ONH- Optic Nerve Head

POAG- Primary Open Angle Glaucoma

RGC- Retinal Ganglion Cells

CHAPTER I

INTRODUCTION TO THE STUDY

Glaucoma

Glaucoma is a group of optic neuropathies affecting approximately 70 million people worldwide (Quigley, 1996). There are multiple types of glaucoma including primary open-angle glaucoma, acute closed angle glaucoma, secondary glaucoma, and congenital glaucoma (Quigley, 1998). At least 3 million Americans have glaucoma, although about one-half of the individuals are not aware that they have the disease because there are no immediate symptoms (Glaucoma Research Foundation). Therefore, glaucoma is also referred to as the silent blinder (Coleman, 1999). With time, an individual with glaucoma will become aware that they are losing their peripheral vision, and as the diseases progresses, as in many cases, total vision is lost.

Primary Open Angle Glaucoma

The most common form of glaucoma is primary open angle glaucoma (POAG). POAG is associated with a loss of retinal ganglion cells (RGC) and their axons, cupping or excavation at the optic nerve head (ONH), notching and thinning of the neuroretinal rim, and disc hemorrhages. (Quigley, 1983; Varela and Hernandez, 1997; Quigley, 1998; Coleman, 1999). The pathogenesis of POAG is unknown, although two mechanisms for the pathogenesis of glaucoma have been suggested: (a) mechanical damage to the ONH

may cause constriction of RGC axons and (b) ischemia may damage RGC (Coleman, 1999; Quigley et al. 1995).

Risk factors for developing glaucoma include family history of glaucoma, diabetes, race (African-American 4 times more likely than Caucasians), long-term glucocorticoid treatment, age (over the age of 45), myopia, and increased IOP (Clark, 1995, Harper and Reeves, 1995; Quigley, 1998; Wordinger & Clark, 1999). The major risk factor for glaucoma is increased IOP that leads to pathological changes at the ONH (Varela and Hernandez, 1997; Kolker, 1983, Hayreh, 1978; Maumenee, 1977; Wilson, 1996).

Aqueous humor, a transparent nutritive fluid, is secreted by the processes of the ciliary body into the posterior chamber, flows around the iris into the anterior chamber, and is finally collected in the trabecular meshwork exiting into the Schlemm's canal and the episcleral venous system (To et al., 2002). The aqueous humor has multiple roles that include providing nutrients and oxygen, removing wastes, creating an IOP, transporting ascorbic acid into the anterior segment, and regulating the local immune responses during inflammation and infection (To et al., 2002). However, in certain individuals with glaucoma, increased IOP develops due to increased resistance of aqueous humor outflow through the trabecular meshwork. The increase in pressure is displaced towards the back of the eye causing cupping at the ONH and damage to the optic nerve. The optic nerve consists of approximately 1 million retinal ganglion cell axons that connect the retina with the visual cortex of the brain (Kaushik et al., 2003; Osborne et al., 1999). Because the optic nerve is responsible for delivering visual signals to the brain via RGC, damage to the ONH leads to vision loss (To et al., 2002).

The Optic Nerve Head, Lamina Cribrosa, and Extracellular Matrix

Three distinct histological regions have been identified in the ONH including the prelaminar region, lamina cribrosa, and postlaminar region (Varela and Hernandez, 1997). The prelaminar region consists of unmyelinated axons of the RGC organized into bundles, ONH astrocytes, and glial processes (Varma & Minkler, 1996). The transition of the prelaminar region into the laminar region has an increase of glial tissue suggesting glial support for the axon bundles is at its highest in the lamina cribrosa (Varma & Minkler, 1996). The lamina cribrosa (LC) region is composed of connective tissue plates lined up to supply the axons openings for passage and support for RGC axons as they exit the eye to form the optic nerve (Anderson, 1969; Birch et al., 1997). Optic nerve head astrocytes are the major cell type residing within the LC and these cells separate the nerve fibers from the connective tissue plates and contribute to the extracellular matrix (ECM) (Anderson, 1969). The postlaminar region is the area where the axons become myelinated (Hogan et al., 1971). Within the postlaminar region, myelination of axons increases the size of the optic nerve twice that compared to the size of the optic disc (Varma & Minkler, 1996).

Within the LC, connective tissue plates are composed of elastin, collagen fibers, glycosaminoglycans (GAGs), and adhesive glycoproteins. GAGs are macromolecules consisting of repeating disaccharide units and adhesive glycoproteins, and include laminin and fibronectin, which are linked to the ECM to maintain the support and strength required by tissues (Acott & Wirtz, 1996). A single gene encodes elastin, however, various elastin isoforms have been documented due to alternative splicing

(Vrhovski et al., 1998). Elastin production is believed to be maintained at the level of translation, since the protein expression of elastin is maximal during development and declines with age, however, the mRNA expression of elastin remains at a constant level despite age (Swee et al., 1995). Collagens fibers and collagen networks are encoded by a family of collagen genes. There are various isoforms of collagen, each encoded by different genes (Alberts et al., 1994). The collagens that predominate within the LC connective tissue plates are fibrillar collagens, collagen I and collagen III (Hernandez and Ye, 1993). Basement membranes consist of collagen types IV and V, laminin, and heparin sulfate (Varma & Minkler, 1996).

Glial Cells and Astrocytes

Glial cells play important roles within the central nervous system (CNS). The role of a glial cell was once described as only providing structural support to neurons; however, more recent studies have shown that they are essential for neuron survival. Neurons are protected by glial cells from mechanical stress, ionic imbalances, pathogens, and plasma proteins (Levison et al., 1996). Glial cells perform a wide range of functions and react to inflammation, repair, and fluid balance. The major glial cells include oligodendrocytes, microglia, and astrocytes. Oligodendrocytes are small, round cells whose processes provide the myelin sheath for axons in the CNS (Junqueira et al., 1995). Microglial cells are small cells with elongated processes, and their role is to remove cellular debris in the CNS (Junqueira et al., 1995).

Astrocytes are star-shaped cells that provide structural support and control the chemical and ionic environment of neurons within the CNS (Junqueira et al., 1995). There are two different types of astrocytes: protoplasmic and fibrous. Protoplasmic astrocytes have many short-branch processes and is found in the gray matter, while fibrous astrocytes have only a few long processes and are found in the white matter of the CNS (Junqueira et al., 1995). Astrocytes are also known to provide growth factors for neurons (Du and Dreyfus, 2002). Astrocytes are characterized by the expression of glial fibrillary acidic protein (GFAP, 50 kDa), a type III intermediate filament protein. In astrocytes, GFAP is responsible in maintaining the cell structure and resistance to mechanical forces (Kaufman and Alm, 2003; Hernandez, 2000; Galou et al., 1997).

Optic Nerve Head Astrocytes

Optic nerve head astrocytes (ONA) are the major glial cell type found in the ONH (Hernandez, 2000). These cells are characterized as large cells that express GFAP, neural cell adhesion molecule (NCAM), ECM proteins, and growth factors with many long and thin processes (Hernandez et al., 1988; Ricard et al., 1999; Agapova et al., 2003). Retinal ganglion cells depend on ONA for their survival (Yorio et al. 2002; Morgan, 2000). Optic nerve head astrocytes play vital roles within the ONH by maintaining the homeostasis environment, regulating potassium and glutamate levels, and providing neurotrophic support for nearby neurons (Yorio et al., 2002; Waniewski and Martin, 1986; Goss et al.,1998).

There are two sub-populations of astrocytes found in the ONH: type 1A and 1B (Varela and Hernandez, 1997). To differentiate between the two cell types, NCAM expression is used as a marker for type 1B astrocytes (Ricard et al., 1999; Varela and Hernandez, 1997). Type 1A astrocytes serve as a structural support for axons, while type 1 B astrocytes are involved in the remodeling of the connective tissues in the ONH (Ricard et al., 1999; Varela and Hernandez, 1997). As previously described, three distinct regions have been identified in the ONH. These include the prelaminar region, LC, and postlaminar region (Varela and Hernandez, 1997). In the prelaminar region, type 1B astrocytes are mainly found and contribute to the glial columns, surround the blood vessels, and line the vitreal surface (Varela and Hernandez, 1997). In the LC, the major astrocyte population are type 1B astrocytes that lay horizontally across the ONH perpendicular to the axons (Agapova et al., 2003, Varela and Hernandez, 1997; Anderson, 1969). In the postlaminar region, type 1A astrocytes primarily contribute to the lining of the pial septa and form the glial membrane (Varela and Hernandez, 1997).

Activated Astrocytes and Gliosis

When there is injury/trauma, neurodegeneration or an inflammatory response, astrocytes undergo gliosis. In gliosis, glial cells respond to neural tissue damage resulting from physical and chemical insults. Stimulated glial cells respond by increasing cell body size and the number of cell processes, proliferation, increased GFAP expression, and/or increased expression of cell surface molecules, cytokines and growth factors (Streit et al., 1999; Wu and Schwartz , 1998). For example, a change in astrocyte

morphology can be due to the inability to maintain membrane potentials. Damaged neurons release glutamate thereby activating receptors on astrocytes causing an influx of potassium, sodium, and chloride resulting in glial hypertrophy (Hudgins and Levison 1998). Activated astrocytes up-regulate growth factors and cytokines such as nerve growth factor (NGF), fibroblast growth factors (FGFs), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), transforming growth factors beta (TGFβ), and tumor necrosis factor alpha (TNF α) (Ridet et al., 1997).

In the injured CNS, myelin and the glial scar (a dense mat of glial processes) are main impediments to axon regeneration due to inhibitory molecules associated with the ECM (Levison et al., 1996). A glial scar has both beneficial and detrimental effects; it acts as a barrier to inhibit any further injury, but also inhibits axon regrowth (Bush et al. 1999; Tardy, 2002). During gliosis, astrocytes receive signals from injured neurons located near the region of injury (Streit et al., 1999). Both vimentin and GFAP are two major intermediate filaments that appear to play a key role in gliosis because in the absence of these proteins, glial reactivity is inhibited thus permitting regeneration of axons (Privat, 2003). Knock out mice for GFAP, vimentin or double knock outs are able to regenerate neuronal axons. Within the CNS, astrocyte gliosis is the primary reason for the inhibition of axon regeneration (Privat, 2003; Ridet et al., 1997).

Growth Factors and Cytokines as Activators of Astrocytes

Growth factors have the ability to stimulate the growth of a cell including cell division and differentiation (Baines, 2004). Cytokines are proteins released by cells

involved in the immune response (The American Heritage Stedman's Medical Dictionary, 2002). Cytokines communicate between cells of the immune response and other cells of the body to produce consequences such as inflammation (Bains, 2004).

Growth factors are defined as signaling molecules that participate in cell-to-cell communication by activating specific growth factor receptors, which elicit responses in cells (Cross and Dexter, 1991). Growth factors play a myriad of roles including proliferation, motility, differentiation, ECM synthesis, development, and survival (Wordinger et al., 1998). All growth factors act through one or more high affinity receptors to initiate signaling pathways. Growth factor responses are influenced by various conditions such as the balance of stimulatory and inhibitory signals, the responding cell type, the concentration of the factor, and the presence of other stimuli (Cross and Dexter, 1991).

The roles of growth factors in the ONH are not completely understood. Therefore, the expression of growth factors and their receptors and their action in the context of the microenvironment of the ONH is important to study. Growth factors and their receptors regulate normal development and homeostasis in ocular tissues. Altered growth factor signaling may be involved in abnormal development or function that may be associated with the pathogenesis of glaucoma (Wordinger et al., 2002). Growth factors/cytokines that are known to be expressed by ONH astrocytes include neurotrophins (NGF, BDNF, NT-3, NT-4), transforming growth factor beta (TGFβs), insulin-like growth factor (IGF-II), and insulin-like growth factor binding protein 1 (IGFBP1), tumor necrosis alpha (TNFα), bone morphogenetic proteins (BMPs), and glial cell line-derived neurotrophic

factor (GDNF), (Lambert et al., 2001; Pena et al., 1999b, c; Hernandez et al., 2002; Yuan and Neufeld, 2000; Wordinger et al., 2002; Wordinger et al., 2003).

As described previously, known processes that activate astrocytes include neuronal insults where damaged neurons send out signals in the vicinity of astrocytes, injury as in the case of ischemic insults, and diseases as in glaucoma within the CNS (Streit et al, 1999). These processes then cause production of cytokines, growth factors, ECM macromolecules, and the expression of different cell surface molecules (Varela and Hernandez, 1997).

This study will focus on the ability of growth factors and cytokines to activate ONA, since understanding in this activation process is lacking. This study will concentrate on two growth factors, one cytokine, and their receptor complexes: (a) ciliary neurotrophic factor (CNTF); (b) fibroblast growth factor -9 (FGF-9); and (c) interleukin -1α (IL-1α). Within the CNS, CNTF, FGF-9, and IL-1α activate glial cells, including astrocytes (Hudgins & Levison, 1998; Levison et al., 1996; Winter et al., 1995; Lisovoski et al., 1997; Monville et al, 2001; Miyamoto et al., 1993; Nakamura et al., 1999; Giulian et al., 1985).

Ciliary Neurotrophic Factor

Ciliary neurotrophic factor (CNTF) (25 kDa) was originally shown to promote the survival of chick ciliary ganglion neurons (Adler et al., 1979; Barbin et al., 1984) and more recently to have a neuroprotective role following spinal cord injury (Ye et al., 2004). Ciliary neurotrophic factor has other roles including protection of neurons and

glial cells within the central nervous system, as well as activating astrocytes during gliosis (Hudgins and Levison, 1998). Ciliary neurotrophic factor lacks a signal sequence and is therefore not a secreted protein. It has been hypothesized that because it lacks a signal sequence, CNTF is released via injury/trauma to cells (Winter et al., 1995; Lin et al., 1989; Stocklit al., 1989; Sendtner et al., 1992).

Ciliary neurotrophic factor belongs to the interleukin-6 (IL-6) family of cytokines that includes IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), interleukin-11 (IL-11) and cardiotrophin-like cytokine (CLC) (Schuster et al., 2003; Grötzinger et al., 1999; Shi et al.,1999; Senaldi et al., 1999). Ciliary neurotrophic factor acts through a tripartite receptor complex involving ciliary neurotrophic receptor alpha (CNTFR-α), leukemia inhibitory factor receptor (LIFR), and glycoprotein 130 (gp 130). For CNTF to illicit a cellular response, it must first act through a signaling complex by binding to its receptor, CNTFRα, which then recruits gp130 and LIFR in a stepwise fashion. This is followed by the activation of the Janus Kinase/signal transducer and activator of transcription 3 (JAK/STAT) pathway (Davis et al., 1991; Rane and Reddy, 2000; He et al., 2002).

Previous literature has shown that CNTF is an activator of brain astrocytes, (Hudgins and Levison, 1998; Levison et al., 1996; Winter et al., 1995; Lisovoski et al., 1997; Monville et al., 2001) producing a variety of effects that include stimulating hypertrophy, cell proliferation, differentiation of O-2A progenitors into type 2 astrocytes, and increasing expression of multiple gliotic genes such as GFAP, vimentin, S100, and clusterin (Hudgins and Levison, 1998; Levison et al., 1996; Winter et al., 1995; Lisovoski

et al., 1997; Monville et al., 2001). Levison and co-workers (1996), reported that recombinant CNTF (rhCNTF) delivered into the motor cortex of rats up-regulated vimentin, clusterin, and GFAP mRNA and induced hypertrophic and hyperplastic astrocytes.

The mechanism responsible for CNTF induced gliosis is unknown (Dallner et al., 2002). Ciliary neurotrophic factor expression is detected in brain astrocytes, optic nerve, olfactory bulb, and optic nerve astrocytes (Dallner et al., 2002; Stockli et al., 1991; Kirsch et al., 1998). Levision and co-workers (1998), reported that brain astrocytes are activated resulting in hypertrophy as well as showing an increase of GFAP in response to CNTF. Ciliary neurotrophic factor and CNTFRα are also up regulated due to injury in rat brain astrocytes (Lee et al., 1997) and in rat optic nerve astrocytes (Kirsch et al., 1998). CNTF also acts directly on neurons as a trophic factor and activates glial cells to protect and support neurons (Hudgins & Levision, 1998). Monville and co-workers (2001) indicated that CNTF is released from astrocytes to protect neurons from further injury and therefore acts as an "injury molecule". There is some controversy whether CNTF activates astrocytes directly because mature brain astrocytes lack CNTFRα. However, there are two possible explanations. Ciliary neurotrophic factor may activate mature astrocytes via direct binding with LIFR. In addition neurons express CNTFRα and may cleave CNTFRα via its GPI anchor, releasing the receptor in the extracellular space enabling the activation of astrocytes (Monville et al., 2001).

Fibroblast Growth Factor-9

Fibroblast growth factor-9 (FGF-9) belongs to a family of at least 23 factors (Ford-Perriss et al., 2001). The fibroblast growth factors (FGF) are a family of heparin binding growth factors that all have a similar structure that includes a 120 amino acid core region and 30-60% amino acid homology (Ford-Perriss et al., 2001; Powers et al., 2000). The FGF family has several functions including cell proliferation, migration and differentiation (Ford-Perriss et al., 2001). Fibroblast growth factor-9 was first identified in human glioma cell (NMC-G1) condition media (Miyamoto et al., 1993). Fibroblast growth factor-9 was originally named glia activating factor (GAF), because of its ability to promote glial cell proliferation (Miyamoto et al., 1993). Fibroblast growth factor-1 (FGF-1) and fibroblast growth factor-2 (FGF-2) are similar to FGF-9 in that they lack an amino-terminal signal sequence (Powers, 2000; Ford-Perriss et al., 2001). Despite lacking a signal sequence, FGF-9 may be secreted through an ER-Golgi-independent pathway (Powers et al., 2000; Ford-Perriss et al., 2001).

Fibroblast growth factor-9 is highly conserved among human, rat, mouse (Miyamoto et al., 1993; Seo and Noguchi, 1995), *Xenopus*. *Xenopus* FGF-9 has 93% amino acid identity with mammalian FGF-9 (Song and Slack, 1996). The high degree of homology suggests that biological functions within the CNS are conserved (Nakamura et al., 1999). Reuss and co-workers (2000) reported that FGF-9 plays a role in the regulation of astroglial gap junction communication in the brain, and recently, FGF-9 has been reported to increase RGC survival compared to FGF-1, FGF-2, and FGF-4 (Kinkl et al., 2003).

Nakamura and colleagues first demonstrated that FGF-9 was present in only neurons, suggesting FGF-9 found in glial cells came from surrounding sources such as neurons or that glial cells produced FGF-9 at undetectable levels (Nakamura et al., 1997). However, further studies demonstrated FGF-9 to be present in some but not all astrocytes within the CNS, but in lower quantities than neurons (Nakamura et al., 1999). They believed FGF-9 is involved in the differentiation, proliferation and activation of astrocytes within the CNS (Nakamura et al., 1999). Fibroblast growth factor-9 has also been reported to activate glial cells by increasing cell proliferation (Naruo et al. 1993). This group proposed FGF-9 acts via paracrine/autocrine signaling mechanisms to cause proliferation and differentiation of glial cells.

The fibroblast growth factor family members act through one or more high affinity receptors. Four transmembrane receptors have been identified including FGFR1, FGFR2, FGFR3, and FGFR4. Each receptor has three immunoglobulin (Ig) like loops (I-III), with the third loop displaying receptor specificity (Ford-Perriss et al., 2001). Splice variants of the III Ig-like loop have been described for FGFR1, FGFR2 and FGFR3 (Ford-Perriss et al, 2001). All four receptors have tyrosine kinase activity and are composed of three domains: an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain (Lovicu and Overbeek, 1998). Dimerization and autophosphorylation occurs once these receptors are activated upon binding to its fibroblast growth factor. Fibroblast growth factor-9 signals through receptors FGFR3 and FGFR2 (Hecht et al., 1995; Santos-Ocampo et al., 1996; Cohen and Chandross, 2000). This binding results in various biological effects through

signaling pathways including the Ras/Raf and phospholipase C dependent pathways (Spivak-Kroizman et al., 1994; Legeai-Mallet et al., 1998).

Interleukin-1α

Interleukins belong to a group of small, active proteins called cytokines that produce biological responses in the picomolar concentration range (Dinarello, 1994). Cytokines are 8-40 kDa molecules included in an 18 member family (Dinarello 2000). The term cytokine is given because almost all nucleated cells synthesize and respond to these molecules (Dinarello, 2000). Cytokines are reported as being mediators of inflammation, host defense reactions, and tissue injury (Lee et al., 1995). Cytokines have been subdivided into proinflammatory and anti-inflammatory groups (Dinarello, 2000). Proinflammatory cytokines induce the disease process, while anti-inflammatory cytokines mitigate inflammation (Dinarello, 2000). It has been suggested that cytokines are a product of stressful environments and that cytokine gene expression is activated by noxious events (Dinarello, 2000).

Interleukin-1α is one of three members that make up the IL-1 gene family (IL-1α, IL-1β, and IL-1 receptor antagonist (Dinarello, 1994). Interleukin-1α, unlike IL-1β, is active in both the pro-IL-1α (31 kDa) and the mature IL-1α (17 kDa) form (Dinarello, 1994). The interleukin 1 receptor gene is composed of IL-1RI, IL-1RII, and T1/ST2/*Fit-1* (Dinarello, 1994). Interleukin-1α acts with high affinity through receptor IL-1RI (Dinarello, 1994). After the binding of IL-1α to IL-1RI, the receptor becomes phosphorylated, followed by phospholipid hydrolysis and/or a release of ceramide from

membrane sphingomyelin (Dinarello, 1994). Within the CNS, the end result of IL-1α may include inflammation, tissue destruction, cell death, or neuroprotection.

Interleukin-1has diverse effects on neuronal cells. Interleukin-1 can reduce neuronal death or contribute to neurodegeneration. Interleukin-1 inhibits neuronal death by preventing Ca^{2+} entry into neurons or preventing the release of glutamate from neurons (Allan & Rothwell, 2001; Plata-Salaman & Ffrench-Mullen, 1992; Murray et al., 1997). Interleukin-1 contributes to neurodegeneration by inducing cyclooxygnease 2 (COX2) and inducible nitric oxide synthase (iNOS) (Allan & Rothwell, 2001; Sercu et al., 1999). Cytokines such as IL-1 activate glial cells by triggering the release of other cytokines; including IL-6, TNFα, and TGFβ (Allan & Rothwell, 2001; Benveniste et al., 1990; Bethea et al., 1992; Da Cunha &Vitkovic, 1992).

Giulian and Lachman first reported that IL-1 stimulated the proliferation of brain astrocytes (Giulian and Lachman, 1985). Furthermore, Lee and co-workers (1995) also showed that fetal astrocytes exposed to IL-1β became activated by displaying increased expression of GFAP and morphologic changes such as the formation of multiple cell processes. These authors believe IL-1 acts as a stress molecule and may play a role in gliosis.

Primary Open Angle Glaucoma, Activated Astrocytes, and Gliosis

Optic nerve head astrocytes have been implicated in the pathophysiology of glaucoma. It has been suggested that the activation of ONA is responsible for RGC death in POAG (Hernandez MR, 2000). Following gliosis, activated astrocytes are known to

secrete growth factors including TGFβs, CNTF, and FGF-2 that may directly or indirectly affect RGC axons (Hernandez, 2000). In POAG, there is considerable damage to the ONH resulting in the remodeling of the prelaminar and LC regions of the ONH by ONA (Hernandez, 2000; Varela and Hernandez, 1997). In the prelaminar region, cellular responses include hypertrophy, formation of thick and long cellular processes and the loss of glial columns (Varela and Hernandez, 1997). In the LC region, changes to ONA include round cell bodies, loss of processes, and the migration of ONA into the nerve bundles (Varela and Hernandez, 1997). TGFβ regulates ECM production and tissue remodeling in the ONH (Hernandez, 2000), including increased synthesis of ECM molecules such as elastin, collagens, and proteoglycans and the release of ECM degrading molecules (Hernandez 2000; Border and Noble, 1994). Agapova and co-workers (2003) hypothesize that in POAG, ONA are able to respond to increased IOP by undergoing a reactive phenotype that includes changes in the cytoskeleton, cell adhesion, migration and synthesis of new ECM (Hernandez, 2000; Pena et al., 2001; Pena et al., 1999a; Varela and Hernandez, 1997).

Hypothesis and Specific Aims

A major risk factor of glaucoma is increased intraocular pressure that leads to pathological changes at the ONH. Astrocytes within the ONH become activated in glaucoma and may create an environment detrimental to RGC axons. The factors that cause activation of ONA are unknown, although there is evidence that CNTF, FGF-9, and IL-1α activate glial cells within the CNS. The **hypothesis** to be tested in these studies is

that exogenous FGF-9, CNTF, and IL-1α activate human ONA. The following **specific aims** have been designed to test this hypothesis: (1) demonstrate ONA isolated from the human ONH express mRNA for CNTF, FGF- 9, and IL- 1 α and their respective receptor complexes, (2) demonstrate ONA express protein for CNTF, FGF- 9, and IL- 1 α and their receptor complexes, and (3) demonstrate that ONA cells become activated by displaying increased expression of GFAP, and undergo hypertrophy, and or hyperplasia following treatment with exogenous FGF-9, CNTF and/or IL-1α.

This research will determine if these growth factors play a critical role in the microenvironment of the ONH. This study proposes that FGF-9, CNTF, and IL-1α act via autocrine/paracrine signaling mechanisms within the ONH to activate ONA and may help us to further understand the pathology of the ONH in glaucoma.

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CHAPTER II

METHODS AND MATERIALS

Cell Culture

Human ONA were obtained from Alcon Laboratories (Fort Worth, Texas) and characterized as described previously (Lambert et al., 2001). Human ONA were cultured in Dulbecco's Modified Low glucose (HyClone Labs, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone Labs), L-glutamine (0.292 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (4 μg/ml). All antibiotics were purchased from Gibco BRL, Grand Island, NY. Media was changed every 2-3 days. Confluent cultures were trypsinized and seeded in 25 cm^2 or 75 cm^2 cell culture flasks (Corning Inc, Corning, NY) and maintained at 37°C in 5% CO_2-95% air.

