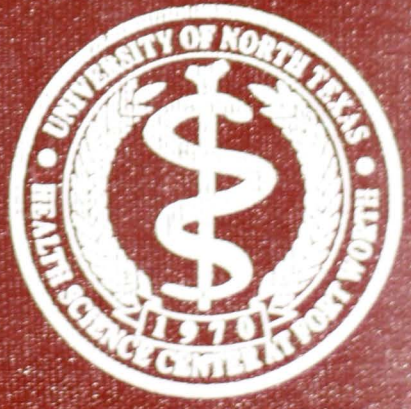
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This research provides important insights into the means by which interleukin-1alpha (IL-1α) regulates TM cell functions and enhances aqueous outflow, thus lowering IOP. The studies reported herein represent the first known characterization of the central role of the AP-1 transcription factor pathway in IL-1α-mediated production of proMMP-3 by TM cells, as well as the first known evidence that IL-1α can also enhance TM phagocytosis. Using these results as a stepping stone, this research has furthermore led to the identification of "AP-1 activators" as a novel compound class which may be useful in the treatment of glaucoma; it also points to the potential for compounds which regulate MEK, p38, and PKCμ activity as additional means of treatment . Based on these results, it is postulated that such compounds would be expected to lower IOP via upregulation of MMP production, followed by ECM degradation, and, potentially, enhanced clearance of degraded ECM via phagocytosis.

INTERLEUKIN-1ALPHA-MEDIATED SIGNALING MECHANISMS

IN THE HUMAN TRABECULAR MESHWORK

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INTERLEUKIN-1ALPHA-MEDIATED SIGNALING MECHANISMS IN THE HUMAN TRABECULAR MESHWORK

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Debra L. Shade, B.S.

Fort Worth, Texas

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CHAPTER 1

INTRODUCTION

Significance

A variety of therapeutic agents have been proposed as having the ability to reduce the elevated intraocular pressures (IOP) often associated with glaucoma. However, the mechanism(s) of action by which many of these agents regulate IOP is not always fully understood. Furthermore, the use of many current "anti-glaucoma" drugs is often associated with unwanted side effects. This project provides important insights into the mechanisms by which interleukin-1α (IL-1α), an endogenous cytokine which can reduce IOP, increases aqueous outflow in the human. Delineation of such mechanisms may allow for identification of novel agents possessing the desirable (i.e. IOP-lowering) effects of IL-1α, but with an increased safety profile in terms of absent or reduced side effects.

Overview of glaucoma

"Glaucoma" is an umbrella term encompassing a heterogeneous group of ocular disorders that are ultimately characterized by a loss of the retinal ganglion cells (RGC) whose axons comprise the optic nerve (Guyton, 1991). It has been estimated that the prevalence of glaucoma will approach as many as 66.8 million people in the year 2000 (Quigley, 1996), hence the ongoing need for novel,

efficacious therapeutic agents in order to better manage the disease, particularly since currently-available IOP-lowering drugs are not effective in all patients.

The majority of adult-onset glaucomas are often classified into two general categories: "angle closure" and "open angle". Both types refer to the apparent patency status of the drainage angle formed in the anterior chamber between the iris and the cornea. "Angle closure" patients thus have a narrowed or otherwise restricted angle region, such as may occur during acute episodes of mydriasis; the resulting contraction of the iris tissue leads to obstruction of the chamber angle. The drainage angles of "open angle" patients, on the other hand, are seemingly "open" and normal in appearance when examined clinically. However, under ultra-microscopic examination, the angles of these patients often demonstrate increased deposition of plaque-like materials within the cribiform region of the trabecular meshwork (TM) as well as in the inner wall of the drainage channel termed Schlemm's canal (Rohen and Witmer, 1972; Lee and Grierson, 1974). The TM is an ocular tissue which occupies the angle's outflow region and is the major site of aqueous humor (AH) drainage (Bill, 1975). The trabecular meshwork region and the drainage angle of the human ocular anterior segment are depicted in the accompanying generalized diagram (see Figure 1).