Primer Design

Primers for GFAP, CNTF, FGF-9, IL-1α and their receptor complexes were designed using Primer 3 available on-line from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Cambridge, MA http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/results from mRNA sequences obtained using the Entrez database (available on-line from National Centers for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov). The specificity of the

primers were verified using BLAST (available on-line from NCBI; http://www.ncbi.nlm.nih.gov/blast). The primer sequences, annealing temperature and expected product base pair are described in Table 1.

Total RNA Extraction and cDNA Synthesis

Total cellular RNA was prepared using the TRIzol reagent (Life Technologies, Carlsbad, CA). Confluent ONA cells were scraped, pelleted by centrifugation and lysed in 1 ml TRIzol reagent. RNA was precipitated from the aqueous phase by adding isopropyl alcohol followed by centrifugation. The RNA pellet was resuspended in 40 µl of water and stored at -80°C. cDNA was generated by incubating 20µg of total RNA and 3µl (0.75 µg) of random primers at 85°C for 3 minutes. The following was then added to the reaction tube: 4µl (80 units) of RNasin, 8 µl (40 units) of avian myeoblastosis virus (AMV) reverse transcriptase, 40 µl (0.625 mM) of dNTPs, 40 µl of 5X RT Buffer (50mM Tris-HCl, 75mM potassium chloride, 10 mM dithiothreitol, and 3 mM magnesium chloride). Reactions were brought up to a final volume of 200µl with DEPC water. Reaction tubes were incubated at 42°C for 30 minutes followed by 94°C for 2 minutes. All cDNA samples were stored at -20°C. All reagents were from Promega, Madison WI.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

Details of the PCR procedure used in our laboratory have been published previously (Wordinger et al., 1998). Amplification took place for each sample with its specific primer pair using a master mix containing all the components in the PCR reaction. In the case of a negative control, water was used to replace the cDNA for each pair of primers. The *Taq* Antibody Hot Start method was used for all PCR reactions (Clonetech Laboratories Inc., Palo Alto, CA). All PCR reactions were then placed in a programmable thermal cycler (PTC-100, M.J. Research, Inc., Watertown, MA) for 40 cycles undergoing three basic steps in the following order: denaturing, primer annealing, and elongation. Denaturation was carried out at 94°C to cause strand separation. The annealing step was carried out at the optimal annealing temperature for each specific primer pair (see Table I) to hybridize the cDNA template to primer sequences. The elongation process was carried out at 72° C to replicate the primers. All PCR reactions were subjected to 2.0 minutes at 94°C, 2.0 minutes at 92°C, [40 cycles of 30 seconds at the optimal annealing temperature, 90 seconds at 72°C, 45 seconds at 92°C], 5 minutes at 72°C, and an indefinite step of 4° C. PCR products (20 μl) were visualized by electrophoresis on a 2.0% agarose gel containing 10 mg/ml ethidium bromide in 1X Tris Acetate EDTA (TAE) buffer. A 100 bp ladder was used as a molecular size standard. All reagents were products of Promega (Madison WI).

Protein Extraction and Western Blot Analysis

Total cellular protein was isolated from cultured cells using a lysis buffer containing 10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM PMSF in ethanol, 1 μg/ml aprotinin, 4 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate (10 μl/ml) (Lambert., 2001). Protein concentration was determined using the Bio-Rad D_c Protein Assay System as described by the manufactures instructions (Bio-Rad Laboratories, Richmond, CA). A standard curve was generated using bovine serum albumin (BSA) and absorbance at 750nm was read within 15 minutes.

Proteins (30-40 ug/ul were loaded per well) were separated on SDS-PAGE denaturing polyacrylamide gels and then transferred by electrophoresis to nitrocellulose membranes. The membranes were stained with ponceau red for 10 minutes to evaluate the transfer. Membranes were incubated in 5% milk with Tris Buffered Saline Tween (TBST - 20mM Tris, 0.5M NaCl, and 0.05% Tween 20, pH 7.4) for 50 minutes in order to block non-specific binding. Blots were incubated with primary antibody (see table 2) in 1% milk. The membranes were washed with TBST, then incubated with appropriate secondary antibodies in 1% milk. Enhance chemiluminescence's reagents (Amersham, Arlington Heights, IL) were used to carry out detection and blots exposed to FUJIFILM (Cuevas, Fort Worth, Texas) for various times depending on the amount of target protein present.

Treatment with Exogenous FGF-9, CNTF and IL-1α

Cell cultures were grown until they reached 80% confluence, at which time they were placed in a serum-free media for 24 hours. Cultured ONA cells were treated with FGF-9 (2ng/ml), CNTF (150ng/ml), and IL-1α (2ng/ml) for 48 hours. In other studies the concentration of CNTF used ranged between 100ng/ml to 250ng/ml (Hudgins and Levison., 1998; Monville et al., 2001), therefore a dose of 150ng/ml was chosen. FGF-9 has been reported to cause activity between the ranges of 1-2ng (R&D Systems, Minneapolis, Minnesota). IL-1α doses that have elicited responses ranged between 0.1ng/ml to 500ng/ml (Kloss, 1997, and Chao, 1996), therefore 2 ng/ml was chosen. Untreated cell lines were used as controls.

CellTiter Proliferation Assays

Cell proliferation was examined using a CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Confluent ONA were trypsinized and plated in quadruplet into Costar 96-well cell culture plates (Fisher Scientific, Pittsburgh, PA). A standard curve was generated at 0, 500, 1000, 2000, 3000, 4000 and 5000 cells per well. An hour later a 20μl volume of a combination of PES/MTS ((phenazine ethosulfate)/(tetrazolium compound (3-(4,5-dimethythiazol-2-ul)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)) was added to each well in culture. Cells were maintained in 5% CO_2/95% air at 37°C and the absorbance was taken 1 hour later. The experimental plate was plated at a density of 2000 cells per 10 ul and consisted of the growth factor in serum free media, serum-free

media alone, or media plus 10% FBS. Cells were maintained in 5% CO_2/95% air at 37°C for 48 hours. At the end of the 48 hour treatment 20µl of PES/MTS per 100 µl culture media was added to each well. Cell were maintained in 5% CO_2/95% air at 37°C and the absorbance was taken 1 hour later. The reagent control consisted of 100 µl volume of culture media without the addition of cells. After incubation at 37°C, colorimetric development was read at 490 nm using a BioRad model 450 microplate reader (BioRad Laboratories, Richmond, CA).

TABLE 1. Expected Sizes of Polymerase Chain Reaction Amplification Products

GF/ Receptor	Size (bp)	Upstream Primer	Downstream Primer	Annealing Temperature
CNTF	195 bp	CAG GGC CTG AAC AAG AAC AT	ATG GAA GTC ACC TTC GGT TG	60. 0°C
CNTFRα	237 bp	CCA CCT ACA TTC CCA ACA CC	GGG CTA CCA CAT TTT CTG GA	60.0°C
gp 130	426 bp	GGT CAC CTC ACA CTC CTC CAA	TAC CAT CAC CGC CAT CTA CAT	55.0°C
LIFR	500 bp	ATC TTC ATT TTT CTG GTC TCG	GCT TTC GTT TGT AGG TGC TT	55.0°C
FGF-9	197 bp	GGG GAG CTG TAT GGA TCA GA	GTG AAT TTC TGG TGC CGT TT	60.0°C
FGFR-1	200 bp	GCC TGT CCA GGA ACT TTT CA	TGG ACA GGT CCA GGT ACT CC	60.0°C
FGFR-2	199bp	CAG AGA CCA ACG TTC AAG CA	GAG GAA GGC ATG GTT CGT AA	60.0°C
FGFR-3	184 bp	GGG CGC TAA CAC CAC CGA CAA	ATA CAC ACT GCC CGC CTC GTC	60.0°C
FGFR-4	240 bp	CTC GAA TAG GCA CAG TTA CCC	GGC ACC ACG CTC TCC ATC ACG	55.0°C
GFAP	207 bp	CTG TCC CTA GGT CAG CTT GC	GAT GTG GAG GGC GAT GTA GT	63.0°C
IL-1α	729 bp	GTT CCA GAC ATG TTT GAA GAC CTG	TGG ATG GGC AAC TGA TGT GAA ATA	55.0°C
IL-1R	390 bp	TGA CAA AAT TGG CCA GAG AG	TTG TGC TAA ACC AGT TTT AA	55.0°C
NCAM 180*	147 bp	GAA CCG AGG AGG AGA GGA CC	TAG TGG TGA CGG TAG TGA CAG	55.0°C
β-Actin	350 bp	AGG CCA ACC GCG AGA AGA TGA CC	GAA GTC CAG GGC GAC GTA GCA C	55.0°C

* Roesler J, Srivatsan E, Moatamed F, Peters J, Livingston EH. Tumor suppressor activity of neural cell adhesion molecule in colon carcinoma. The American Journal of Surgery. 1997; 174: 251-257.

TABLE 2. Primary Antibodies used in the Study

Antibody	Catalog number	pAb/mAb	Source	Antibody Concentration
GFAP	sc-9065	rabbit pAb	Santa Cruz Biotechnology Inc.	1:400
FGF-9	MAB273	mouse mAb	R&D Systems	1:100
FGFR-1	sc7945	rabbit pAb	Santa Cruz Biotechnology	1:150
FGFR-2	MAB 684	mouse mAb	R&D Systems	1:100
FGFR-3	MAB 13121	mouse mAb	Santa Cruz Biotechnology Inc	1:100
FGFR-4	sc-9006	rabbit pAb	Santa Cruz Biotechnology Inc.	1:100
CNTF	338	mouse mAb	Chemicon International	1:1000
CNTFRα	MAB 303	mouse mAb	R&D Systems	1:100
gp130	sc-655	rabbit pAb	Santa Cruz Biotechnology Inc.	1:500
LIFR	sc-659	rabbit pAb	Santa Cruz Biotechnology Inc.	1:400
IL-1α	sc-7929	rabbit pAb	Santa Cruz Biotechnology Inc.	1:1000
IL-1RI	sc-688	rabbit pAb	Santa Cruz Biotechnology Inc	1:1000
β-actin	MAB1501	mouse mAb	Chemicon International	1:1000

CHAPTER III

IN VITRO EFFECT OF CNTF, FGF-9, AND IL-1α ON HUMAN OPTIC NERVE HEAD ASTROCYTES

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Purpose. Glaucoma is a leading cause of blindness worldwide. A major risk factor for glaucoma is increased intraocular pressure that leads to pathological changes in the optic nerve head (ONH). Astrocytes within the ONH become activated in glaucoma and may create an environment detrimental to retinal ganglion cell axons. The factors that cause activation of the ONH astrocytes (ONA) are unknown, although there is evidence that CNTF, FGF-9, and IL-1α activate glial cells within the CNS. The purpose of this research was to determine if exogenous CNTF, FGF-9, and/or IL-1α activate human ONA.

Methods. Human ONA cell lines were grown until approximately 80% confluent and treated for 48 hours with either CNTF (150ng/mL), FGF- 9 (2ng/mL), or IL-1α (2ng/mL) in serum-free media. Untreated cell lines in media plus 10% FBS (S+) acted as controls. Activation of ONA in response to exogenous CNTF, FGF-9, and IL-1α was determined by (a) RT-PCR analysis of the ONA markers GFAP and NCAM, as well as Western Blot analysis of GFAP, (b) phase contrast microscopy of cell morphology, and (c) cell proliferation as measured by a MTS cell titer proliferation assays. RT-PCR and Western blotting were used to determine mRNA and protein expression of CNTF, FGF- 9, and IL-1α, and their respective receptors.

Results. Glial fibrillary acidic protein mRNA appeared to increase following IL-1α treatment, however, GFAP expression was not increased by exogenous CNTF and FGF-9 treatment. Glial fibrillary acidic protein expression in ONA was increased following exogenous FGF-9, and IL-1α treatment. Exogenous IL-1α caused human ONA to extend more processes than in control culture conditions. Treatment with IL-1α also

resulted in a significant increase in ONA cell proliferation compared to cells in serum containing media. Human ONA expressed mRNA and protein for CNTF and its receptor complex. Treatment with CNTF, FGF-9, and IL-1α did not increase the expression of mRNA for CNTF and its receptors, although protein expression of CNTF and its receptors was increased by treatment with CNTF, FGF-9 and IL-1α in the ONA cell lines. With the exception of FGFR3, ONA expressed message and protein for FGF-9 and its receptors. This expression was not increased by treatment with CNTF, FGF-9, and IL-1α. Human ONA expression of mRNA for IL-1α and its receptor was expressed for all ONA cell lines and increased mRNA expression for IL-1α following treatment with IL-1α. Treatment with FGF-9 and IL-1α increased the protein expression of IL-1α.

Conclusions. This study demonstrated that exogenous IL-1α treatment of ONA resulted in responses consistent with astrocyte activation including increased expression of GFAP, morphological changes, and increased proliferation. These results suggests that IL-1α may play a role in the activation of ONA. In addition, ONA express mRNA and protein for factors known to activate astrocytes in the CNS. Finally, ONA express receptors for these factors and respond to exogenous CNTF, FGF-9 and IL-1α treatment by increased expression of CNTF and IL-1α.

INTRODUCTION

Glaucoma is a group of optic neuropathies affecting approximately 70 million people worldwide (Quigley, 1996). POAG is associated with elevated intraocular pressure (IOP) due to a reduction of aqueous flow through the trabecular meshwork.

Elevated IOP results in a remodeling of the optic nerve head (ONH), including compression, stretching, and rearrangement of the cribriform plates of the lamina cribrosa (Agapova et al, 2003; Pena et al., 2001; Quigley et al., 1983). Cells within the lamina cribrosa of the ONH are believed to be responsible for protecting RGC from injury.

The astrocyte is the main glial cell within the ONH and is known to become activated in glaucoma (Hernandez, 2000). Following insult or injury, astrocytes increase their expression of glial fibrillary acidic protein (GFAP) and/or change morphology (Ridet et al., 1997; Wu & Schwartz., 1998). In addition, activated astrocytes are characterized by hypertrophy, hyperplasia, and an increase in the number of processes (Hernandez, 2000; Varela and Hernandez, 1997).

Growth factors play a myriad of roles that include, but are not limited to, proliferation, motility, differentiation, ECM synthesis, development, and survival (Wordinger et al., 1998). The roles of growth factors in the ONH are not completely understood. Therefore the expression of growth factors and their receptors and their actions within the microenvironment of the ONH are worthy to investigate. Growth factors that are known to be expressed in ONH astrocytes (ONA) include the neurotrophins (NGF, BDNF, NT-3. NT-4), transforming growth factor beta (TGFβs), and insulin-like growth factor (IGF-II) (Lambert et al., 2001; Pena et al., 1999b, c; Hernandez et al., 2002). However, there is no clear data that indicates these growth factors are involved in ONA activation.

Within the CNS, ciliary neurotrophic factor (CNTF), fibroblast growth factor-9 (FGF-9), and interleukin-1 alpha (IL-1α) have been reported to activate glial cells,

including astrocytes (Hudgins & Levison, 1998; Levison et al., 1996; Winter et al., 1995; Lisovoski et al., 1997; Monville et al, 2001; Miyamoto et al., 1993; Nakamura et al., 1999; Giulian et al., 1985). The purpose of this study was to determine if exogenous CNTF, FGF-9, and IL-1α activate ONA.

METHODS

Cell Culture

Human ONA were obtained from Alcon Laboratories (Fort Worth, Texas) and characterized as described previously (Lambert et al., 2001). Human ONA were cultured in Dulbecco's Modified Low glucose supplemented with 10% fetal bovine serum (FBS) (both from HyClone Labs, Logan, UT), L-glutamine (0.292 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (4 μg/ml). Antibiotics purchased from Gibco BRL, Grand Island, NY. Media was changed every 2-3 days. Confluent cultures were trypsinized and seeded in 25 cm^2 or 75 cm^2 cell culture flasks (Corning Inc, Corning, NY) and maintained at 37°C in 5% CO_2-95% air. A total of four adult human ONH astrocyte cell lines from donors ranging from 66 to 95 years of age were used in the following experiments.

Primer Design

Primers for GFAP, CNTF, FGF-9, IL-1α and their receptor complexes were designed using Primer 3 available on-line from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Cambridge, MA http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/results from mRNA sequences

obtained using the Entrez database (available on-line from National Centers for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov). The specificity of the primers were verified using BLAST (available on-line from NCBI; http://www.ncbi.nlm.nih.gov/blast). The primer sequences, annealing temperature and expected product base pair are described in Table 1.

Total RNA Extraction and cDNA Synthesis

Total cellular RNA was prepared using the TRIzol reagent (Life Technologies, Carlsbad, CA). Confluent ONA cells were scraped, pelleted by centrifugation and lysed in 1 ml TRIzol reagent. RNA was precipitated from the resulting aqueous phase by adding isopropyl alcohol followed by centrifugation. The RNA pellet was resuspended in 40 µl of water and stored at -80°C. cDNA was generated by incubating 20µg of total RNA and 3µl (0.75 µg) of random primers at 85°C for 3 minutes. The following was then added to the reaction tube: 4µl (80 units) of RNasin, 8 µl (40 units) of avian myeoblastosis virus (AMV) reverse transcriptase, 40 µl (0.625 mM) of dNTPs, 40 µl of 5X RT Buffer (50mM Tris-HCl, 75mM potassium chloride, 10mM dithiothreitol, and 3 mM magnesium chloride). Reactions were brought up to a final volume of 200µl with DEPC water. Reaction tubes were incubated at 42°C for 30 minutes followed by 94°C for 2 minutes. All cDNA samples were stored at -20°C. All reagents were from Promega, Madison WI.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

Details of the PCR procedure used in our laboratory have been published previously (Wordinger et al., 1998). Amplification took place for each sample with its specific primer pair using a master mix containing all the components in the PCR reaction. In the case of a negative control, water was used to replace the cDNA for each pair of primers. The *Taq* Start Antibody Hot Start method was used for all PCR reactions (Clonetech Laboratories Inc., Palo Alto, CA). All PCR reactions were then placed in a programmable thermal cycler (PTC-100, M.J. Research, Inc., Watertown, MA) for 40 cycles undergoing three basic steps in the following order: denaturing, primer annealing, and elongation. Denaturation was carried out at 94°C to cause strand separation. The annealing temperature was carried out at the optimal annealing temperature for each specific primer pair (see Table I) to hybridize the cDNA template to primer sequences. The elongation process was carried out at 72°C to replicate the primers. All PCR reactions were subjected to 2.0 minutes at 94°C, 2.0 minutes at 92°C, [40 cycles of 30 seconds at the optimal annealing temperature, 90 seconds at 72 C, 45 seconds at 92°C], 5 minutes at 72°C, and an indefinite step of 4°C. PCR products (20 µl) were visualized by electrophoresis on a 2.0% agarose gel containing 10 mg/ml ethidium bromide in 1X Tris Acetate EDTA (TAE) buffer. A 100 bp ladder was used as the molecular size standard. All reagents were products of Promega (Madison WI).

Protein Extraction and Western Blot Analysis

Total cellular protein was isolated from cultured cells using a lysis buffer containing 10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM PMSF in ethanol, 1 μg/ml aprotinin, 4 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate (10 μl/ml) (Lambert., 2001). Protein concentration was determined by using the Bio-Rad D_c Protein Assay System as described by the manufacturers instructions (Bio-Rad Laboratories, Richmond, CA). A standard curve was generated using bovine serum albumin (BSA) and absorbance at 750nm was read within 15 minutes.

Proteins (30-40 ug/ul were loaded per well) were separated on SDS-Page denaturing polyacrylamide gels and then transferred by electrophoresis to nitrocellulose membranes. The membranes were stained with ponceau red for 10 minutes to evaluate the transfer. Membranes were incubated in 5% milk with Tris Buffered Saline Tween (TBST - 20mM Tris, 0.5M NaCl, and 0.05% Tween 20, pH 7.4) for 50 minutes in order to block non-specific binding. Blots were incubated with primary antibody (see table 2) in 1% milk. The membranes were washed with TBST, then incubated with appropriate secondary antibodies in 1% milk. Enhance chemiluminescence's reagents (Amersham, Arlington Heights, IL) were used to carry out detection and blots exposed to FUJIFILM (Cuevas, Fort Worth, Texas) for various times depending on the amount of target protein present.

Treatment with Exogenous FGF-9, CNTF and IL-1α

Cell cultures were grown until they reached 80% confluence, at which time they were placed in a serum-free media for 24 hours. Cultured ONA cells were treated with FGF-9 (2ng/ml), CNTF (150ng/ml), and IL-1α (2ng/ml) for 48 hours. In other studies the concentration of CNTF used ranged between 100ng/ml to 250ng/ml (Hudgins and Levison., 1998; Monville et al., 2001), therefore a dose of 150ng/ml was chosen. FGF-9 has been reported to cause activity between the ranges of 1-2ng (R&D Systems, Minneapolis, Minnesota). IL-1α doses that have elicited responses ranged between 0.1ng/ml to 500ng/ml (Kloss, 1997, and Chao, 1996), therefore 2 ng/ml was chosen. Untreated cell lines in media plus 10% FBS (S+) were used as controls.

CellTiter Proliferation Assays:

Cell proliferation was examined using a CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Confluent ONA were trypsinized and plated in quadruplet into Costar 96-well cell culture plates (Fisher Scientific, Pittsburgh, PA). A standard curve was generated at 0, 500, 1000, 2000, 3000, 4000 and 5000 cells per well. An hour later a 20μl volume of a combination of PES/MTS ((phenazine ethosulfate)/(tetrazolium compound (3-(4,5-dimethythiazol-2-ul)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)) was added to each well in culture. Cells were maintained in 5% CO_2/95% air at 37°C and the absorbance was taken 1 hour later. The experimental plate was plated at a density of 2000 cells per 10 ul and consisted of the growth factor in serum free media, serum-free

media alone, or media plus 10% FBS. Cells were maintained in 5% CO_2/95% air at 37°C for 48 hours. At the end of the 48 hour treatment 20µl of PES/MTS per 100 µl culture media was added to each well. Cell were maintained in 5% CO_2/95% air at 37°C and the absorbance was taken 1 hour later. The reagent control consisted of 100 µl volume of culture media without the addition of cells. After incubation at 37°C, colorimetric development was read at 490 nm using a BioRad model 450 microplate reader (BioRad Laboratories, Richmond, CA).

STATISTICS:

Statistical significance, ($p<0.05$ or $p<0.005$), for the cell proliferation assays was evaluated using a statistical software SPSS-10 for Macintosh. A student's t-test was used for analysis between two groups with cells cultured in media plus 10% FBS (S+) serving as the control.

RESULTS

Expression of Astrocyte Markers GFAP and NCAM 180 by Human ONA Following Exogenous CNTF, FGF-9, and IL-1α Treatment

Amplification products of expected size for GFAP primer pairs in one normal and one glaucomatous cell line are shown in Figure 1. Both cell lines express GFAP mRNA in control (e.g. serum) cell culture conditions indicating astrocyte cell specificity. Interestingly, exogenous treatment with IL-1α appeared to increase mRNA expression of GFAP in both cell lines (Figure 1, Lane 5). Amplification products for NCAM 180

primer pairs, another marker for astrocytes, are shown in Figure 2. Both cell lines express low levels of NCAM 180 mRNA in control (e.g. serum) cell culture conditions (Figure 2, Lane 1). There were no obvious changes in NCAM 180 mRNA expression following exogenous administration of CNTF, FGF-9, or IL-1α (Figure 2, Lanes 3-5). Control reactions without cDNA did not result in amplification products indicating that reagents and primers were free of DNA or RNA contamination. The β-actin PCR primers yielded a single product of 350 bp.

Figure 3 represents chemiluminescent immunoblot detection of GFAP (50 kDa) protein in human ONA. GFAP protein was observed in all cell lines again confirming the cell specificity of the ONH astrocyte. In normal ONH astrocytes, exogenous treatment with CNTF, FGF-9, and IL-1α did not appear to have a pronounced effect for GFAP protein expression (Figure 3, Lanes 3-5). In the glaucomatous ONA cell lines, GFAP protein expression appears to be slightly increased with FGF-9 and IL-1α treatment (Figure 3, Lanes 4-5). All western blots were re-probed for β-actin which served as a loading control.

Human ONA Morphology Following Exogenous CNTF, FGF-9, and IL-1α Treatment

Optic nerve head astrocytes grown in control (e.g. serum) culture conditions are characterized as large cells with many long thin processes (Figure 4, Plate A). No significant changes in morphology were observed when ONH astrocytes were cultured in serum-free media for up to 48 hours (Figure 4, Plate B). There were no apparent

morphology changes following exogenous administration of CNTF or FGF-9 (Figure 4, Plates C and D). However, as shown in Figure 4, Plate E, IL-1α treatment appeared to alter ONH astrocyte morphology by increasing the number of cellular processes. There was no change in the overall size of the ONH astrocytes with any treatment.

Human ONA Cell Proliferation Following Exogenous CNTF, FGF-9, and IL-1α Treatment

The effect of exogenous CNTF, FGF-9, and IL-1α on cell proliferation was examined in three separate cell lines. All experiments were repeated three times. Figures 5 and 6 represent results from normal ONH astrocyte cell lines. IL-1α caused increased cell proliferation at 2 ng/ml but at no other concentrations (ONA 66 years) compared to S+. There was no significant cell proliferation with any other treatment. Figure 7 represent results from a glaucomatous ONH astrocyte cell line. IL-1α caused significant cell proliferation at all the various concentrations compared to S+ as shown in figure 7.

mRNA and Protein Expression of CNTF and its Tripartite Receptor Complex by ONH Astrocytes Following Exogenous CNTF, FGF-9, and IL-1α Treatment

PCR amplification products of expected sizes for CNTF, CNTFRα, gp130 and LIFR primer pairs in human ONA are shown in Figure 8. Amplification product for CNTF and the tripartite receptor complex were expressed by ONA in control (e.g. serum) cell culture conditions (Figure 8, Lane 1). Serum-free culture conditions (Figure 8, Lane 2) did not appear to affect mRNA expression of CNTF or the tripartite receptor complex.

Exogenous treatment with CNTF, FGF-9, or IL-1α did not appear to affect mRNA expression (Figure 8, Lanes 3-5). Control reactions without cDNA did not result in amplification products indicating that reagents and primers were free of DNA or RNA contamination (Figure 8, Lane 6). The β-actin PCR primers yielded a single product of 350 bp.

To confirm that message for CNTF and its tripartite receptor complex is translated into protein, Western blot analysis was performed (Figure 9). A representative ONA cell line (ONA 66 years) is shown. Recombinant CNTF protein was run as a positive control and was observed at 22 kDa and 77 kDa (Figure 9, Lane 1). The CNTF protein is usually found to be approximately 22 kDa, however, our Western blot analysis showed a band at approximately 77 kDa in all human ONA cell lines examined. Protein expression for CNTF and the tripartite receptor complex was observed in control (e.g. serum) cell culture conditions (Figure 9, Lane 2). Interestingly, serum-free culture conditions decreased the protein expression of both CNTF and CNTFRα (Figure 9, Lane 3). Exogenous treatment with CNTF, FGF-9, and IL-1α appeared to increase CNTF protein expression above that seen in serum-free conditions (Figure 9, Lanes 4-6). Interestingly, exogenous FGF-9 and IL-1α tended to increase protein expression of CNTF towards control levels.

Exogenous treatment with CNTF, FGF-9 or IL-1α failed to restore CNTFRα protein (72 kDa) expression (Figure 9, Lanes 4-6). Protein expression for gp130 (130 kDa) was expressed by ONH astrocytes in low levels (Figure 9, Lane 2-6). Protein expression of LIFR (61 kDa) was not altered either by serum-free culture

conditions (Figure 9, Lane 3) or by exogenous treatment with CNTF, FGF-9, or IL-1α (Figure 9, Lanes 4-6). All Western blots were re-probed for β-actin (42 kDa) which served as a loading control. Blocking peptides for CNTF, gp 130, and LIFR eliminated protein identification (Data not shown).

mRNA and Protein Expression of FGF-9 and FGF Receptors by ONH Astrocytes Following Exogenous CNTF, FGF-9, and IL-1α Treatment

Amplification products of expected size for FGF-9 and the four FGFR primer pairs in human ONA are shown in Figure 10. Amplification products for FGF-9 and the four FGF receptors were expressed in ONH astrocytes in control (e.g. serum) cell culture conditions (Figure 10, Lane 1). There was variable FGF-9 mRNA expression with all exogenous treatment compared to the control (S+, Figure 10, Lane 2-6). FGFR1 and FGFR2 were expressed in ONA but there appeared to be minimal expression FGFR3 and FGFR4. Control reactions without cDNA did not result in amplification products indicating that reagents and primers were free of DNA or RNA contamination (Figure 10, lane 6). The β-actin PCR primers yielded a single product of 350 bp.

To confirm that message for FGF-9 and the four FGF receptors was translated into protein by ONA, Western blot analysis was performed (Figure 11). Protein expression for FGF-9 and FGF receptors was observed in control (e.g. serum) cell culture conditions (Figure 11, Lane 2). Recombinant FGF-9 protein was run as a positive control and was observed at 23 kDa and 88 kDa (Figure 11, Lane 1). The FGF-9 protein is usually found to be approximately 23 kDa, however, our Western blot analysis showed a

band at approximately 88 kDa in all human ONA cell lines examined. With respect to FGF-9 expression, serum-free media significantly reduced expression of FGF-9 (Figure 11, Lane 3). Exogenous treatment with CNTF, FGF-9, and IL-1α did not restore FGF-9 protein expression (Figure 11, Lanes 4-6).

Protein expression of FGFR1 (90 kDa) appeared to be uniformly expressed throughout all ONA cell lines examined with no changes observed with exogenous treatment (Figure 11, Lanes 4-6). Similar to FGF-9, the protein expression of FGFR2 (75 kDa) was significantly reduced in serum-free media and was not restored by exogenous growth factor treatment (Figure 11, Lanes 3-6). With respect to FGFR3 (120 kDa), no protein appeared to be expressed by ONA cells (Figure 11, Lanes 2-6). A positive control for FGFR3 protein (e.g. K562 cell line protein) was used to confirm reactivity of the antibody (Figure 11, Lane 1). Protein for FGFR4 (93 kDa) appeared to be uniformly expressed with no changes observed with exogenous CNTF, FGF-9 or IL-1α treatment. All Western blots were re-probed for β-actin which served as a loading control. Blocking peptide for FGF-9 eliminated protein identification (Data not shown).

mRNA and Protein Expression of IL-1α and IL-1RI by ONH Astrocytes Following Exogenous CNTF, FGF-9, and IL-1α Treatment

Amplification products of expected size for IL-1α and the IL-R1 primer pairs in human ONA are shown in Figure 12. Amplification products for IL-1α and IL-1RI receptor were expressed in all ONH astrocytes (Figure 12, Lane 1). Exogenous treatment with IL-1α appeared to greatly increase mRNA expression of IL-1α (Figure 12, Lane 5). The

expression of IL-1RI mRNA did not appear to be altered by serum-free culture conditions or exogenous treatment with CNTF, FGF-9, or IL-1α (Figure 12, Lanes 2-6). Control reactions without cDNA did not result in amplification products indicating that reagents and primers were free of DNA or RNA contamination (Figure 12, lane 6). The β-actin PCR primers yielded a single product of 350 bp.

Western blot analysis was performed to confirm that message or IL-1α and IL-1RI was translated into protein (Figure 13). Recombinant IL-1α protein was run as a positive control and was observed at 60 kDa (Figure 13, Lane 1). The IL-1α protein is usually found to be approximately 18 kDa, however, Western blot analysis showed a band at approximately 60 kDa in all human ONA cell lines examined. Protein expression for IL-1α and IL-1RI was observed in control (e.g. serum) cell culture conditions (Figure 13, Lane 2). With respect to normal human ONA, exogenous treatment with CNTF, FGF-9, and IL-1α all appeared to increase IL-1α protein expression. Interestingly, the increase was greatest with exogenous IL-1α treatment (Figure 13, Lane 6). IL-1RI protein is usually found to be 80 kDa in size, however, Western blot analysis showed a band at approximately 218 kDa in all human ONA cell lines examined. With respect to the expression of IL-1RI, IL-1α administration appeared to decrease protein expression (Figure 13, Lane 6). Exogenous FGF-9, CNTF or serum-free media appeared to have no effect on protein expression of IL-1RI. All Western blots were re-probed for β-actin which served as a loading control.

DISCUSSION

The pathophysiology of activated astrocytes is believed to play a significant role within the CNS. Ciliary neurotrophic factor, FGF-9 and IL-1α have been shown to activate astrocytes within the CNS. To determine if these factors stimulate ONA to become activated, ONA were treated with CNTF, FGF-9 and IL-1α and various biological responses were observed. The purpose of this study was to demonstrate mRNA and protein expression for CNTF, FGF-9, IL-1α, and their respective complexes in untreated ONA and in ONA treated with these factors using semi-quantitative RT-PCR and western blot analysis. To determine ONA activation in response to CNTF, FGF-9, IL-1α,increased GFAP expression, increased NCAM 180 expression, altered cell morphology, and increased cell proliferation were observed following treatment.

Within the CNS, it has been described that CNTF provides neurotrophic activity (Sendtner et al., 1990; Oppenheim et al., 1991; Tutle et al., 1994; and Meyer-Franke et al., 1995) to neurons and has stimulatory effects on glial cells (Winter et al., 1995). The expression of mRNA and protein for CNTF along with its tripartite receptor complex was detected in human ONA, and no differences in expression were observed in any ONA cell lines examined. Treatment of ONA with CNTF, FGF-9 or IL-1α did not appear to alter mRNA levels of CNTF or its receptor complex. With respect to CNTF and CNTFRα, serum-free media seemed to decrease protein expression in ONA cell lines. However, CNTF protein levels increased in ONA following treatment with CNTF, FGF-9 and IL-1α with respect to serum-free conditions. As described earlier, the CNTF protein showed a band at about 77 kDa in all human ONA cell lines examined. A 61 kDa

CNTF band was observed in corneal endothelial cells (Koh, 2002). A decrease in this CNTF isoform along with a concomitant increase in secreted CNTF (22 kDa) in the media was observed. This suggests CNTF may be processed from a pro- or prepro- form similar to neurotrophins (Lee et al., 2001). Additionally, it has been reported that after ischemic conditions, CNTF and CNTFRα expression levels are increased in brain astrocytes (Park et al., 2000). Ischemia of the ONH has been suggested as a mechanism of RGC damage in POAG (Fechtner et al., 1994). Therefore, it may be important to determine if ONA under ischemic conditions increase the expression of CNTF or its tripartite receptor complex resulting in activation of ONA.

It has been reported that FGF-9 activates glial cells within the CNS (Naruo et al. 1993). The expression of FGF-9 and FGFR1 through FGFR4 mRNA were detected in ONA. Using semi-quantitative RT-PCR, FGF-9 and its receptors were expressed for mRNA. However, both FGF-9 and FGFR2 protein were not detected in ONA under serum free conditions, and treatment with CNTF, FGF-9, or IL-1αcould not restore expression to control levels. Western blot analysis also revealed protein expression in ONA cell lines for all FGF receptors examined with the exception of FGFR3. FGF-9 signals mainly act through receptors FGFR3 and FGFR2 (Hecht et al., 1995; Santos-Ocampo et al., 1996). As mentioned previously FGFR2 protein was only expressed in S+ conditions, and FGFR3 protein expression was absent in all ONA cell lines examined. Therefore, it is unlikely exogenous FGF-9 treatment could stimulate any response in these cells.

Within the CNS, IL-1α is elevated following brain injury (Giulian and Lachman 1985; Giulian et al., 1988). Message and protein for IL-1α were expressed in ONA, and treatment with IL-1α significantly increased IL-1αmRNA expression. Treatment of normal ONA cell lines with CNTF, FGF-9, and IL-1α increased IL-1α protein expression as well. Also treatment with IL-1α decreased IL-1RI protein expression. A possible explanation for this observation is that the cell may regulate its expression of IL-1RI via autocrine signaling in order not to over-stimulate itself.

Activated astrocytes are depicted as undergoing morphology changes, cell proliferation, and/or increased GFAP expression (Ridet et al., 1997; Wu et al., 1998). We treated human ONA with factors known to activate astrocytes within the CNS and determined ONA activation using the following endpoints: an upregulation of GFAP and NCAM180 mRNA, increased expression of GFAP protein, changes in ONA morphology, and increased cell proliferation. Expression of NCAM180, a marker of activated astrocytes, did not increase in ONA following treatment with CNTF, FGF-9 or IL-1α. Ricard and co-workers (1999) detected the NCAM 180 isoform in glaucomatous ONH tissue via immunohistochemistry and RT-PCR. Another astrocyte marker upregulated in activation is GFAP. Treatment with CNTF and FGF-9 did not up-regulate GFAP mRNA. However, GFAP mRNA was increased in ONA following treatment with IL-1α. There also appeared to be a greater increase in GFAP protein expression following IL-1α treatment of glaucomatous ONA cell lines compared to untreated ONA.

Activated astrocytes undergo hypertrophy and an increase in the number of processes. Similar to our GFAP expression results, no changes in ONA morphology

were observed following treatment with CNTF or FGF-9. Treatment with IL-1α elicited morphological changes in ONA that included an increase in the number of ONA processes. The apparent increase in the number of processes observed for IL-1α within our cells is similar to what has been reported for brain astrocytes (Lee et al., 1995). The increase in the number of processes could ultimately lead to more signals being sent out in order for ONA to communicate with RGC in POAG. Alternatively, the increase in ONA processes may indicate an increase in release of toxins (eg. NO) to RGC resulting in RGC death.

Cell proliferation is another hallmark of glial activation. Within the CNS, glial cells are known to proliferate due to insult/trauma, diseased states (Norton et al., 1992). Glial proliferation has been observed in early and moderate glaucoma (Ritch et al., 1996; Hernandez and Ye, 1993; Minckler and Spaeth, 1981). An explanation for the glial proliferation observed may be that it is a consequence of RGC loss, and ONA may fill the void caused by RGC death (Ritch et al., 1996; Hernandez and Ye, 1993). Another explanation for glial proliferation may be to participate in synthesizing basement membrane components in response to RGC death (Ritch et al., 1996; Hernandez and Ye, 1993). Our results demonstrated IL-1α increased cell proliferation compared to control (S+) conditions in glaucomatous cell lines, although no ONA proliferation were observed following CNTF, FGF-9, and IL-1α treatment compared to control (S+) conditions in normal ONA cell lines. The IL-1α stimulation of glaucomatous cell lines was similar to the IL-1α induced proliferation of brain astrocytes (Giulian and Lachman, 1985; Giulian et al., 1988).

Based on the endpoints we used to determine astrocyte activation, our data suggest that IL-1α may act within the ONH to activate ONA. Treatment with IL-1α resulted in an increase in its own expression for message and protein, increase GFAP message and protein expression, increase number of ONA cell processes, and increase cell proliferation. The role of IL-1α in the ONH in POAG is not known, although previous reports indicate a possible role for immunoregulatory events associated with glaucoma (Yang et al., 2001; Tezel et al., 2004). Possible immunoregulatory events may occur in normal pressure glaucoma where elevated IOP does not play a role and may be due to an autoimmune disease resulting in damage to RGC and their axons (Tezel et al., 2004). In POAG, an increase in IOP may be related to the immune system in response to "stress" placed upon neurons (Tezel et al., 2004). In glaucoma, activated astrocytes show increased expression of cytokines such as TNFα (Neufeld, 2000) and an increase in NOS-2 (Liu and Neufeld, 2000). The end result of increased NOS-2 and cytokine expression may be an environment detrimental for RGC axons resulting in the death of RGC (Liu and Neufeld 2000; Neufeld et al., 1997; Neufeld et al., 1999).

In conclusion, this study was an initial attempt to identify growth factors that may activate ONH astrocytes. Optic nerve head astrocytes expressed mRNA for CNTF, FGF-9, and IL-1α and their respective receptors. The activation of ONA, as measured by increased GFAP expression, changes in cell morphology, and increased ONA cell proliferation was observed following exogenous IL-1α treatment, suggesting IL-1α may play a role in ONA activation in POAG. Treatment with CNTF and FGF-9 resulted in an up-regulation of CNTF. These events may play a role in the activation signaling pathway

in ONA, although the responses elicited by CNTF and FGF-9 were not as robust as IL-1 α. Therefore, further studies to confirm CNTF and FGF-9 activation is required in ONA. Future experiments studying the IL-1αsignaling pathway in ONA could determine potential therapeutic targets in POAG. Another possibility is experimenting with co-cultures of IL-1α treated ONA and RGC to determine if activated ONA create an environment detrimental to RGC survival. The ultimate goal of these studies is to provide a better understanding of the mechanisms of ONH injury in POAG. Using this knowledge, better therapies can be developed for the treatment of glaucoma such as preventing astrocyte activation by blocking potential growth factors whose expression may act to stimulate this process.

Figure 1. Ethidium bromide-stained gel of GFAP PCR reaction products from one normal and one glaucomatous ONH astrocyte cell line (ONA 84 years and ONA 69 years respectively) following exogenous treatment with CNTF, FGF-9, and IL-1α. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.

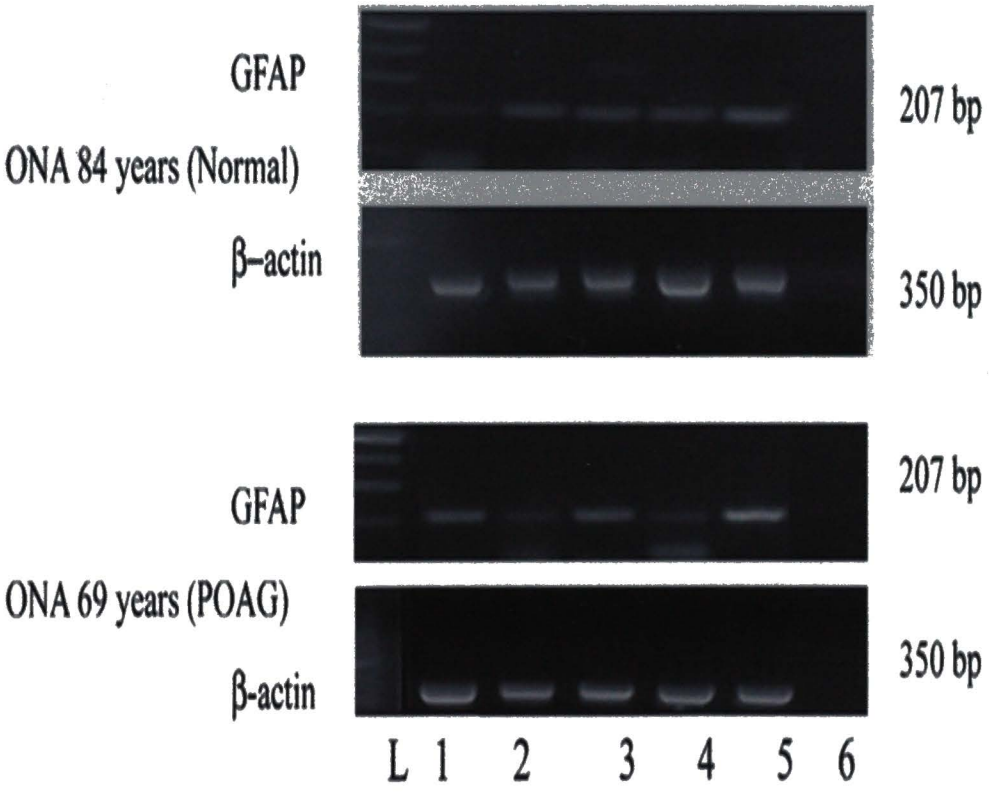

GFAP
ONA 84 years (Normal)
β–actin
207 bp
350 bp
GFAP
ONA 69 years (POAG)
β-actin
207 bp
350 bp
L 1 2 3 4 5 6

Figure 2. Ethidium bromide-stained gel of NCAM 180 PCR reaction products from one normal and one glaucomatous ONH astrocyte cell line (ONA 84 years and ONA 69 years respectively) following exogenous treatment with CNTF, FGF-9, and IL-1α. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.

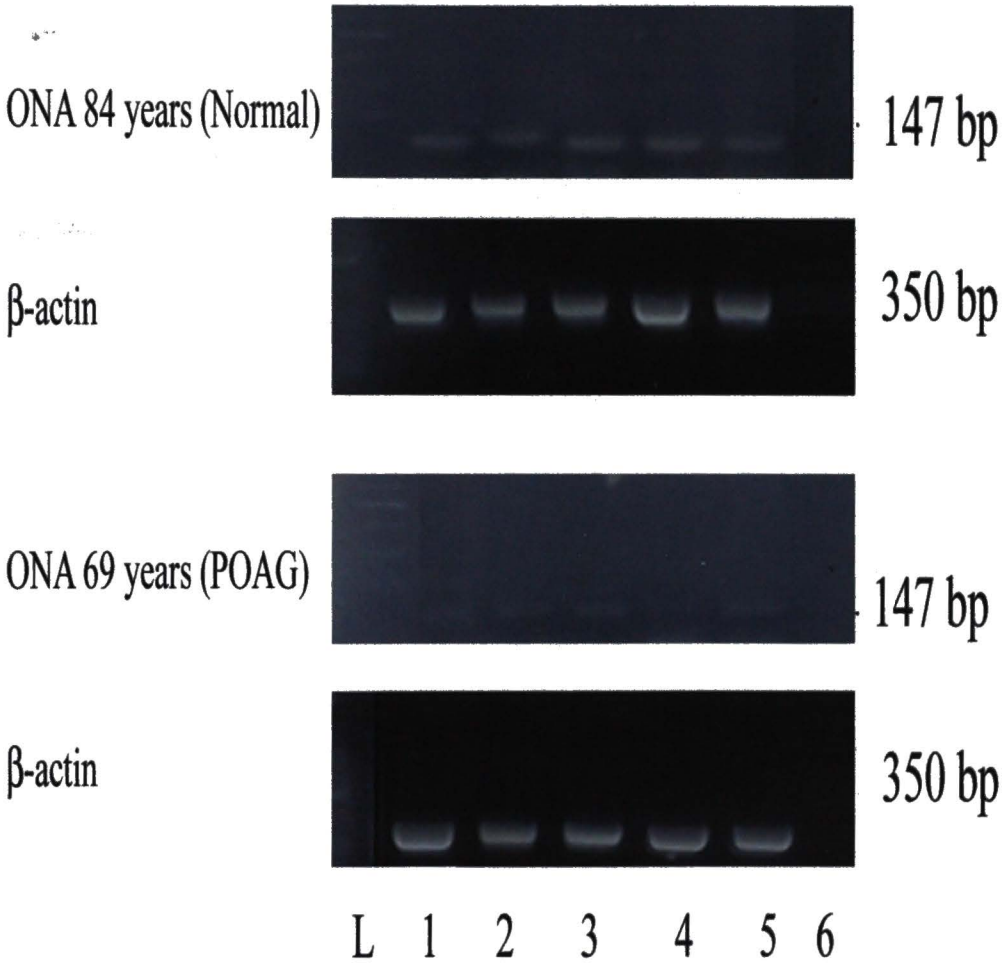

ONA 84 years (Normal)
147 bp
β-actin
350 bp
ONA 69 years (POAG)
147 bp
β-actin
350 bp
L 1 2 3 4 5 6

Figure 3. Chemiluminescent detection of GFAP following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal and one glaucomatous ONH astrocyte cell line (ONA 81 years and ONA 95 years respectively). Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha). The density of each band was measured for each blot. A representative Western blot and densitiometry graph are shown for each ONH astrocyte cell line.

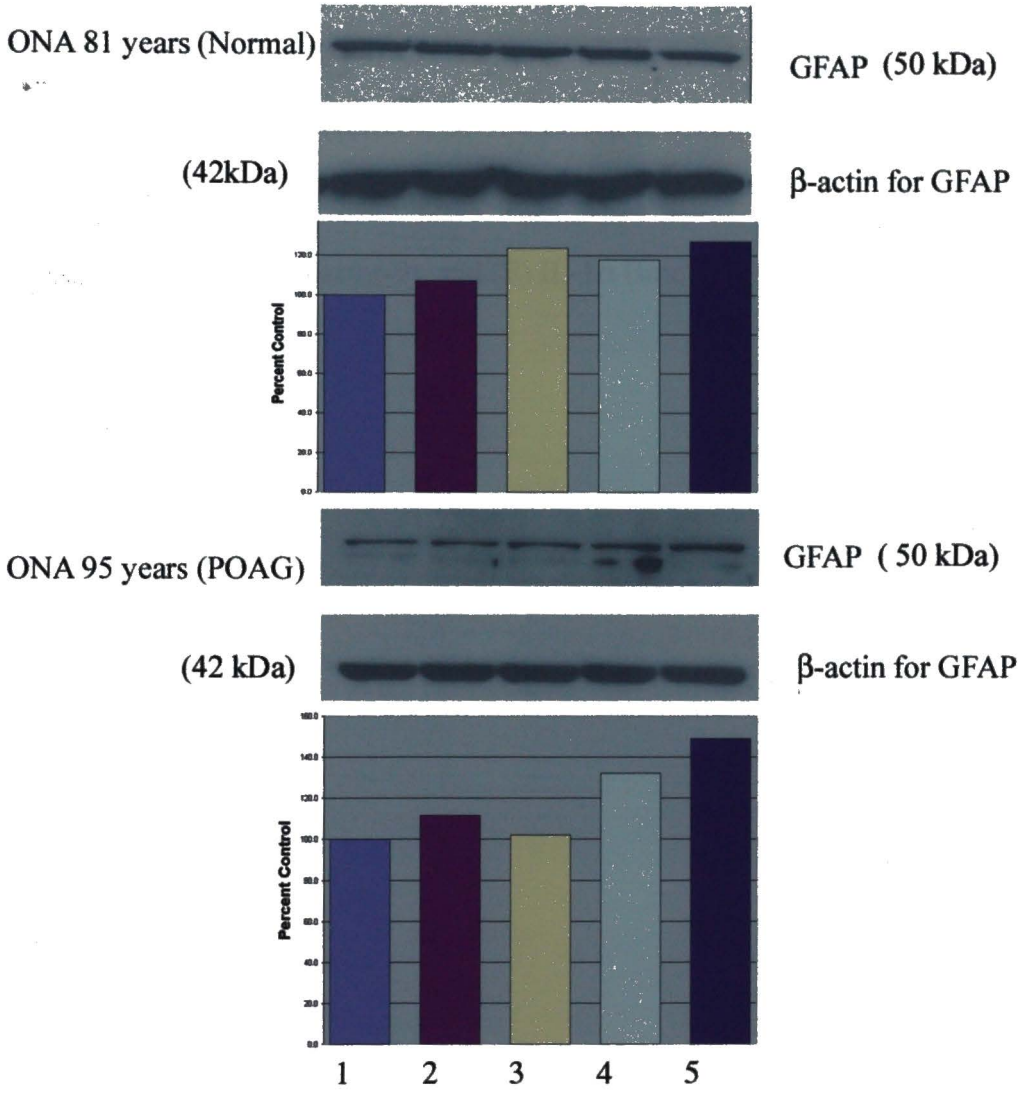
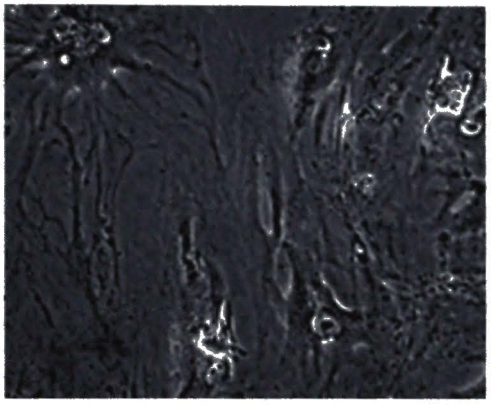
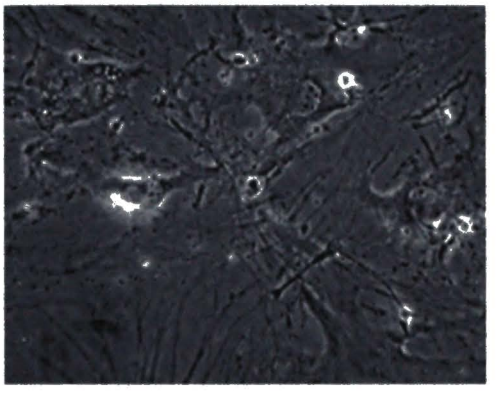

ONA 81 years (Normal)
GFAP (50 kDa)
(42kDa)
β-actin for GFAP
Percent Control
ONA 95 years (POAG)
GFAP (50 kDa)
(42 kDa)
β-actin for GFAP
Percent Control
1
2
3
4
5

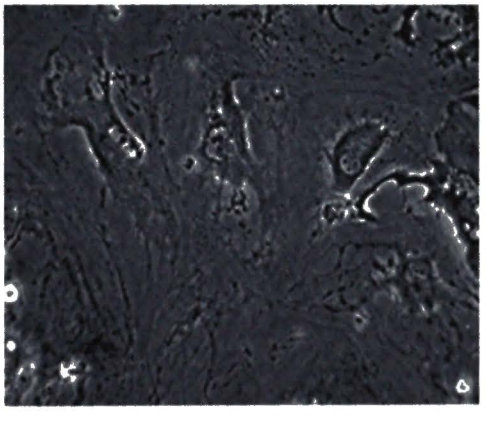
Figure 4. Changes in ONH astrocyte morphology following exogenous treatment with CNTF, FGF-9, and IL-1α. Phase constract microscopy of ONA 95 years (POAG) following treatment with [150 ng/ml] CNTF, [2 ng/ml] FGF-9, or [2 ng/ml] IL-1α. A-E represent the following: (A) S+ (control; ONA cultured in media plus 10% FBS), (B) SF (ONA cultured in serum free media), (C) CNTF (Ciliary Neurotrophic Factor), (D) FGF-9 (Fibroblast Growth Factor-9), and (E) IL-1α (Interleukin-1 alpha).