Figure 1

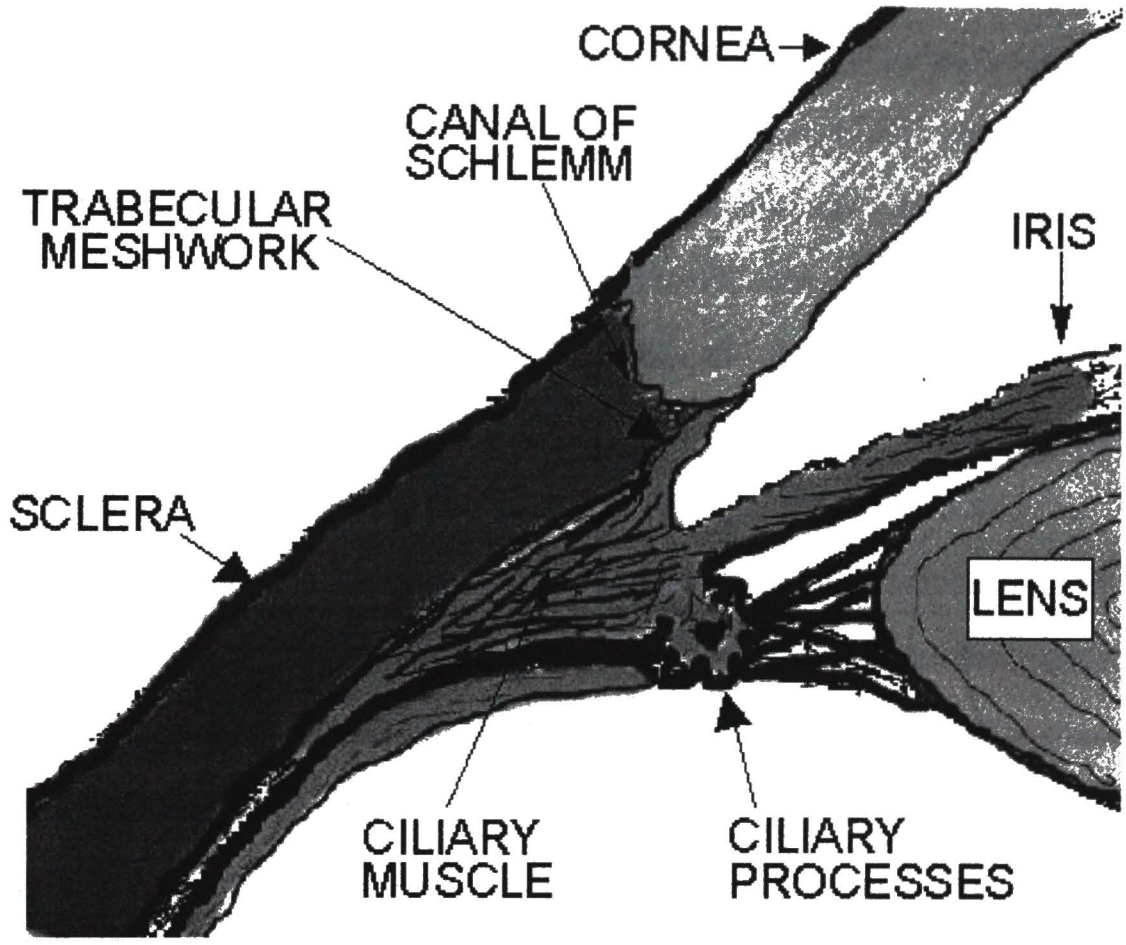


Figure 1: Tissues of the human aqueous outflow pathway. Aqueous humor is produced by the ciliary processes, flows between the lens and iris, then enters the anterior chamber via the pupillary opening (not shown). The majority of aqueous humor then exits via the trabecular meshwork/Canal of Schlemm region.

Although no clear-cut mechanisms have been definitively recognized as being the underlying cause of glaucoma, several factors have been identified which are associated with an increased risk of its development. Of these, the most prevalent risk factors include increasing age, ethnicity (African-Americans are at higher risk), and high IOP (> 21 mm Hg) (Coleman, 1999). Family history also plays an important role and recent discoveries implicate a strong genetic component in many types of glaucoma (Craig and Mackey, 1999).

Among the risk factors, elevated IOP's are the most often cited as being predictive of glaucoma, even though it is possible for pressures to be "high" in an individual without evident glaucomatous-type damage, a condition known as ocular hypertension (Stromberg, 1962). Furthermore, glaucomatous damage can occur within the so-called "normal" IOP range, i.e. "low-tension" or "normal-tension" glaucoma (Shields, 1992a). Despite these contradictions, an increase in IOP remains one of the most commonly-accepted risk factors for the progression of the disease, and, although not proven conclusively, the mechanical forces associated with IOP increases may contribute to changes in the structure of the optic nerve head (Hernandez, 2000).