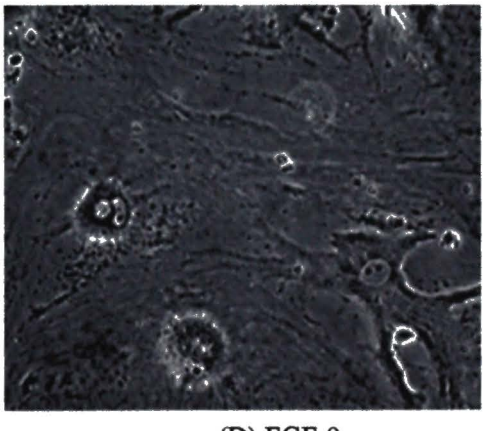
(A) S+



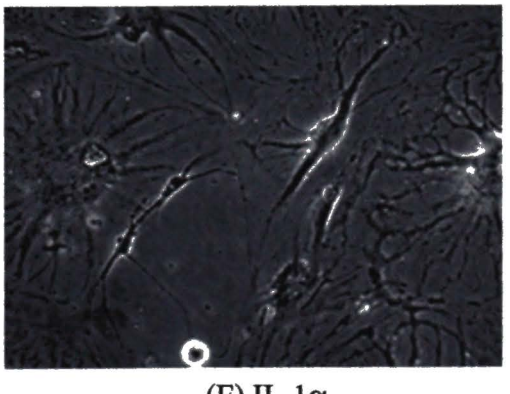
(B) SF



(C) CNTF

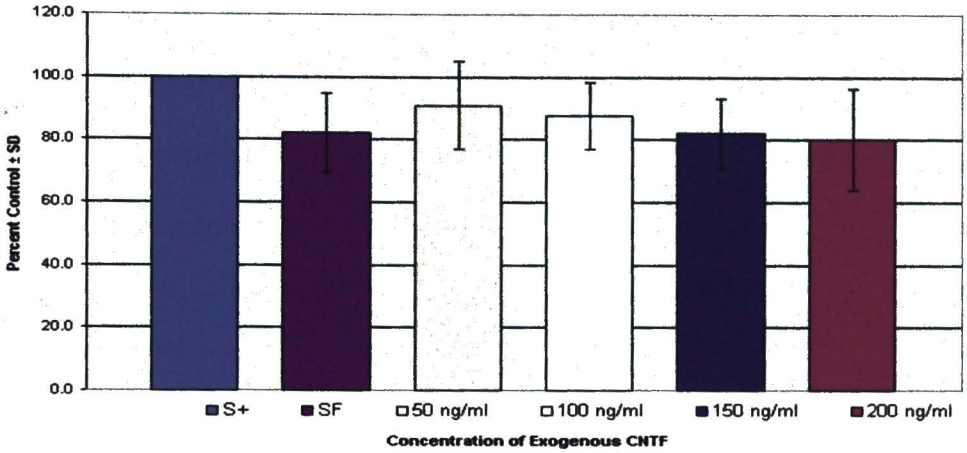


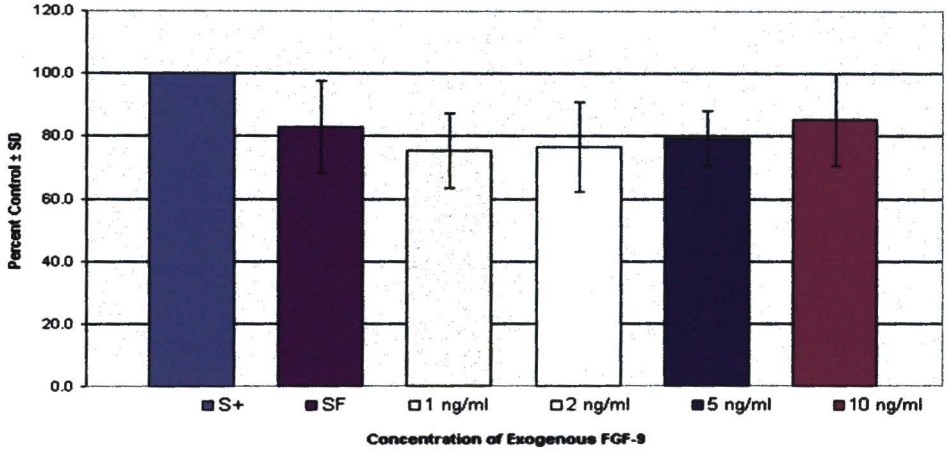
(D) FGF-9



(E) IL-1α

Figure 5. ONH astrocyte cell proliferation following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal ONA cell line (ONA 66 years). No proliferation was observed in ONH astrocytes following CNTF or FGF-9 treatment at any concentration when compared to the control (S+). Exogenous IL-1α at a concentration of 2 ng/ml demonstrated an increase in proliferation compared to the control (S+). Cells were treated for 48 hours. A standard curve for each cell line was generated. ** $p<0.005$ was determined by student t-test.


120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
50 ng/ml
100 ng/ml
150 ng/ml
200 ng/ml
Concentration of Exogenous CNTF


120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous FGF-9

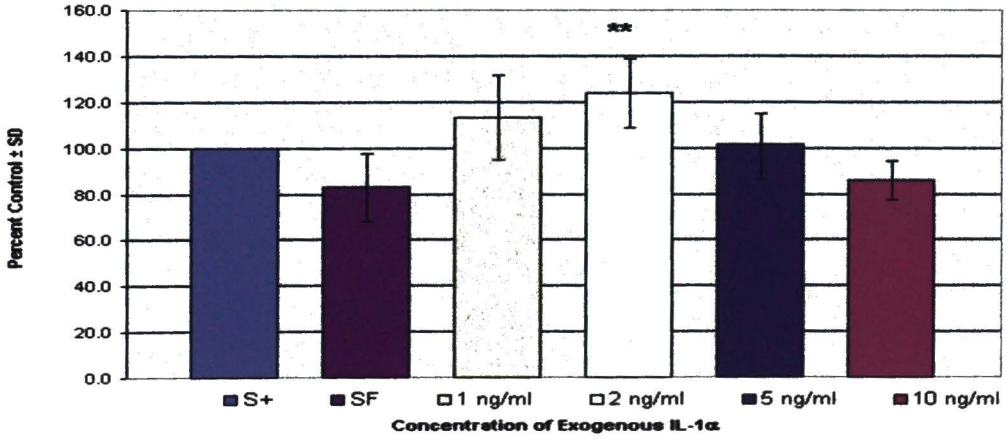
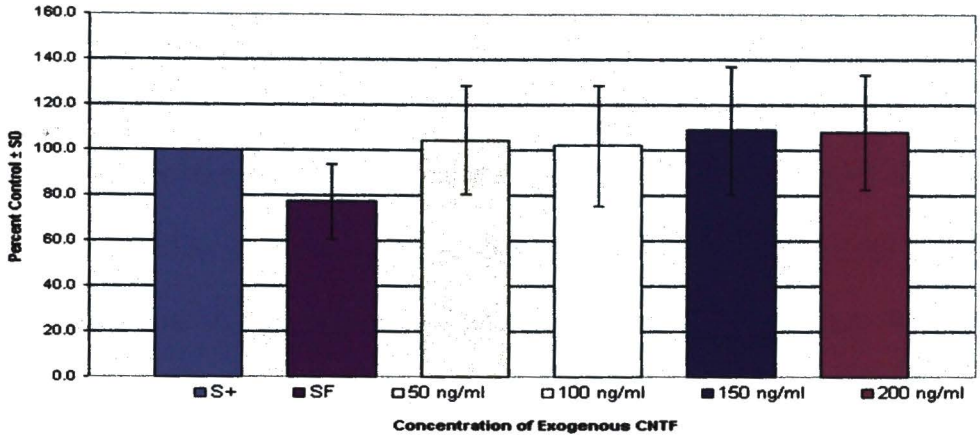
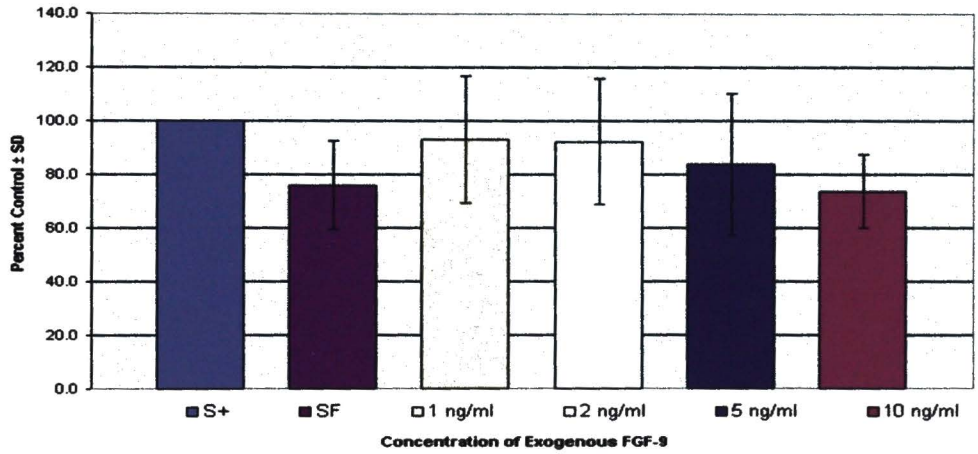

160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
**
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous IL-1α

Figure 6. ONH astrocyte cell proliferation following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal ONA cell line (ONA 87 years). No proliferation was observed in ONH astrocytes following CNTF, FGF-9 or IL-1α treatment at any concentration when compared to the control (S+). Cells were treated for 48 hours. A standard curve for each cell line was generated.


160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
50 ng/ml
100 ng/ml
150 ng/ml
200 ng/ml
Concentration of Exogenous CNTF


140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous FGF-9

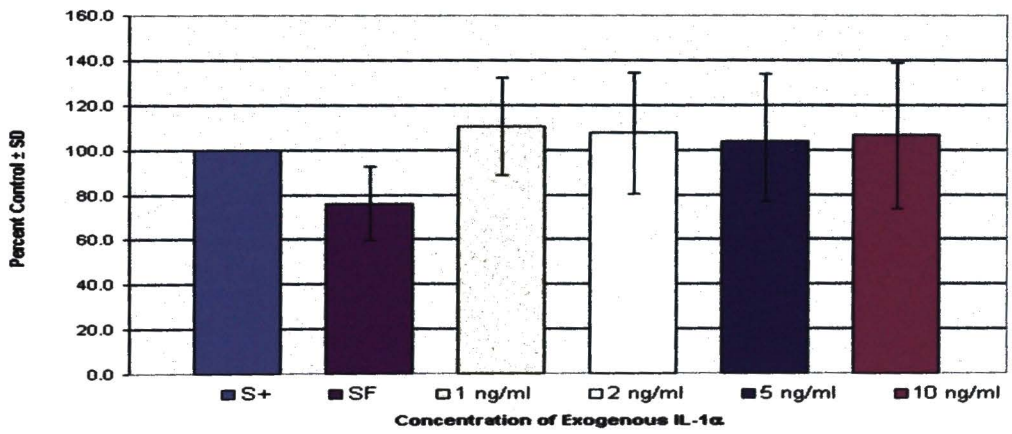
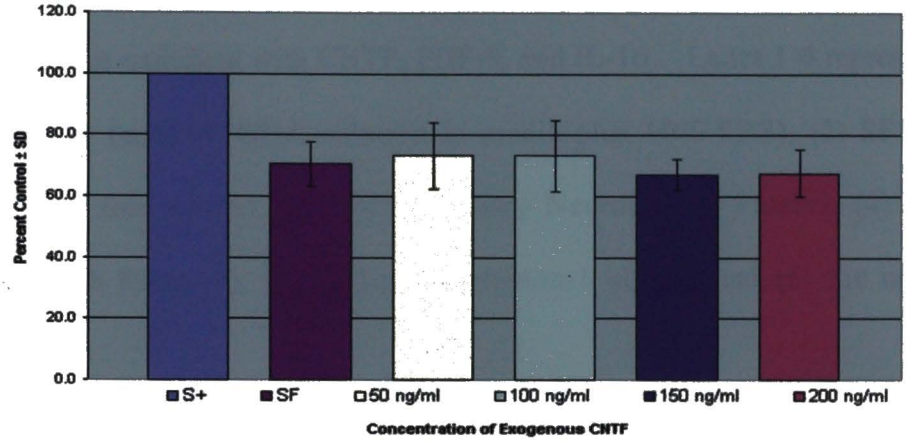
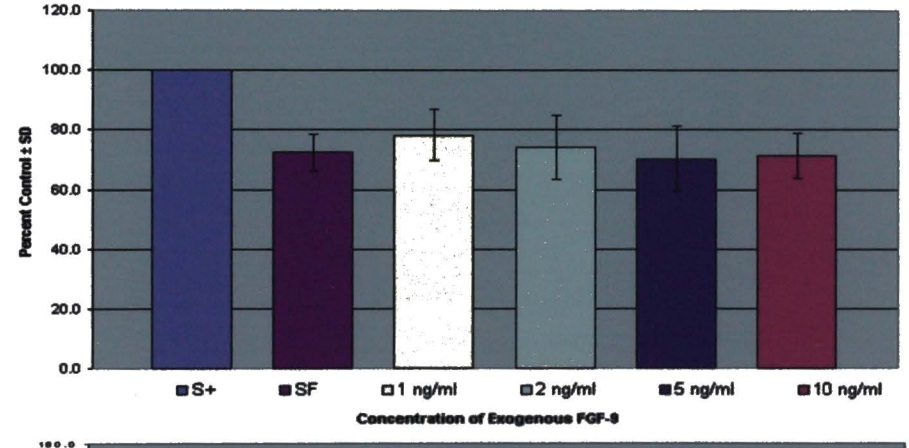

160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous IL-1α

Figure 7. ONH astrocyte cell proliferation following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA cell line (ONA 69 years). No proliferation was observed in ONH astrocytes following CNTF or FGF-9 treatment at any concentration when compared to the control (S+). Exogenous IL-1α at all concentrations tested demonstrated an increase in proliferation compared to the control (S+). Cells were treated for 48 hours. A standard curve for each cell line was generated. *$p<0.05$ or ** $p<0.005$ was determined by student t-test.


120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
50 ng/ml
100 ng/ml
150 ng/ml
200 ng/ml
Concentration of Exogenous CNTF


120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous FGF-9

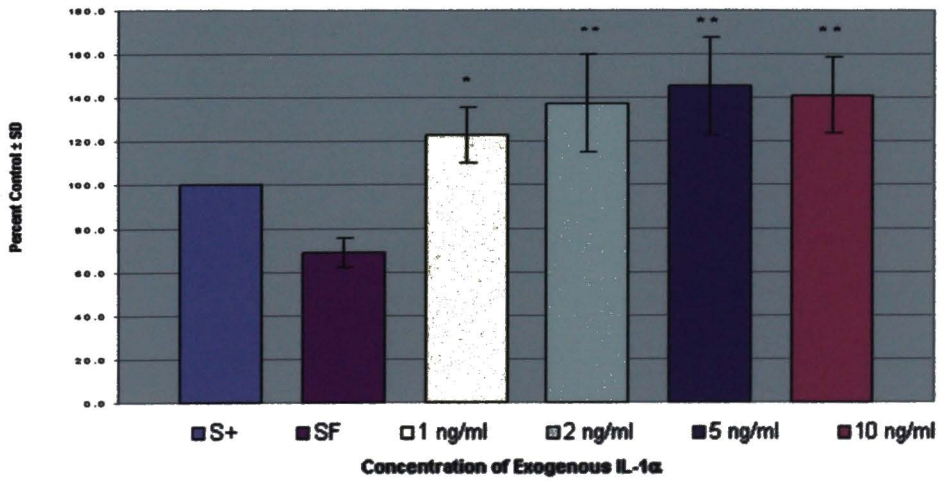

Percent Control ± SD
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous IL-1α

Figure 8. Ethidium bromide-stained gel of CNTF and its tripartite receptor complex PCR reaction products from a representative ONH astrocyte cell line (ONA 69 years) following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.

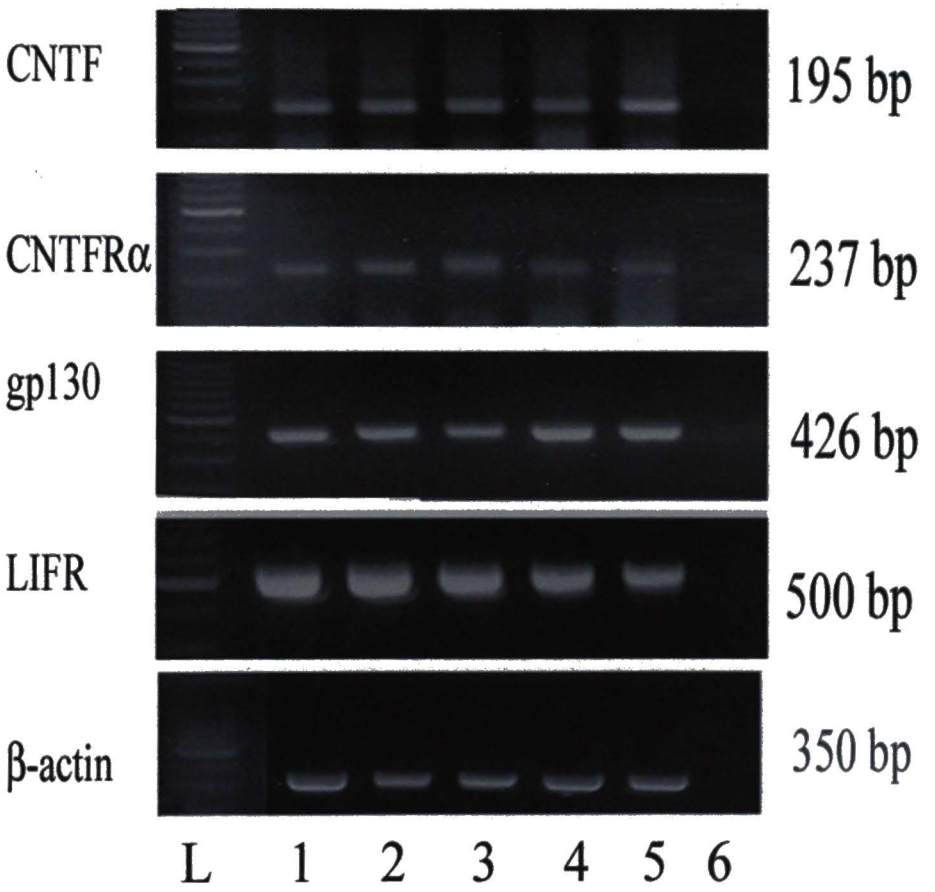
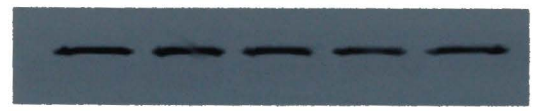

CNTF
195 bp
CNTFRα
237 bp
gp130
426 bp
LIFR
500 bp
β-actin
350 bp
L 1 2 3 4 5 6

Figure 9. Chemiluminescent detection of CNTF and its tripartite receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one ONA cell line. Lanes 1-6 represent the following: (1) r.h. CNTF (recombinant Ciliary Neurotrophic Factor), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha). The density of each band was measured for each blot. A representative Western blot and densitiometry graph are shown for each ONH astrocyte cell line.

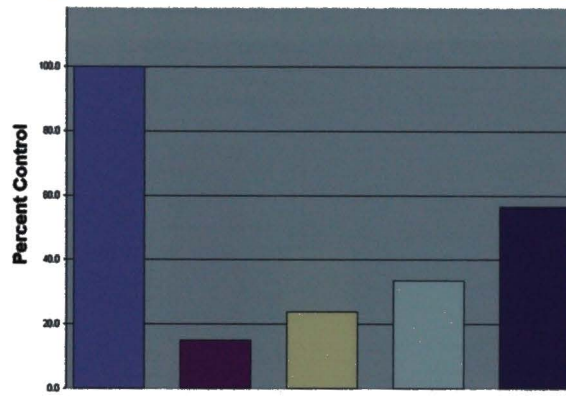
CNTF

77 kDa

β-actin for CNTF



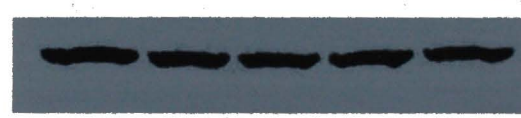
42 kDa



CNTFRα

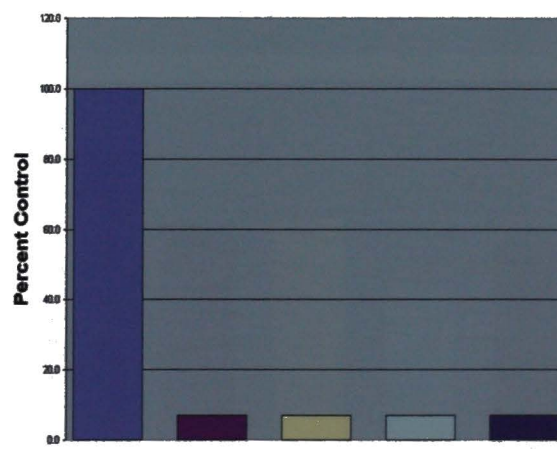
72 kDa

β-actin for CNTFRα



42 kDa

1= rh. protein
2= S+
3= SF
4= CNTF
5= FGF-9
6= IL-1α



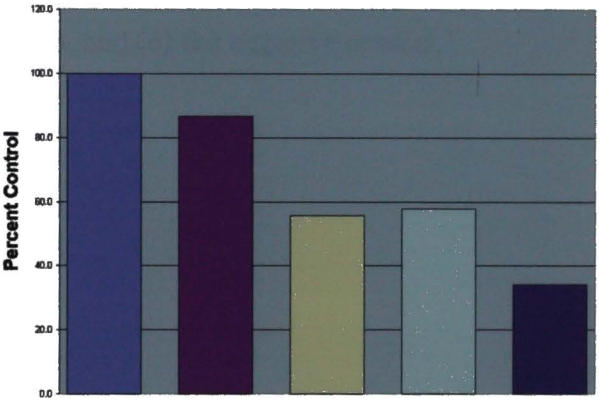
1 2 3 4 5 6

gp 130

130 kDa

β-action for gp130

42 kDa

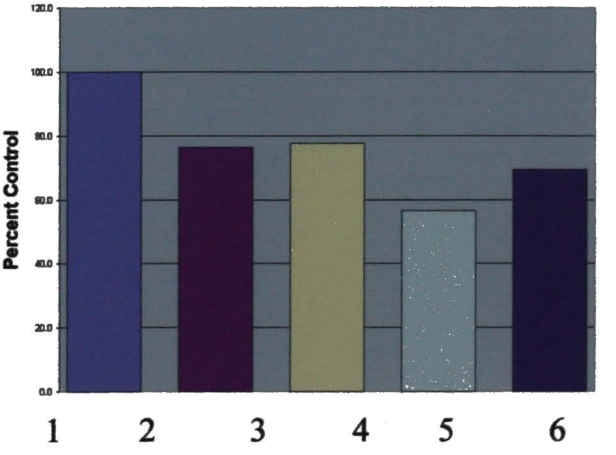


LIFR

61 kDa

β-actin for LIFR

42 kDa



1= rh. protein
2= S+
3= SF
4= CNTF
5= FGF-9
6= IL-1α

Figure 10. Ethidium bromide-stained gel of FGF-9 and its receptor complex PCR reaction products from a representative ONH astrocyte cell line following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.

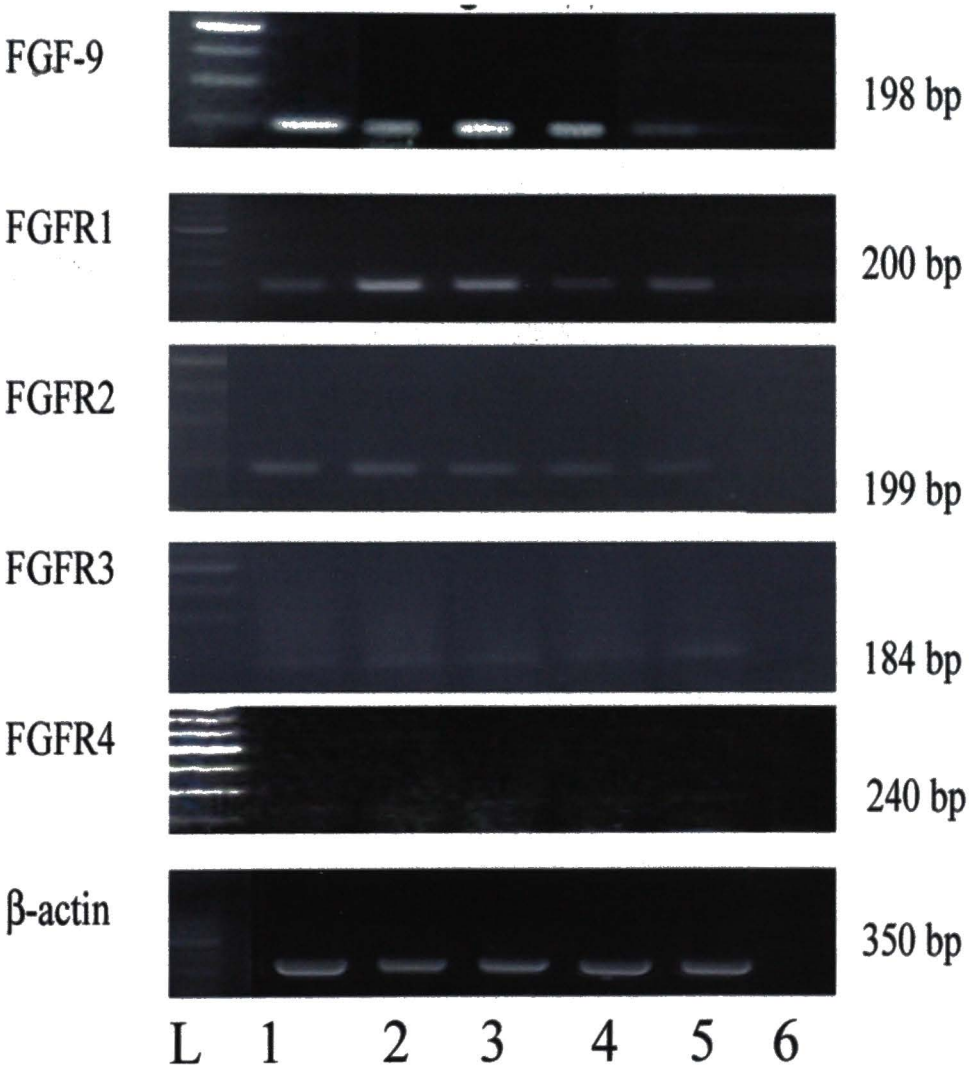
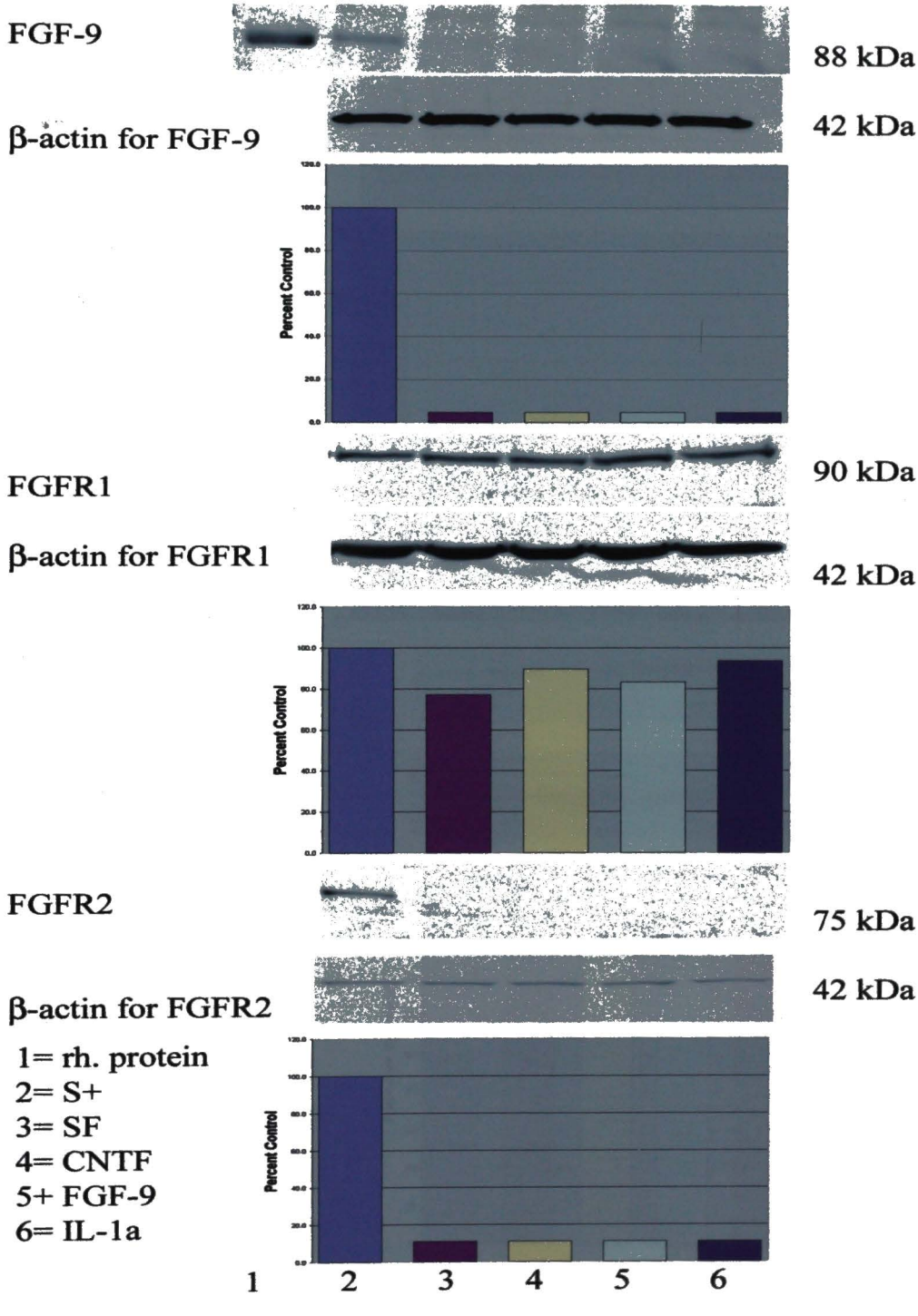

FGF-9
198 bp
FGFR1
200 bp
FGFR2
199 bp
FGFR3
184 bp
FGFR4
240 bp
β-actin
350 bp
L 1 2 3 4 5 6

Figure 11. Chemiluminescent detection of FGF-9 and its receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one ONA cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha). The density of each band was measured for each blot. A representative Western blot and densitiometry graph are shown for each ONH astrocyte cell line.

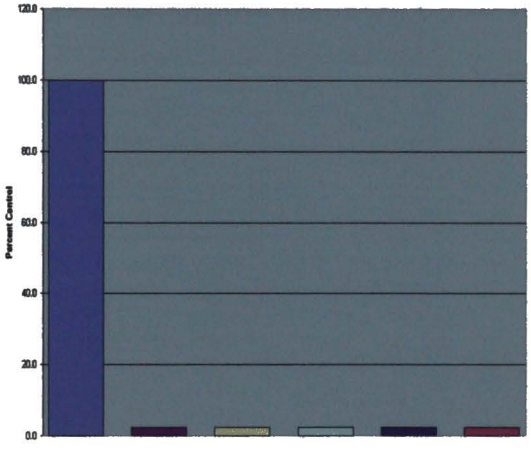

FGF-9
88 kDa
β-actin for FGF-9
42 kDa
Percent Control
FGFR1
90 kDa
β-actin for FGFR1
42 kDa
Percent Control
FGFR2
75 kDa
β-actin for FGFR2
42 kDa
1= rh. protein
2= S+
3= SF
4= CNTF
5+ FGF-9
6= IL-1a
Percent Control
1
2
3
4
5
6

FGFR3

120 kDa

β-actin for FGFR3

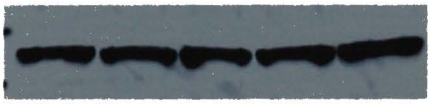
42 kDa



FGFR4

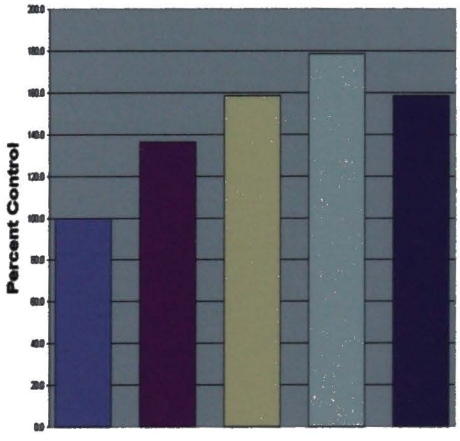
93 kDa

β-actin for FGFR4



42 kDa

1= + control
2= S+
3= SF
4= CNTF
5+ FGF-9
6= IL-1a



1 2 3 4 5 6

Figure 12. Ethidium bromide-stained gel of IL-1α and IL-1RI PCR reaction products from a representative ONH astrocyte cell line following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.

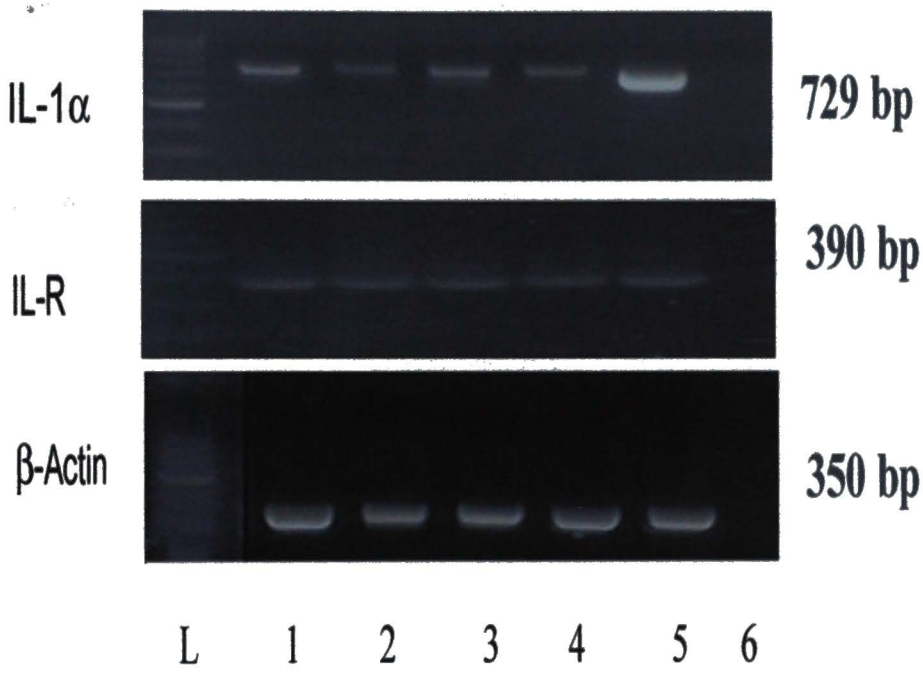

IL-1α
729 bp
IL-R
390 bp
β-Actin
350 bp
L 1 2 3 4 5 6

Figure 13. Chemiluminescent detection of IL-1α and IL-1RI following exogenous treatment with CNTF, FGF-9, and IL-1α in one ONA cell line. Lanes 1-6 represent the following: (1) r.h IL-1α (recombinant Interleukin-1 alpha), (2) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha). The density of each band was measured for each blot. A representative Western blot and densitiometry graph are shown for each ONH astrocyte cell line.

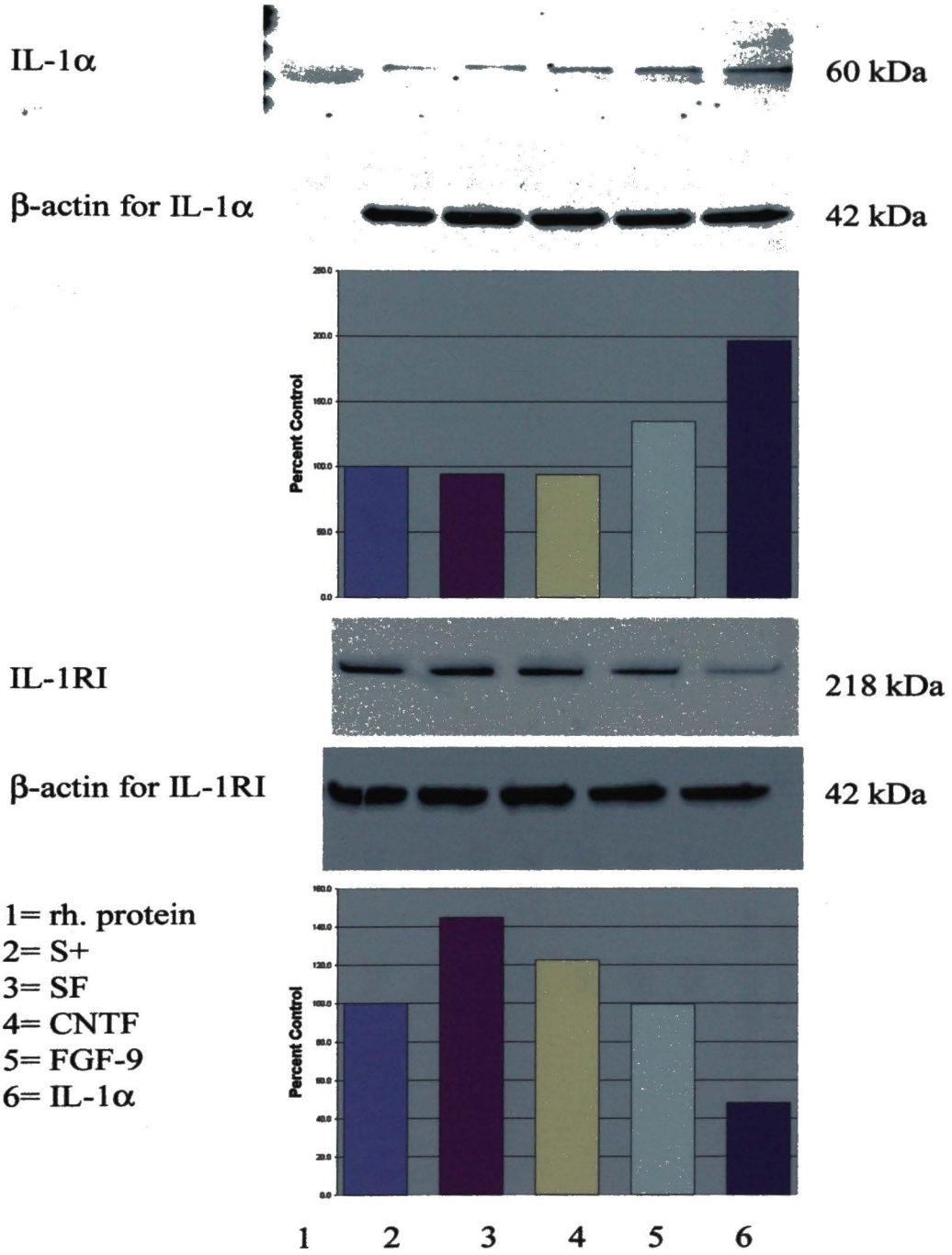

IL-1α
60 kDa
β-actin for IL-1α
42 kDa
Percent Control
IL-1RI
218 kDa
β-actin for IL-1RI
42 kDa
1= rh. protein
2= S+
3= SF
4= CNTF
5= FGF-9
6= IL-1α
Percent Control
1
2
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TABLE 4. Changes in ONH Astrocytes Protein Expression in Response to Exogenous CNTF, FGF-9, and IL-1α Treatment Compared to the S+ Control (media plus 10% FBS)

Growth Factor/ Receptor	SF	CNTF	FGF-9	IL-1α
CNTF	D	I (w/r/t SF)	I (w/r/t SF)	I (w/r/t SF)
CNTFRα	D	D	D	D
gp 130*	*	*	*	*
LIFR	NC	NC	NC	NC
FGF-9	D	D	D	D
FGFR-1	NC	NC	NC	NC
FGFR-2	D	D	D	D
FGFR-3	ND	ND	ND	ND
FGFR-4	NC	NC	NC	NC
IL-1α	NC	NC	I	I
IL-1RI	NC	NC	NC	D
GFAP	NC	NC	I	I

w/r/t SF- with respect to serum free
I- Increase D- Decrease NC- No Change ND- Not Detected
*Expression varied across multiple ONA cell lines.

TABLE 5. Proliferation of ONH Astrocytes in Response to Exogenous CNTF, FGF-9, and IL-1α Treatment Compared to the S+ Control (media plus 10% FBS)

Cell Line	SF	CNTF	FGF-9	IL-1α
ONA 66 years (Normal)	NP	NP	NP	Proliferation observed at 2ng/ml only
ONA 87 years (Normal)	NP	NP	NP	NP
ONA 69 years (POAG)	NP	NP	NP	Proliferation observed at all concentrations

NP- No Proliferation

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CHAPTER IV

SUMMARY

This study has shown that human optic nerve astrocytes express CNTF, FGF-9, IL-1α, and express their receptors. The hypothesis that was tested in this study is that exogenous FGF-9, CNTF, and/or IL-1α activate human ONA. Based on literature reserach, this is the first time that activation of human ONA following treatment with these growth factors has been attempted. Interestingly, activation of ONA in response to IL-1α was shown via GFAP mRNA and protein expression, change in ONA morphology, and cell proliferation.

The source of IL-1α within the ONH is not known. ONA express the ligand and receptor for IL-1α, thus are capable of secreting and responding to IL-1α. Within the CNS, astrocytes make and secrete IL-1 (Aschner, 1998; Benveniste, 1993). Giulian and Lachman (1985), reported that brain astrocytes secreted IL-1 after brain injury. Although the role of NO is controversial, it has been previously reported that cytokines such as IL-1 can trigger nitric oxide (NO) synthase to produce NO, which can result in eye diseases such as glaucoma (Chiou, 2000; Chiou, 2001). ONH degeneration and vision loss in glaucoma may result from neurotoxic effects of NO (Chiou 2001; Neufeld et al., 1999; Kaufman, 1999). Furthermore, NOS-2 has been found in activated astrocytes of the

ONH, therefore causing an environment detrimental to RGC axons and ultimately the death of RGC (Liu and Neufeld 2000; Neufeld et al., 1997; Neufeld et al., 1999).

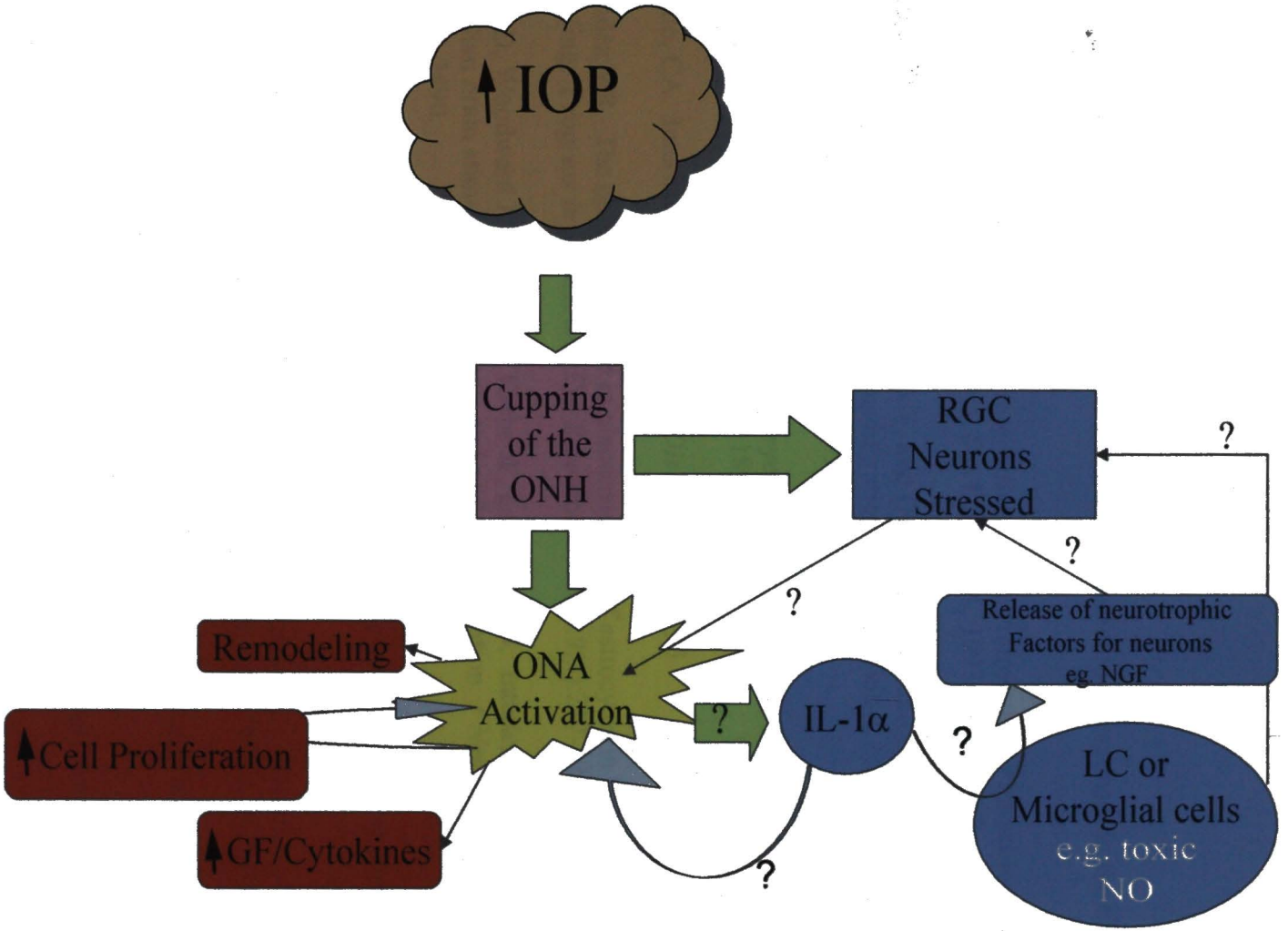
Glial cells other than ONA are found within the ONH and include lamina cribrosa and microglia cells (Hernandez, 2000). Within the CNS, microglia cells are known to be the primary source of IL-1 (Aschner, 1998; Benveniste, 1993; Pinteaux et al., 2002). Microglia have multiple roles within the ONH including release of cytotoxic mediators, tissue remodeling, and phagocytosis (Yuan and Neufeld, 1999). Streit and co-workers (1999), discussed the possible role of cytokines and growth factors involved in inter-glial communications, specifically in astroglia-microglia communication. Microglia and astrocytes are the two major reactive glial cells within the CNS (Streit et al., 1999). In glaucoma, activated microglia have been described (Yuan & Neufeld, 2001; Neufeld, 1999). Gilulian and co-workers (1988b), hypothesized astrocyte proliferation due to IL-1α is a process regulated by microglia cells. Thus, one possibility is that ONA activation in POAG may result from IL-1α secreted by microglia within the ONH. To conclude this project, Figure 1 illustrates possible mechanisms involving activated ONA and IL-1α. On the left of this schematic are mechanisms known to be involved for activated ONA and to the right are speculations for IL-1α.

Future studies will investigate other factors that mimic gliosis in the ONH such as ischemic conditions and/or elevated IOP. These studies may determine a more exact role for IL-1α and other factors within the ONH in terms of their affects on RGCs and ONA. Co-culture of RGC and ONA/or other glial cells such as microglia or lamina cribrosa

cells may determine if growth factors such as IL-1α are up-regulated due to stress environments.

As described previously, the devastating outcome in glaucoma is vision loss due to the loss of RGC via apoptosis. Thus, numerous studies focused on RGC and how to enhance RGC survival. Interestingly, both CNTF and FGF-9 have been documented to increase RGC survival in animal models (Mey and Thanos, 1993; Weise et al., 2000; Kinkl et al., 2003). The sources of CNTF and FGF-9 may be astrocytes or other glial cells. As shown in this study, both CNTF and FGF-9 are expressed in ONA and it would be interesting to determine if ONA secrete these growth factors. Another model that may test RGC survival is an in vivo model that knocks down expression of CNTF or FGF-9 that may compromise RGC survival under high IOP, or an animal model which CNTF and FGF-9 are over-expressed that may save RGC under high IOP. In conclusion, the understanding gained from these studies could be used to develop effective therapies to treat glaucoma patients.

Figure 1. Possible mechanisms involved in the effects of IL-1α on ONA. To the left of this schematic are mechanisms that are known to be involved for activated ONA and to the right are speculations for IL-1α. In glaucoma there is elevated IOP resulting in cupping of the ONH and finally ONA activation. Previous literature indicates ONA undergo tissue remodeling, cell proliferation, and an increase of growth factors/cytokines within the ONH. This study has shown that ONA express IL-1α and its receptor IL-1RI, and may be capable of secreting IL-1α. Interleukin-1α may then act in an autocrine mechanism and activate ONA or in a paracrine mechanism, activating other cells within the ONH which may include microglial and lamina cribrosa cells. Interleukin-1 α may result in the release of toxic, such as NO, causing a detrimental effect to RGC. Retinal ganglion cells in return will release signals in the vicinity of ONA. Finally, IL-1 α may cause the release other growth factors, for example NGF, to provide neurotrophic support to RGC.


IOP
Cupping of the ONH
RGC Neurons Stressed
?
?
?
Release of neurotrophic Factors for neurons eg. NGF
Remodeling
ONA Activation
?
IL-1α
?
LC or Microglial cells e.g. toxic NO
Cell Proliferation
GF/Cytokines
?

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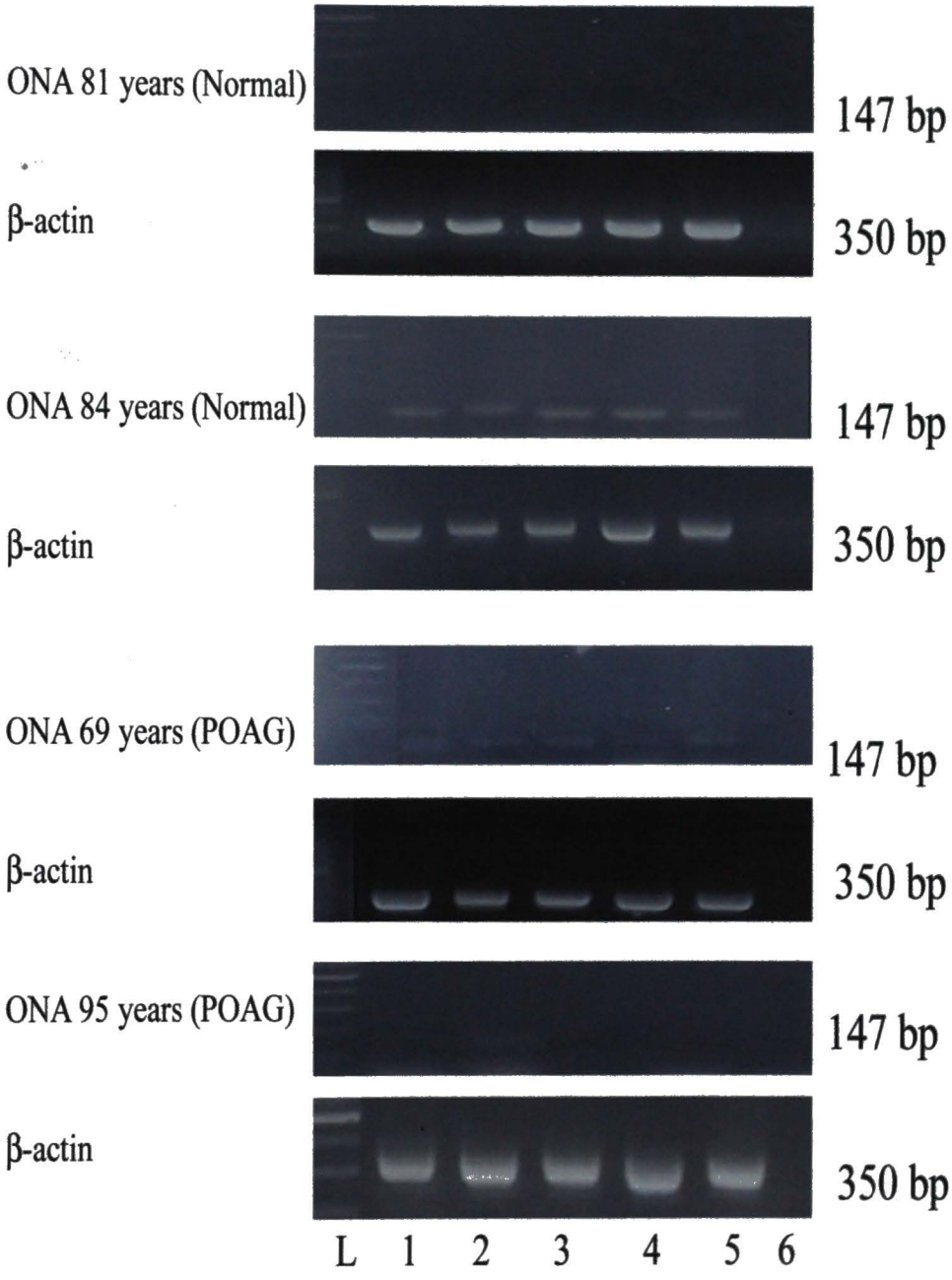
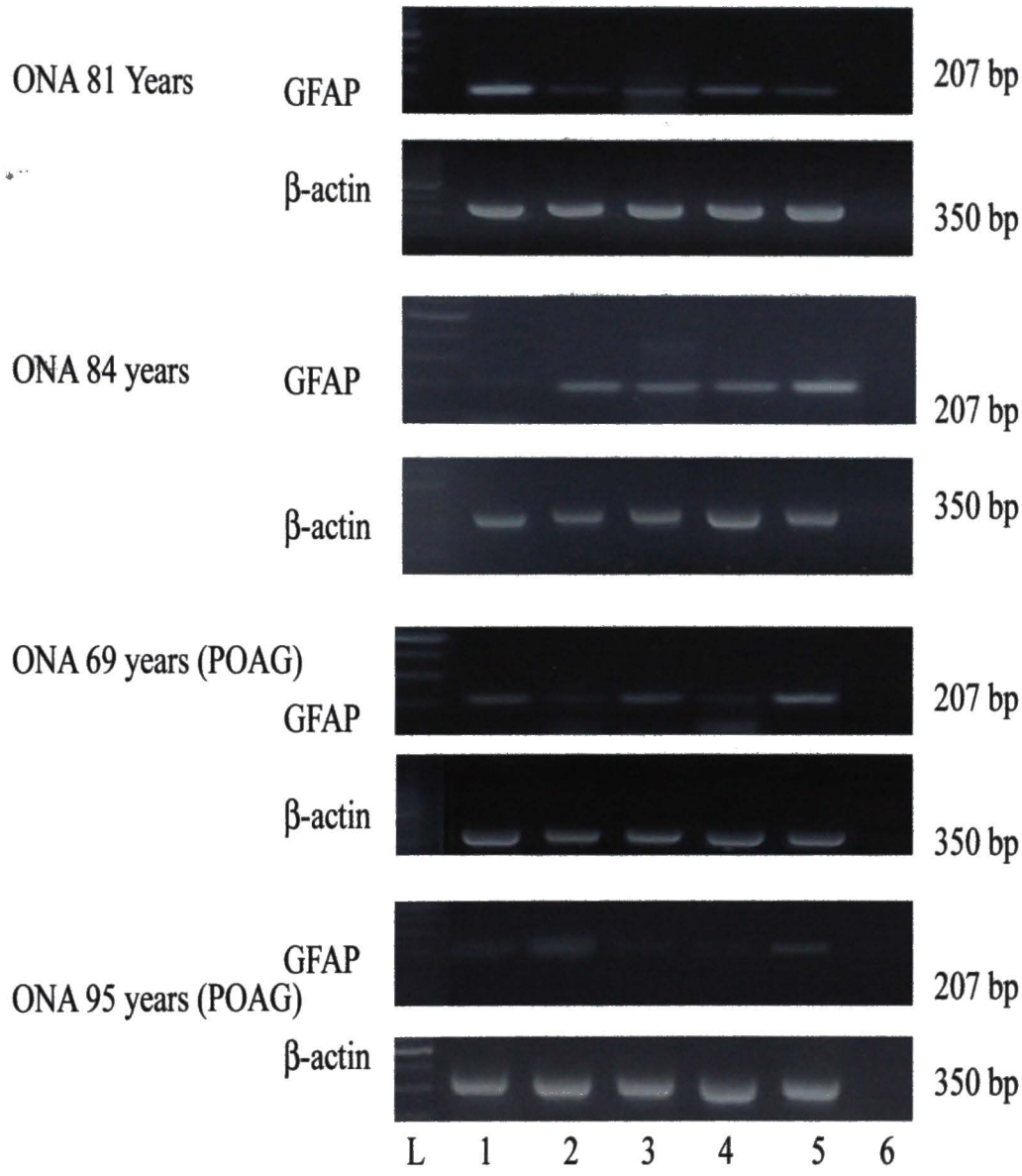
CHAPTER V

APPENDIX

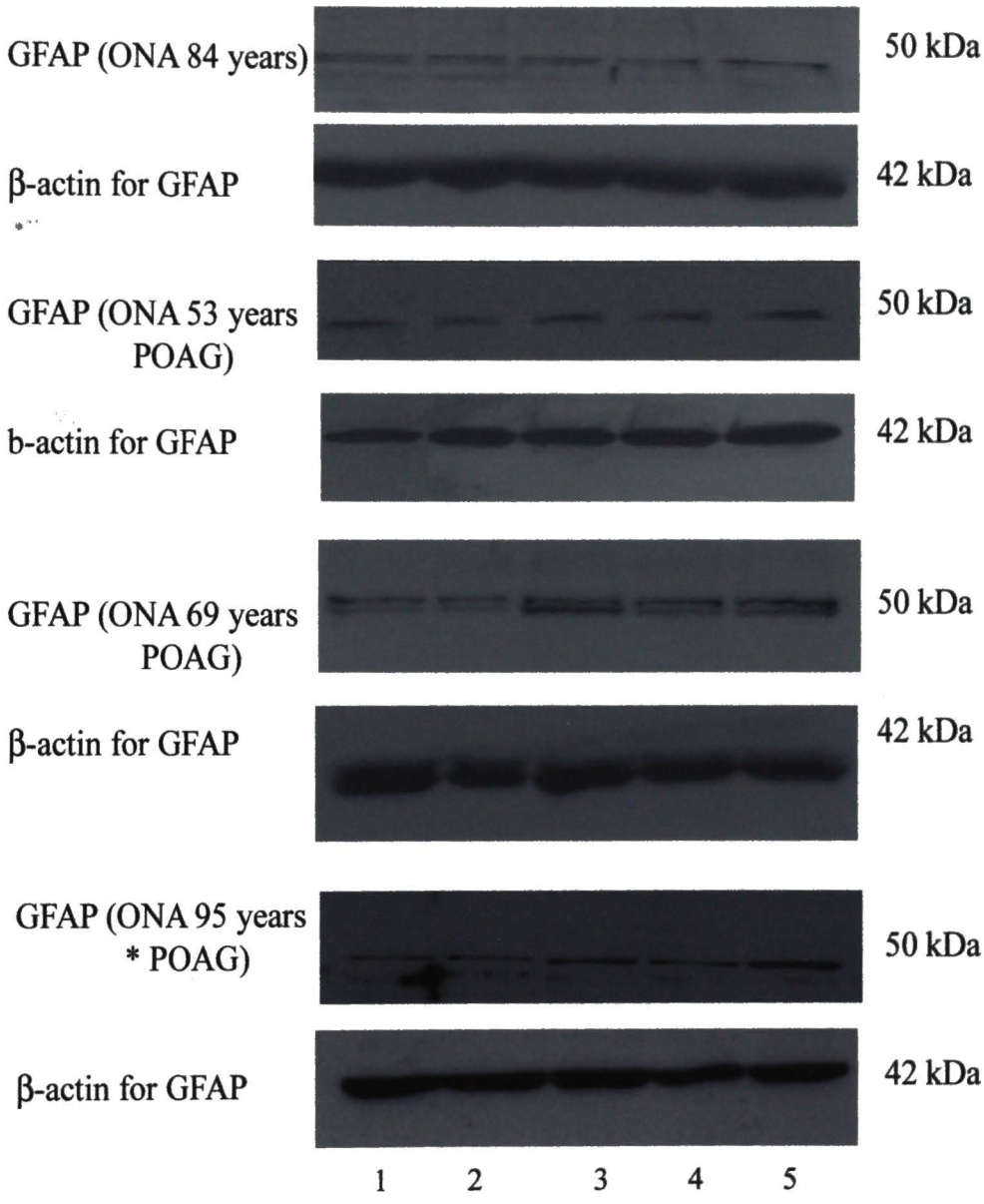
Ethidium bromide-stained gel of NCAM 180 PCR reaction products from ONH astrocytes following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.


ONA 81 years (Normal)
147 bp
β-actin
350 bp
ONA 84 years (Normal)
147 bp
β-actin
350 bp
ONA 69 years (POAG)
147 bp
β-actin
350 bp
ONA 95 years (POAG)
147 bp
β-actin
350 bp
L 1 2 3 4 5 6

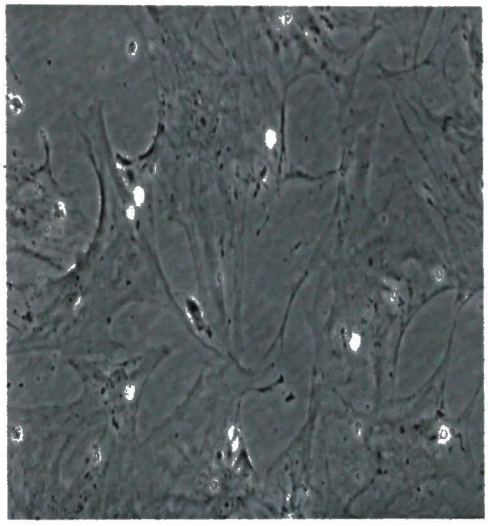
Ethidium bromide-stained gel of GFAP PCR reaction products from ONH astrocytes following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.


ONA 81 Years
GFAP
207 bp
β-actin
350 bp
ONA 84 years
GFAP
207 bp
β-actin
350 bp
ONA 69 years (POAG)
GFAP
207 bp
β-actin
350 bp
GFAP
ONA 95 years (POAG)
207 bp
β-actin
350 bp
L 1 2 3 4 5 6

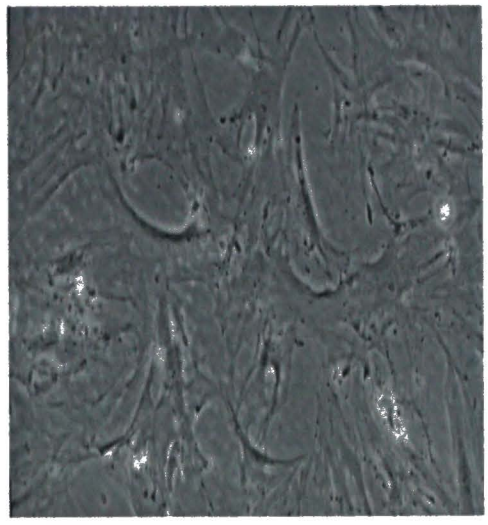
Chemiluminescent detection of GFAP following exogenous treatment with CNTF, FGF-9, and IL-1α in ONH astrocytes. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha). *Denotes treatment with exogenous growth factors for 72 hours rather than 48 hours.


GFAP (ONA 84 years)
50 kDa
β-actin for GFAP
42 kDa
GFAP (ONA 53 years POAG)
50 kDa
b-actin for GFAP
42 kDa
GFAP (ONA 69 years POAG)
50 kDa
β-actin for GFAP
42 kDa
GFAP (ONA 95 years * POAG)
50 kDa
β-actin for GFAP
42 kDa
1
2
3
4
5

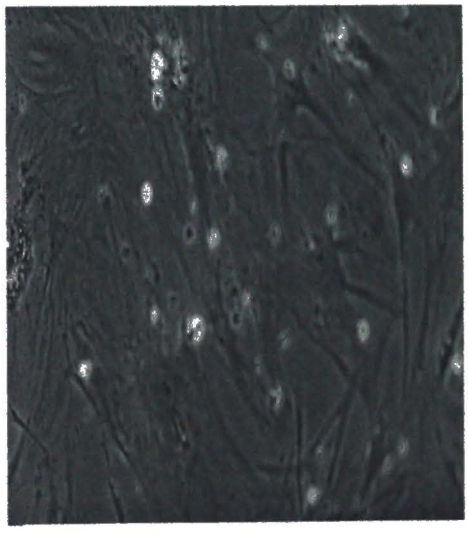
Changes in ONH astrocyte morphology following exogenous treatment with CNTF, FGF-9, and IL-1α. Phase constrast microscopy of ONA 81 years following treatment with [150 ng/ml] CNTF, [2 ng/ml] FGF-9, or [2 ng/ml] IL-1α. A-D represent the following: (A)) SF (ONA cultured in serum free media), (B) CNTF (Ciliary Neurotrophic Factor), (C) FGF-9 (Fibroblast Growth Factor-9), and (D) IL-1α (Interleukin-1 alpha).



(A) SF



(B) CNTF

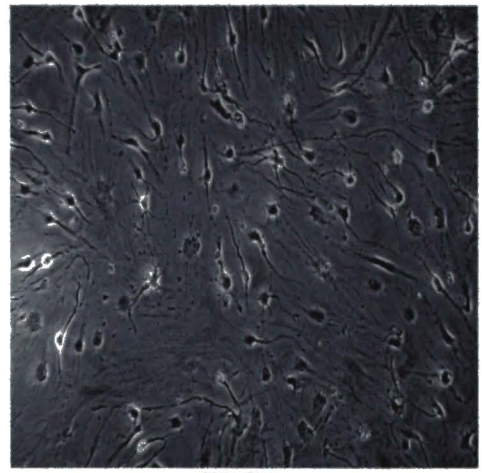


(C) FGF-9

(D) IL-1α

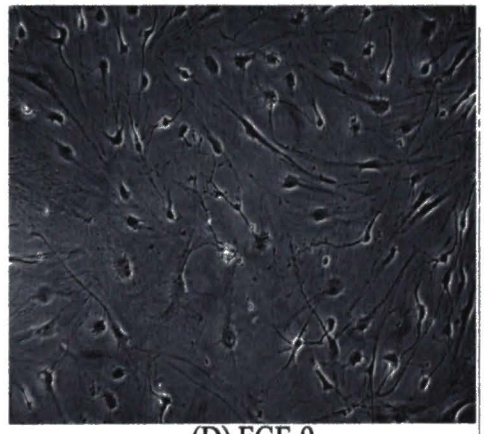
Changes in ONH astrocyte morphology following exogenous treatment with CNTF, FGF-9, and IL-1α. Phase constract microscopy of ONA 69 years (POAG) following treatment with [150 ng/ml] CNTF, [2 ng/ml] FGF-9, or [2 ng/ml] IL-1α. A-E represent the following: (A) S+ (control; ONA cultured in media plus 10% FBS), (B) SF (ONA cultured in serum free media), (C) CNTF (Ciliary Neurotrophic Factor), (D) FGF-9 (Fibroblast Growth Factor-9), and (E) IL-1α (Interleukin-1 alpha).

(A) S+



(B) SF

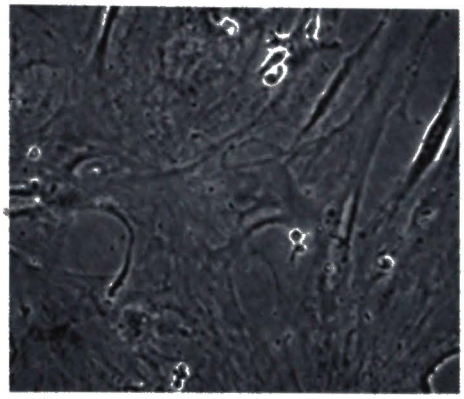
(C) CNTF



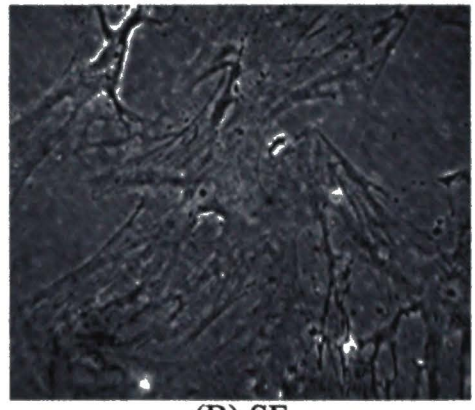
(D) FGF-9

(E) IL-1α

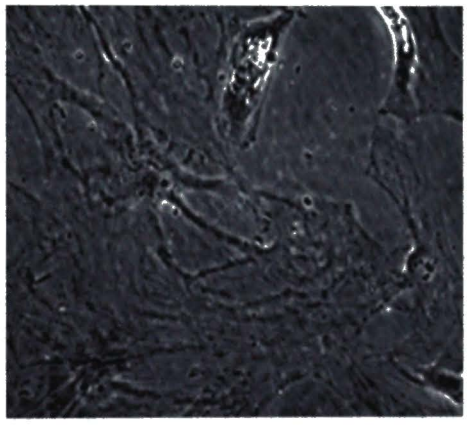
Changes in ONH astrocyte morphology following exogenous treatment with CNTF, FGF-9, and IL-1α. Phase constract microscopy of ONA 53 years (POAG) following treatment with [150 ng/ml] CNTF, [2 ng/ml] FGF-9, or [2 ng/ml] IL-1α. A-E represent the following: (A) S+ (control; ONA cultured in media plus 10% FBS), (B) SF (ONA cultured in serum free media), (C) CNTF (Ciliary Neurotrophic Factor), (D) FGF-9 (Fibroblast Growth Factor-9), and (E) IL-1α (Interleukin-1 alpha).



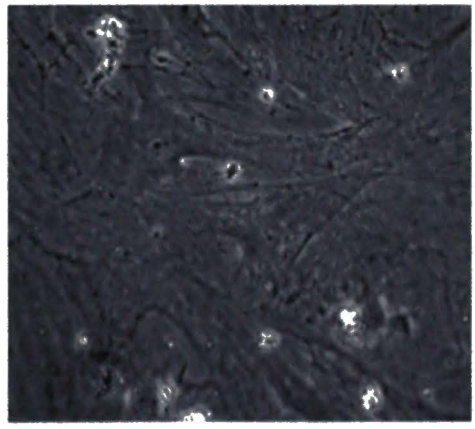
(A) S+



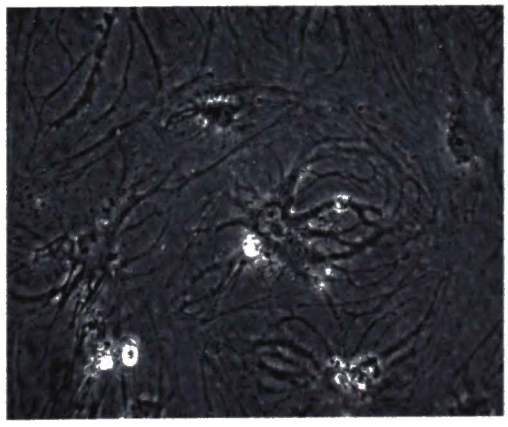
(B) SF



(C) CNTF

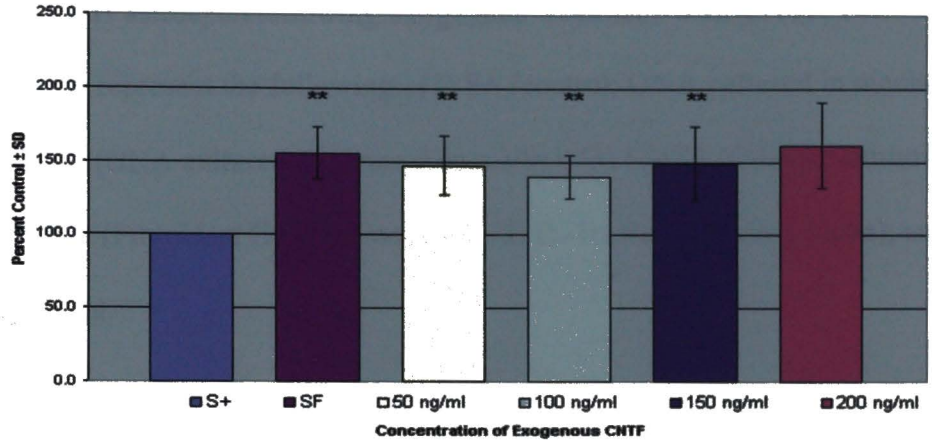


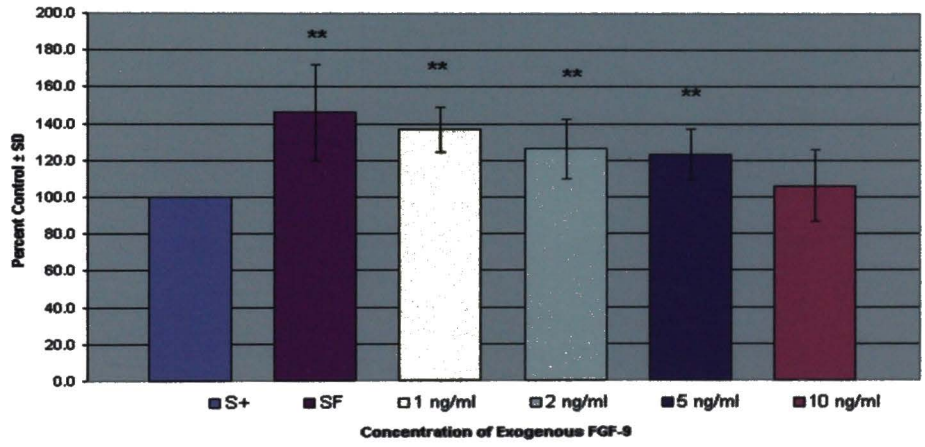
(D) FGF-9

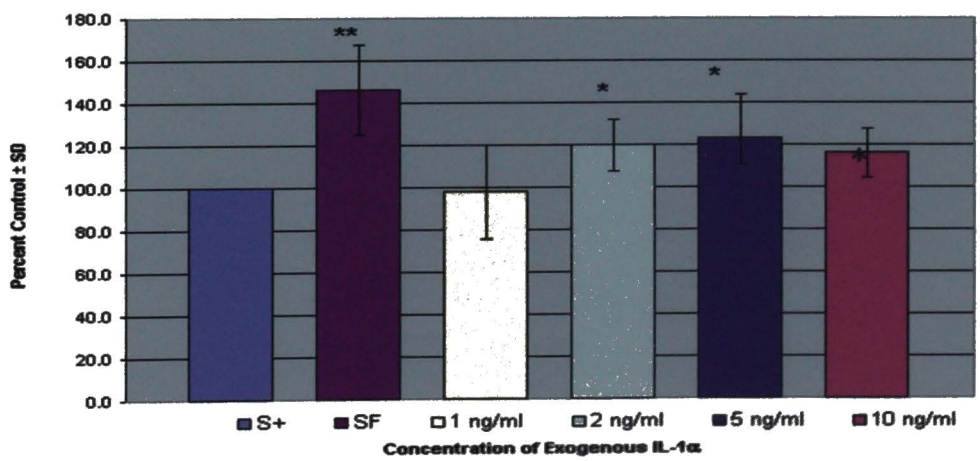


(E) IL-1α

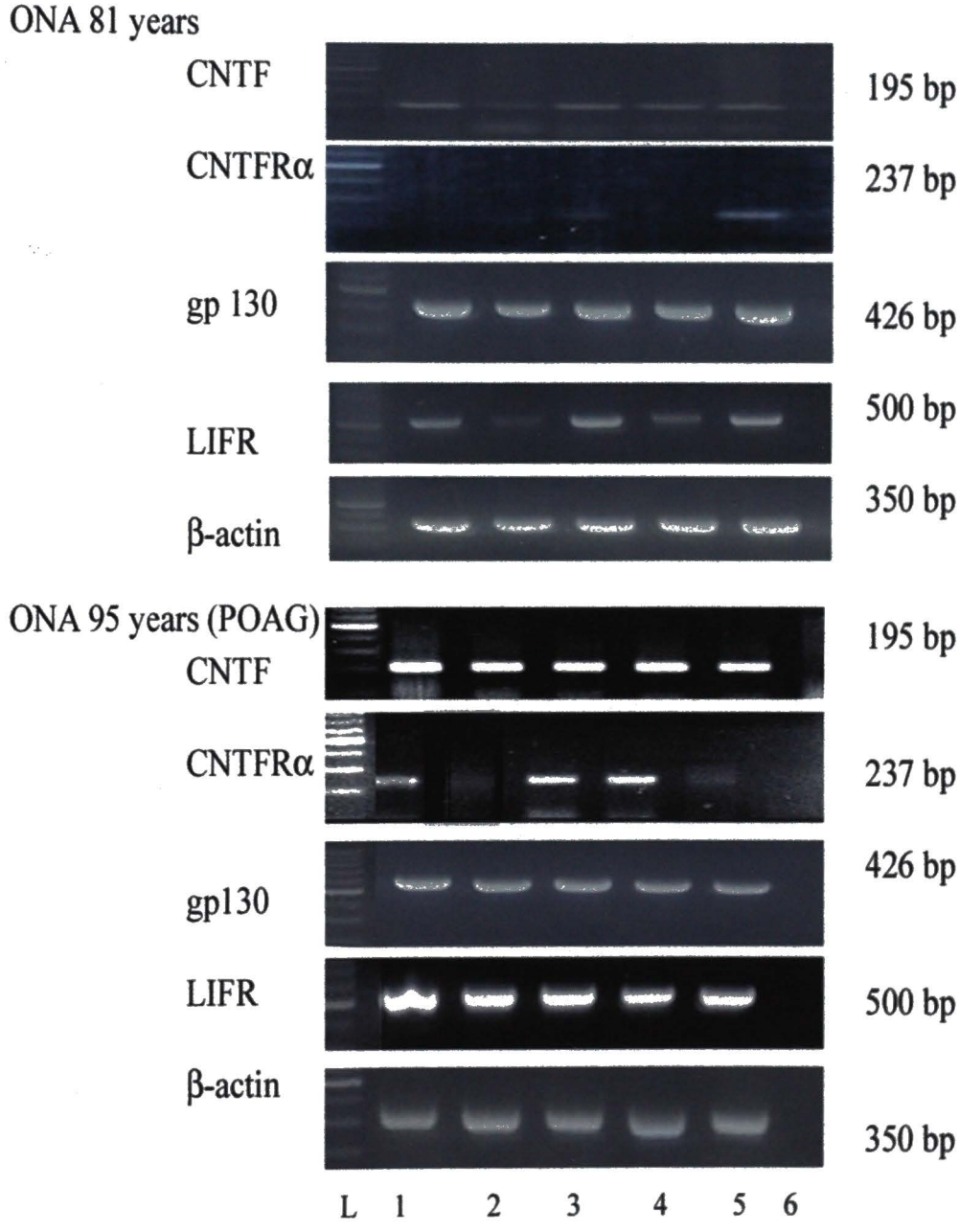
ONH astrocyte cell proliferation following exogenous treatment with CNTF, FGF-9, and IL-1αin one glaucomatous ONA cell line (ONA 53 years). Cell proliferation was observed in ONH astrocytes following CNTF, FGF-9, and IL-1α treatment when compared to the control (S+). Cells were treated for 48 hours. A standard curve for each cell line was generated. *$p<0.05$ or ** $p<0.005$ was determined by student t-test.


250.0
200.0
150.0
100.0
50.0
0.0
Percent Control ± SD
**
**
**
**
S+
SF
50 ng/ml
100 ng/ml
150 ng/ml
200 ng/ml
Concentration of Exogenous CNTF

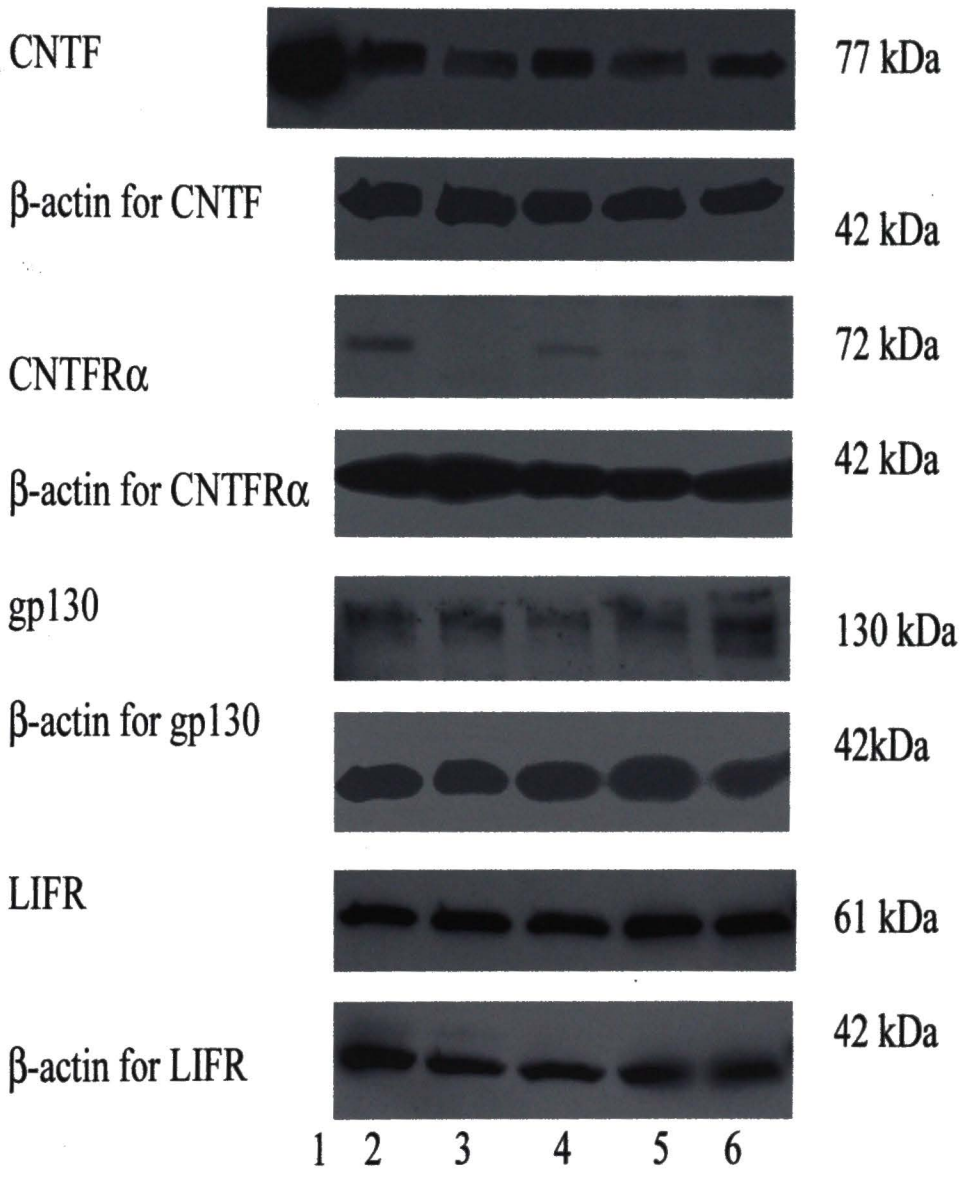

200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
**
**
**
**
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous FGF-8


180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Percent Control ± SD
**
*
*
S+
SF
1 ng/ml
2 ng/ml
5 ng/ml
10 ng/ml
Concentration of Exogenous IL-1α

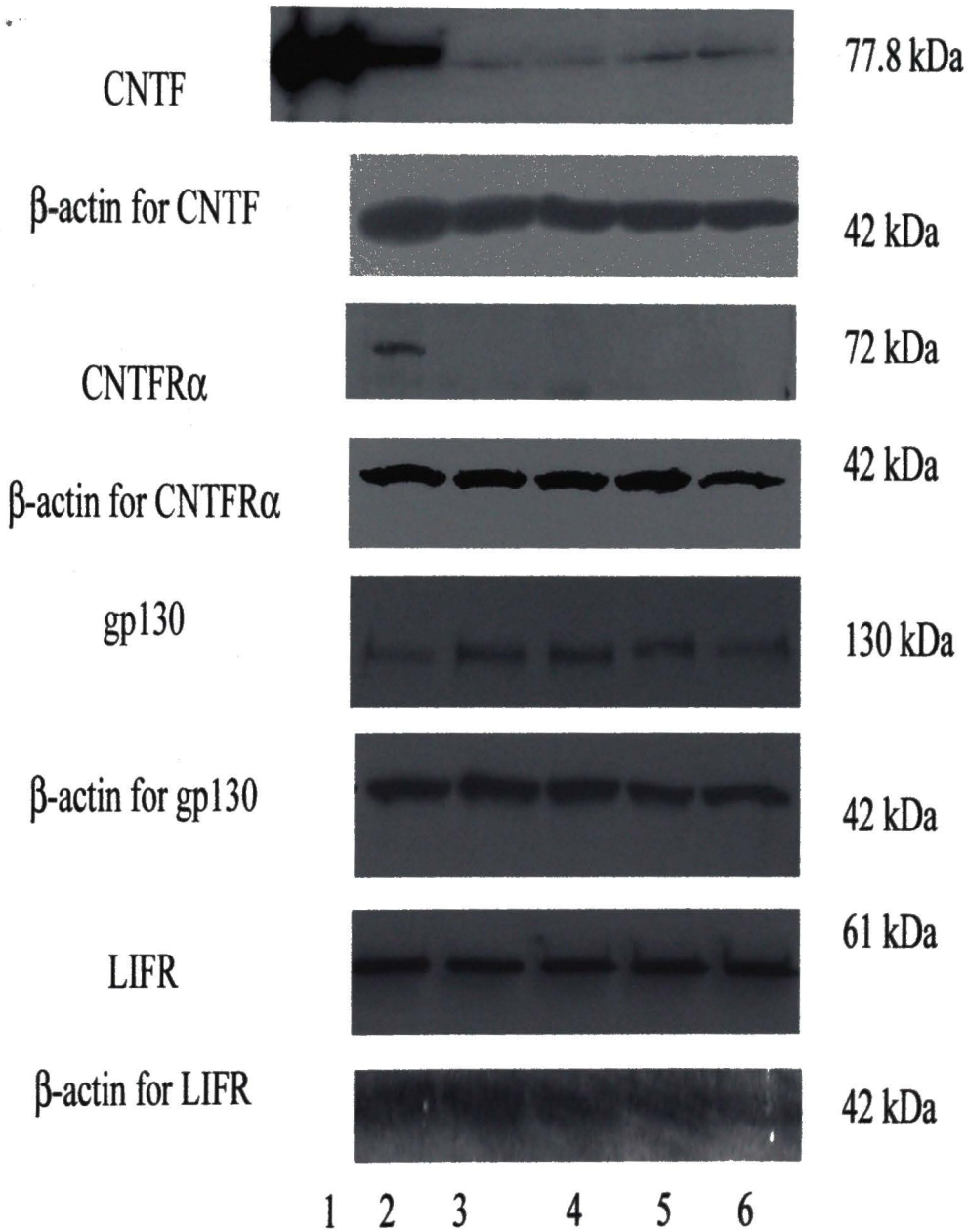
Ethidium bromide-stained gel of CNTF and its tripartite receptor complex PCR reaction products from ONH astrocytesfollowing exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.


ONA 81 years
CNTF
CNTFRα
gp 130
LIFR
β-actin
195 bp
237 bp
426 bp
500 bp
350 bp
ONA 95 years (POAG)
CNTF
CNTFRα
gp130
LIFR
β-actin
195 bp
237 bp
426 bp
500 bp
350 bp
L 1 2 3 4 5 6

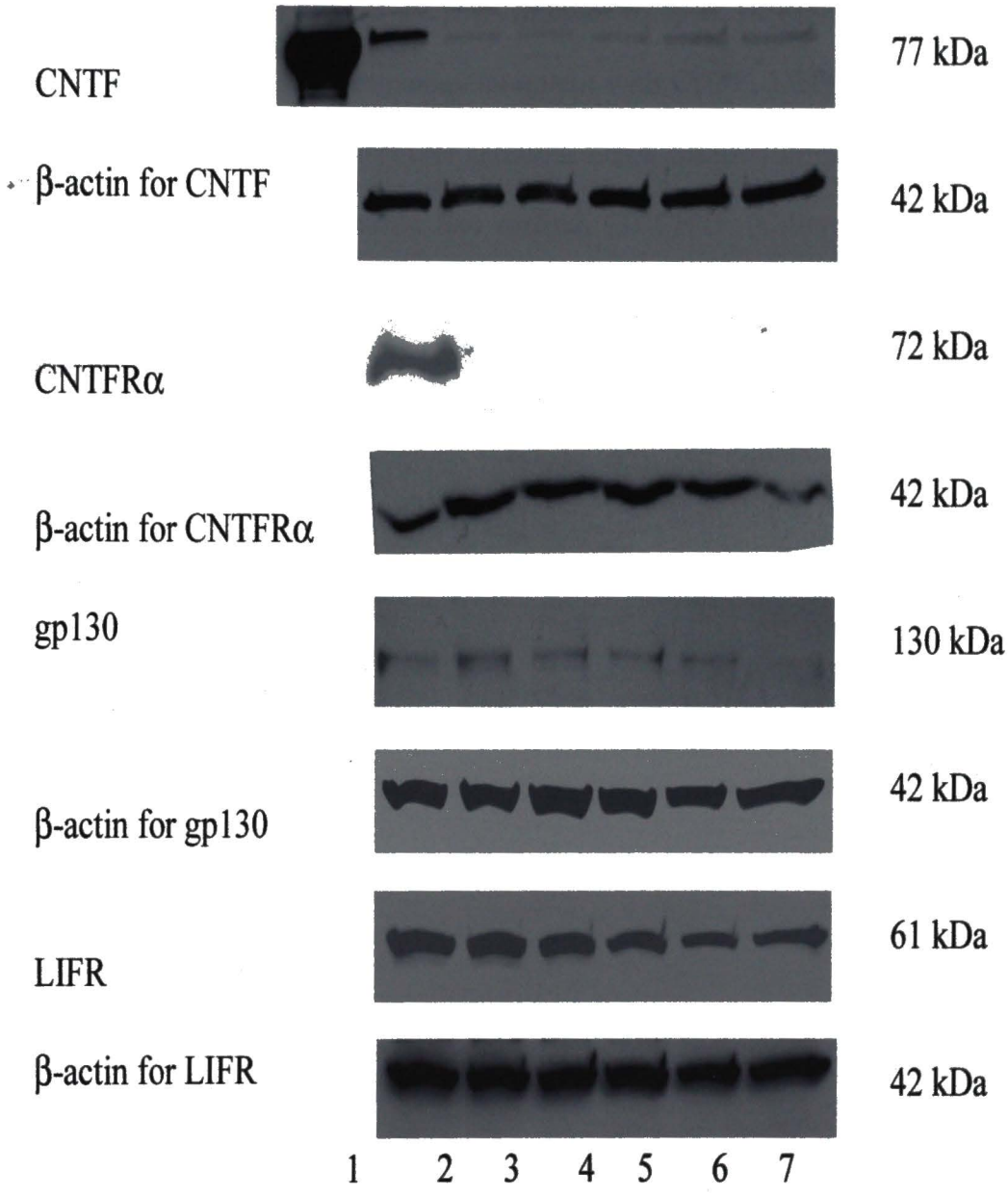
Chemiluminescent detection of CNTF and its tripartite receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal ONA 81 year old cell line. Lanes 1-6 represent the following: (1) r.h. CNTF (recombinant Ciliary Neurotrophic Factor), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


CNTF
77 kDa
β-actin for CNTF
42 kDa
CNTFRα
72 kDa
β-actin for CNTFRα
42 kDa
gp130
130 kDa
β-actin for gp130
42kDa
LIFR
61 kDa
β-actin for LIFR
42 kDa
1 2 3 4 5 6

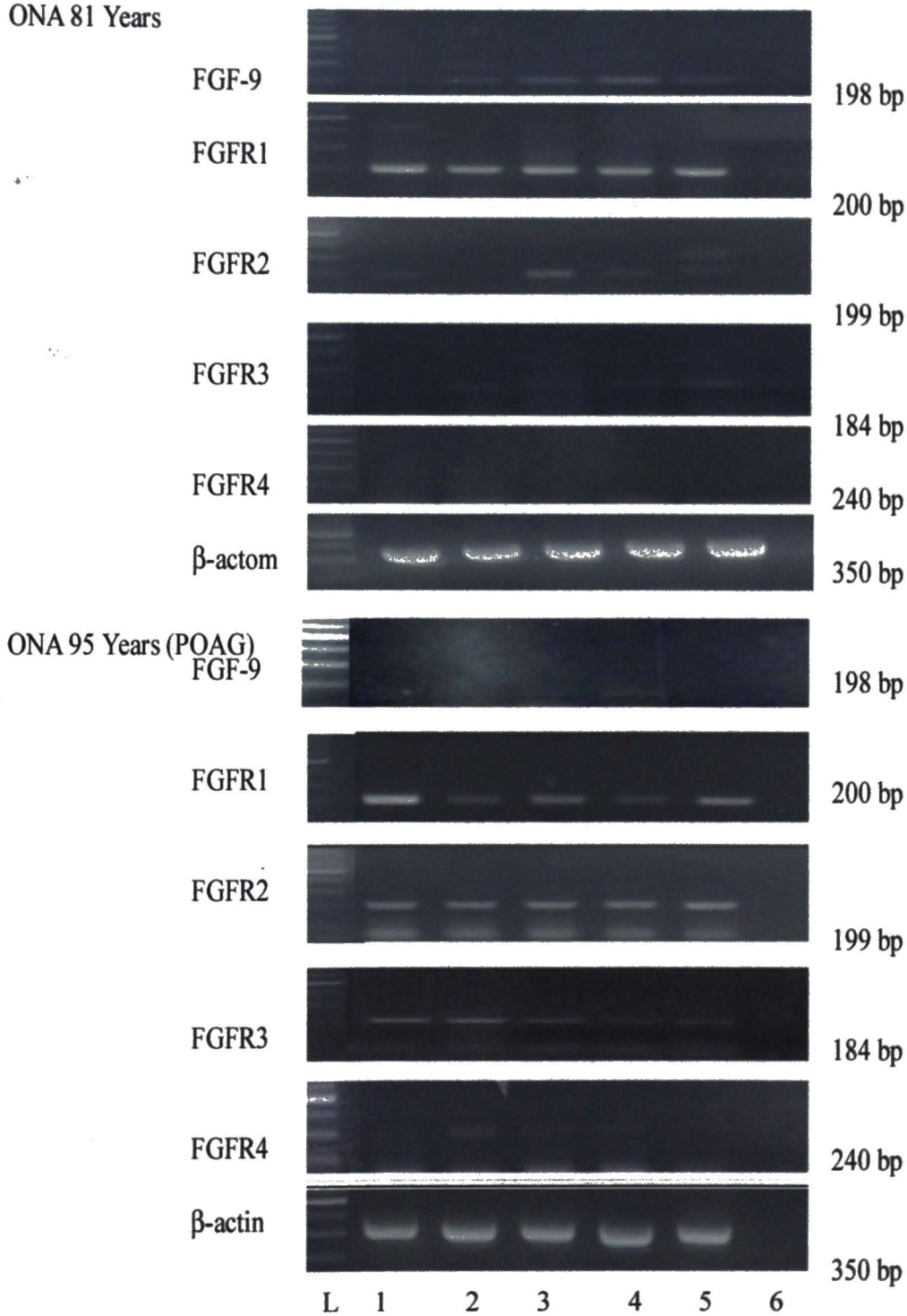
Chemiluminescent detection of CNTF and its tripartite receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA 69 year old cell line. Lanes 1-6 represent the following: (1) r.h. CNTF (recombinant Ciliary Neurotrophic Factor), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


CNTF
77.8 kDa
β-actin for CNTF
42 kDa
CNTFRα
72 kDa
β-actin for CNTFRα
42 kDa
gp130
130 kDa
β-actin for gp130
42 kDa
LIFR
61 kDa
β-actin for LIFR
42 kDa
1 2 3 4 5 6

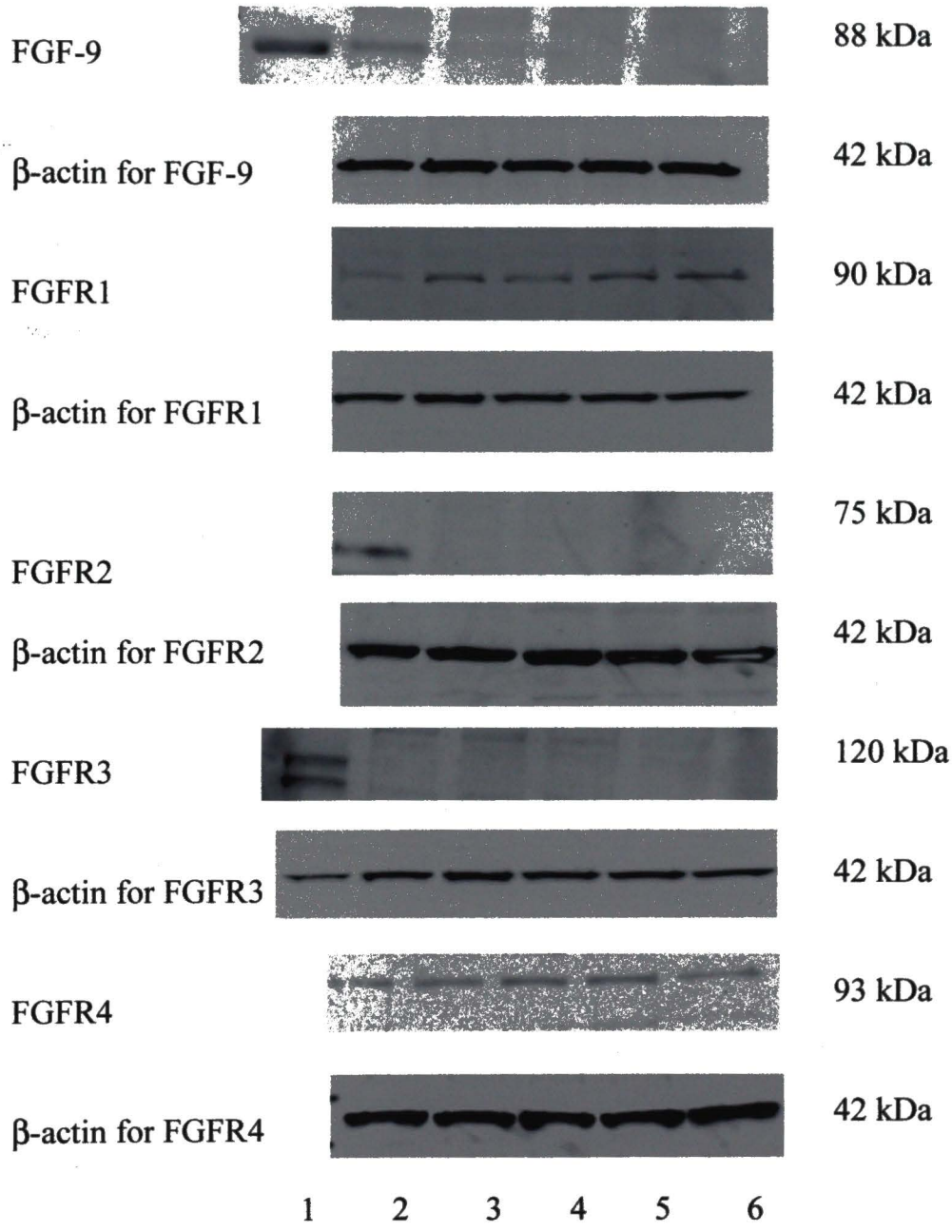
Chemiluminescent detection of CNTF and its tripartite receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA 95 year old cell line. Lanes 1-6 represent the following: (1) r.h. CNTF (recombinant Ciliary Neurotrophic Factor), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


CNTF
77 kDa
β-actin for CNTF
42 kDa
CNTFRα
72 kDa
β-actin for CNTFRα
42 kDa
gp130
130 kDa
β-actin for gp130
42 kDa
LIFR
61 kDa
β-actin for LIFR
42 kDa
1 2 3 4 5 6 7

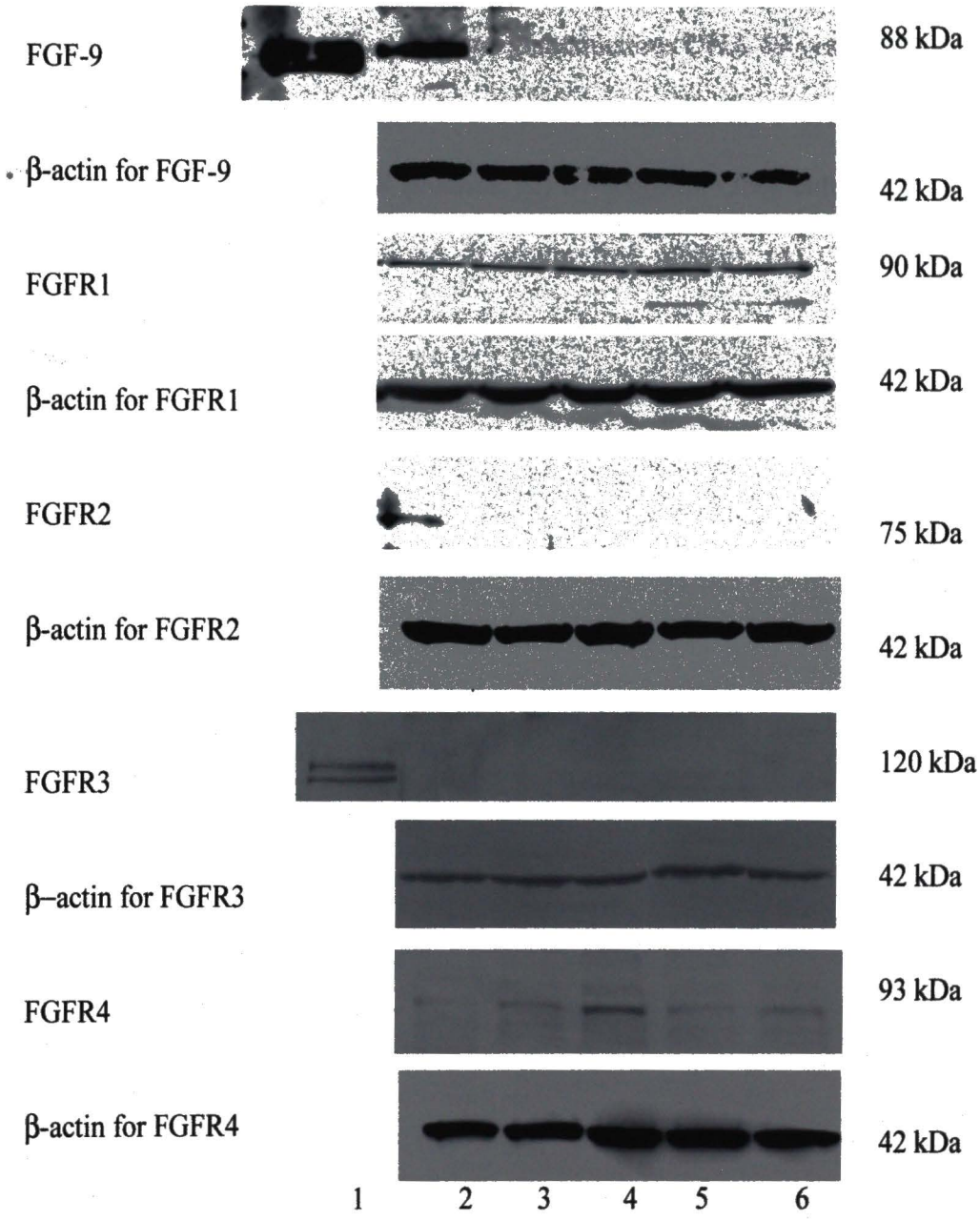
Ethidium bromide-stained gel of FGF-9 and its receptor complex PCR reaction products from ONH astrocytes following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.


ONA 81 Years
FGF-9
198 bp
FGFR1
200 bp
FGFR2
199 bp
FGFR3
184 bp
FGFR4
240 bp
β-actom
350 bp
ONA 95 Years (POAG)
FGF-9
198 bp
FGFR1
200 bp
FGFR2
199 bp
FGFR3
184 bp
FGFR4
240 bp
β-actin
350 bp
L 1 2 3 4 5 6

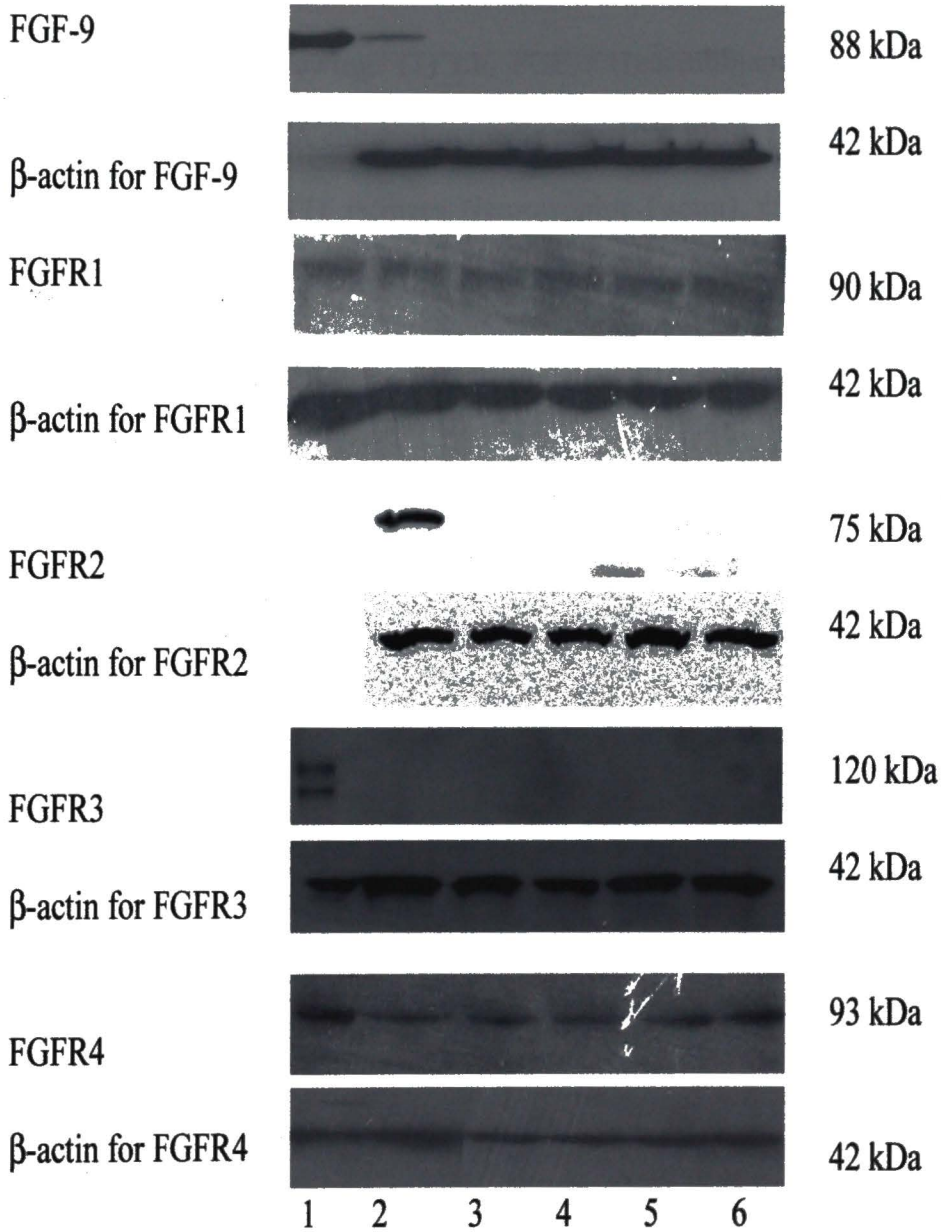
Chemiluminescent detection of FGF-9 and its receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal ONA 66 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


FGF-9
88 kDa
β-actin for FGF-9
42 kDa
FGFR1
90 kDa
β-actin for FGFR1
42 kDa
FGFR2
75 kDa
β-actin for FGFR2
42 kDa
FGFR3
120 kDa
β-actin for FGFR3
42 kDa
FGFR4
93 kDa
β-actin for FGFR4
42 kDa
1 2 3 4 5 6

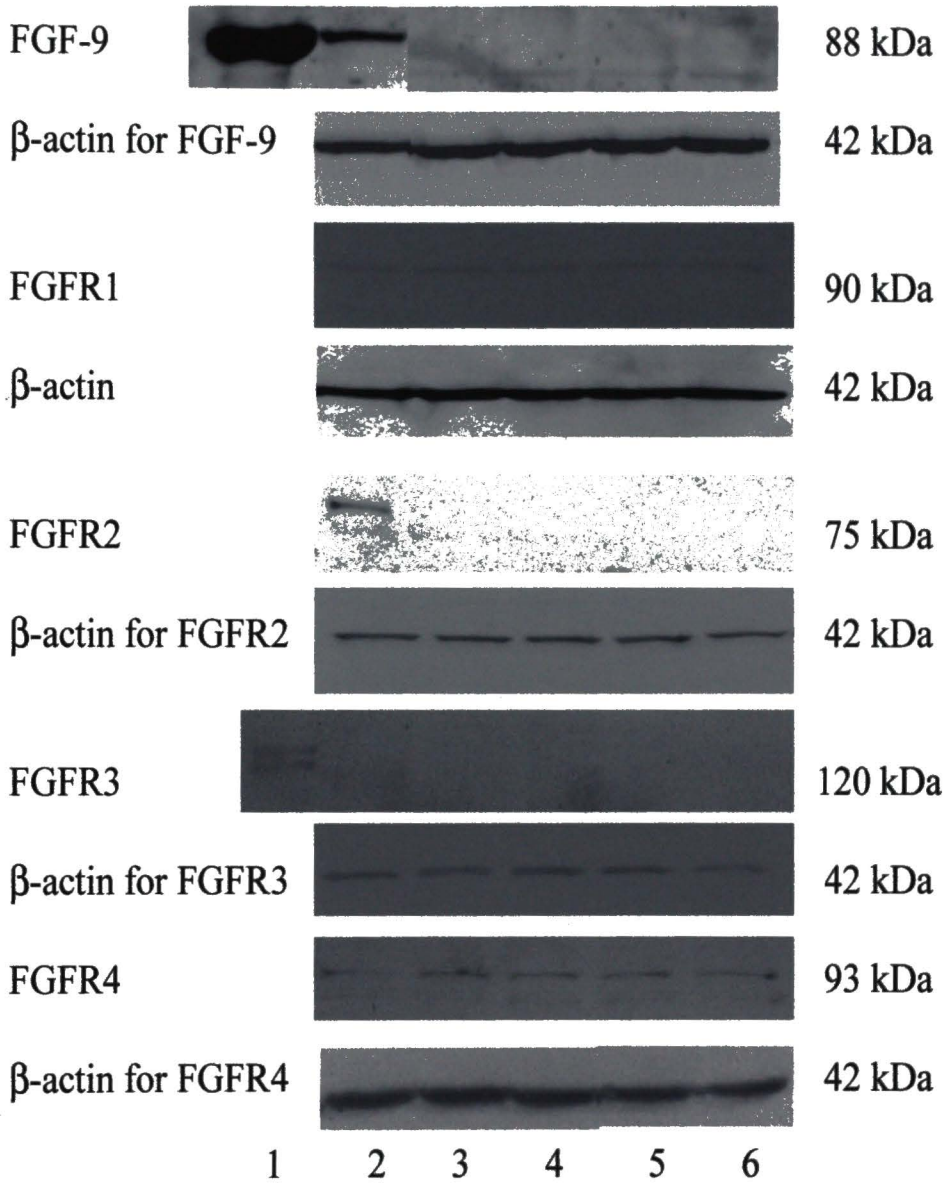
Chemiluminescent detection of FGF-9 and its receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal ONA 81 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


FGF-9
88 kDa
β-actin for FGF-9
42 kDa
FGFR1
90 kDa
β-actin for FGFR1
42 kDa
FGFR2
75 kDa
β-actin for FGFR2
42 kDa
FGFR3
120 kDa
β–actin for FGFR3
42 kDa
FGFR4
93 kDa
β-actin for FGFR4
42 kDa
1
2
3
4
5
6

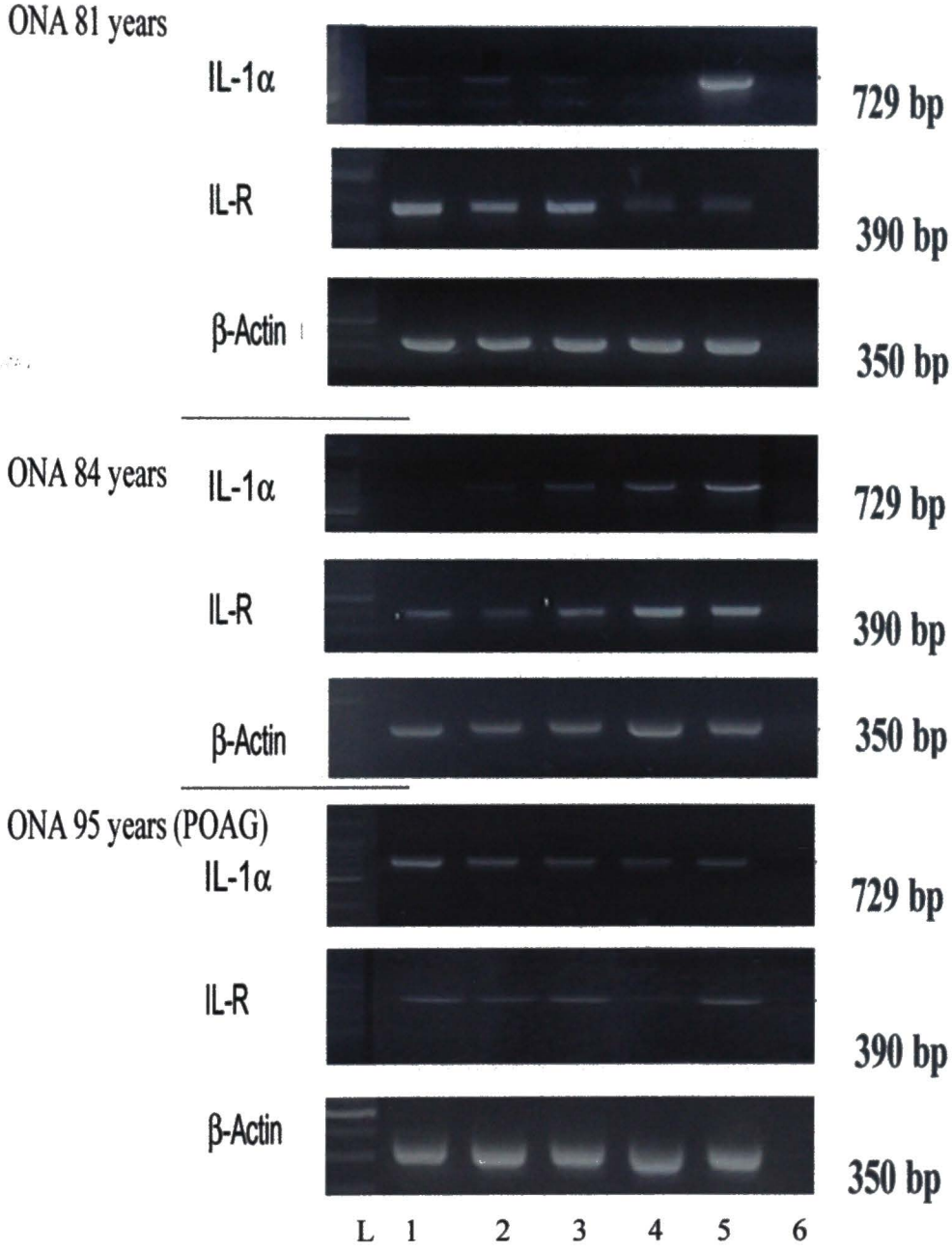
Chemiluminescent detection of FGF-9 and its receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA 69 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9) or positive control for FGFR proteins, (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


FGF-9
88 kDa
β-actin for FGF-9
42 kDa
FGFR1
90 kDa
β-actin for FGFR1
42 kDa
FGFR2
75 kDa
β-actin for FGFR2
42 kDa
FGFR3
120 kDa
β-actin for FGFR3
42 kDa
FGFR4
93 kDa
β-actin for FGFR4
42 kDa
1 2 3 4 5 6

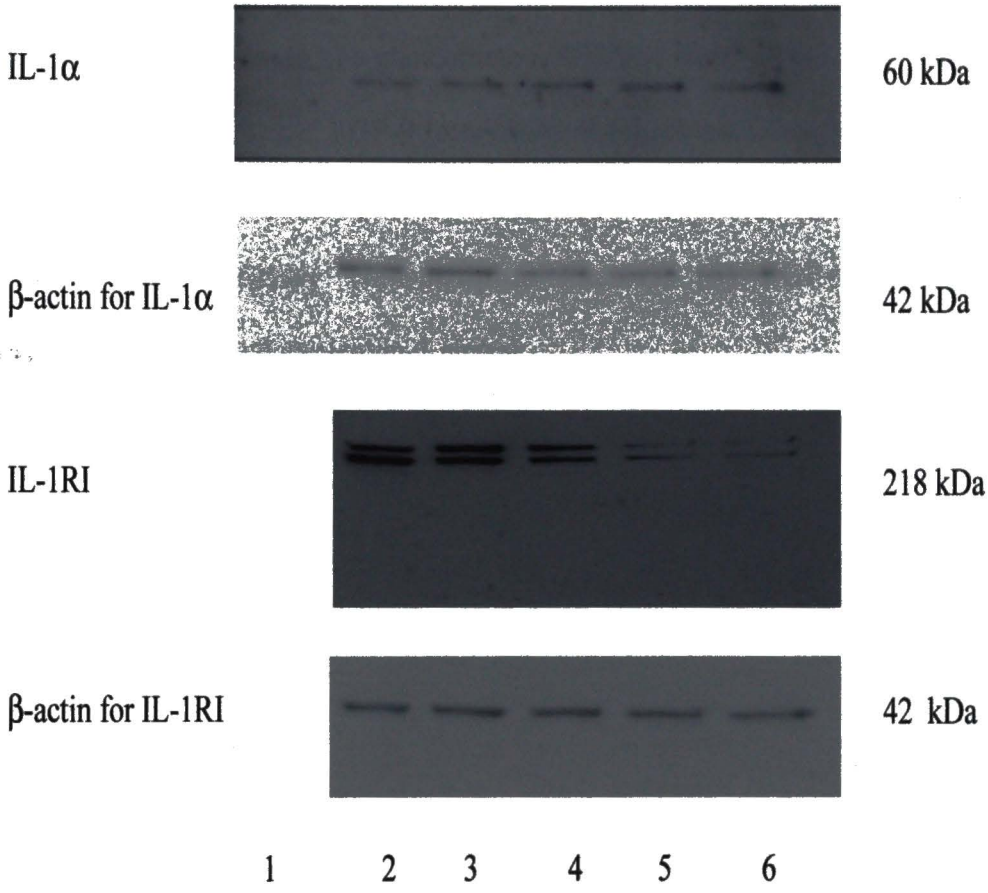
Chemiluminescent detection of FGF-9 and its receptor complex following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA 95 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


FGF-9
88 kDa
β-actin for FGF-9
42 kDa
FGFR1
90 kDa
β-actin
42 kDa
FGFR2
75 kDa
β-actin for FGFR2
42 kDa
FGFR3
120 kDa
β-actin for FGFR3
42 kDa
FGFR4
93 kDa
β-actin for FGFR4
42 kDa
1
2
3
4
5
6

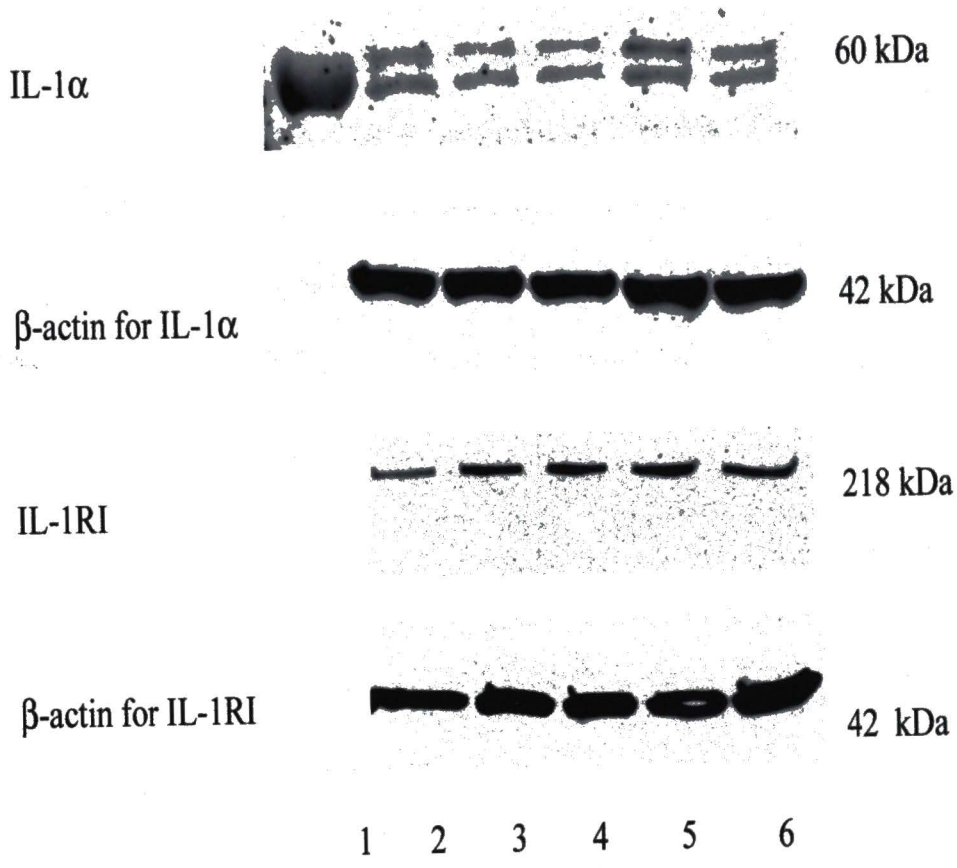
Ethidium bromide-stained gel of IL-1α and IL-1RI PCR reaction products from ONH astrocytes following exogenous treatment with CNTF, FGF-9, and IL-1α.. Lanes 1-6 represent the following: (1) S+ (control; ONA cultured in media plus 10% FBS), (2) SF (ONA cultured in serum free media), (3) CNTF (Ciliary Neurotrophic Factor), (4) FGF-9 (Fibroblast Growth Factor-9), (5) IL-1α (Interleukin-1 alpha), and (6) the negative control.


ONA 81 years
IL-1α
729 bp
IL-R
390 bp
β-Actin
350 bp
ONA 84 years
IL-1α
729 bp
IL-R
390 bp
β-Actin
350 bp
ONA 95 years (POAG)
IL-1α
729 bp
IL-R
390 bp
β-Actin
350 bp
L 1 2 3 4 5 6

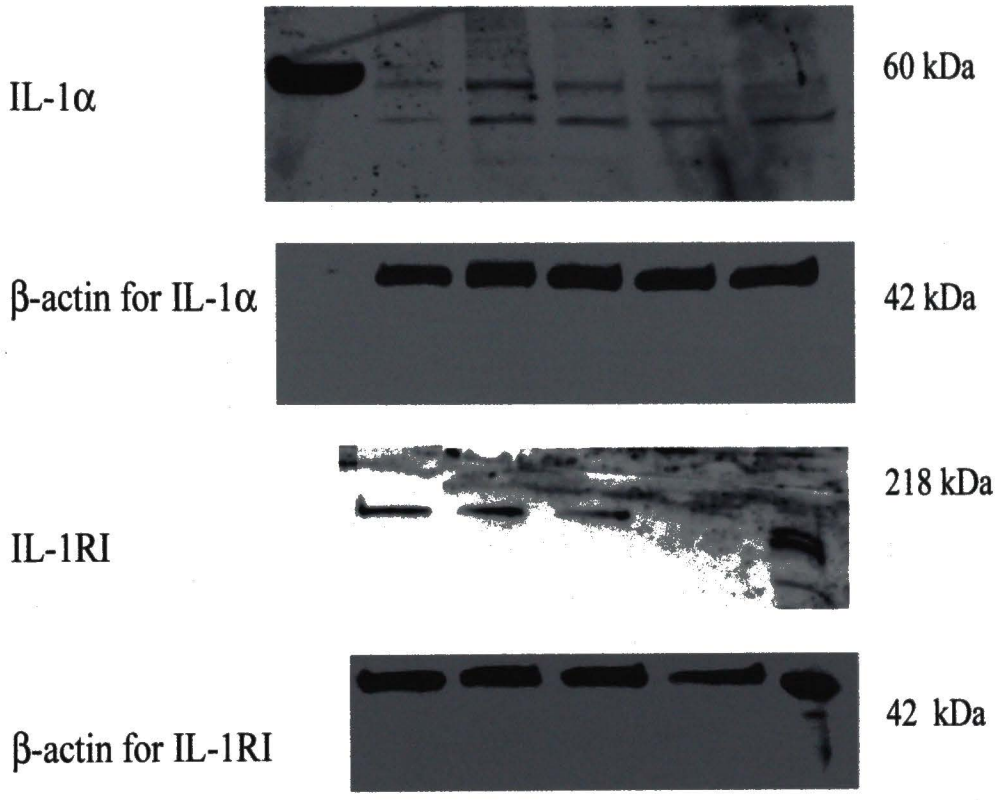
Chemiluminescent detection of IL-1α and its receptor following exogenous treatment with CNTF, FGF-9, and IL-1α in one normal ONA 66 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


IL-1α
60 kDa
β-actin for IL-1α
42 kDa
IL-1RI
218 kDa
β-actin for IL-1RI
42 kDa
1
2
3
4
5
6

Chemiluminescent detection of IL-1α and its receptor following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA 53 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


IL-1α
60 kDa
β-actin for IL-1α
42 kDa
IL-1RI
218 kDa
β-actin for IL-1RI
42 kDa
1 2 3 4 5 6

Chemiluminescent detection of IL-1α and its receptor following exogenous treatment with CNTF, FGF-9, and IL-1α in one glaucomatous ONA 95 year old cell line. Lanes 1-6 represent the following: (1) r.h. FGF-9 (recombinant Fibroblast Growth Factor-9), (2) S+ (control; ONA cultured in media plus 10% FBS), (3) SF (ONA cultured in serum free media), (4) CNTF (Ciliary Neurotrophic Factor), (5) FGF-9 (Fibroblast Growth Factor-9), (6) IL-1α (Interleukin-1 alpha).


IL-1α
60 kDa
β-actin for IL-1α
42 kDa
IL-1RI
218 kDa
β-actin for IL-1RI
42 kDa