Role of the trabecular meshwork

As stated previously, an increase in IOP accompanies many forms of glaucoma: IOP increases associated with glaucoma are believed due to an impairment of the outflow systems(s) by which AH, the fluid that bathes and provides nutrients for the ocular anterior segment tissues, exits the eye. Despite

a pronounced diurnal variation in output (production is decreased at night), AH is continually produced by ocular ciliary epithelial tissues (Brubaker, 1991), thus any reduction in its outflow results in aqueous volume build-up and increased fluid pressure within the anterior chamber. Outflow of AH can occur via two routes – the "conventional" pathway, and the "unconventional", or uveoscleral, route (Hart, 1992): although the bulk of outflow in humans occurs via the conventional pathway, enhancement of either route has been shown to successfully reduce elevated IOP (Forrester et al, 1996).

The conventional pathway is intimately associated with the TM, an anterior chamber tissue that, along with the Canal of Schlemm, forms the primary site of AH outflow. Among the other known functions of the cells comprising the TM are the remodeling and phagocytosis of debris and surrounding extracellular matrix (ECM) materials (Yue, 1996). Such functions are highly important because increased deposition or over-accumulation of ECM (and other) materials may exacerbate aqueous outflow resistance and therefore elevate IOP (Rohen, 1983).

Detection and treatment of glaucoma

If undetected or left untreated, glaucoma results in a gradual decline of the visual field (peripheral vision is diminished first, followed by central vision), and, ultimately, can lead to blindness. Thus early detection and intervention are vital to preserving the patient's remaining visual function. Unfortunately, the symptoms of most forms of glaucoma are rarely detected by the patients themselves during the early stages and generally are discovered only during the course of an ocular examination. First-line identification of glaucoma suspects is often based on IOP measurements, cup-to-disc ratio by fundus examination, and perimetry. However, a more definitive diagnosis requires the use of specialized equipment such as scanning laser ophthalmoscopes, optical coherence tomographs, or related devices, in order to examine the optic nerve head for signs of tissue damage. Assessment of visual function is also accomplished via objective techniques such as electroretinography (ERG), in which retinal dysfunction manifests as a reduction in wave amplitude in response to stimuli such as checkerboard patterns (pattern ERG), light flashes (flash ERG), or flickering lights (flicker ERG) (Shields, 1992b). Subjective evaluation of function may include visual field testing, in which the patient's visual response to pinpoint flashes of lights is recorded; flashes of varying intensities are presented in areas corresponding to both central and peripheral visual regions (Coleman, 1999).

Presently, no "cure" exists for glaucoma, and currently-available strategies can only delay the progression of the disease. Although neuroprotection of the

RGC is the desired, ultimate goal (Schumer and Podos, 1994; Osborne et al, 1999a), existing "anti-glaucoma" drugs and therapies largely function indirectly via their actions on anterior segment tissues in order to lower IOP and can involve both pharmaceutical agents and/or surgical intervention. Glaucoma therapeutic agents generally lower IOP by one of two means: either by reducing the production of aqueous humor by the ciliary epithelium, or by increasing the rate of aqueous outflow (Brubaker, 1991) through either the conventional or unconventional routes. These agents encompass a wide variety of drug classes, including beta adrenergic receptor antagonists, carbonic anhydrase inhibitors, alpha adrenergic receptor agonists, cholinomimetic agents (both direct and indirect), and, most recently, prostaglandins.

Although useful, "anti-glaucoma" drugs often produce side effects that can limit their utility and tolerability from patient to patient. For example, the use of eyedrops containing the direct-acting cholinomimetic pilocarpine may produce local effects such as myopia, loss of accommodation (blurred vision), lacrimation, and brow ache (Kaufman et al, 1984). Pilocarpine may also produce potential systemic side effects such as bradycardia (Olson, 1994) and – due to its ability to cross the blood-brain barrier -- CNS disturbances (Mycek et al, 1992). Side effects of other anti-glaucoma medications can include mydriasis (alpha adrenergic receptor agonists), bronchospasms (beta adrenergic receptor antagonists), potassium depletion and dermatitis (orally-administered carbonic anhydrase inhibitors) (Forrester et al, 1996).

Surgical management of glaucoma can be divided into two broad categories: incisional and laser. Similar to pharmacological treatments, the techniques may be used either to reduce aqueous production or increase aqueous outflow (Shields, 1992c). For example, argon laser trabeculoplasty (ALT) is a technique commonly used to increase aqueous outflow in the management of primary open angle glaucoma. The process entails the careful placement of laser burns on the TM tissue. "Wounding" of the TM in this manner was originally believed to result in an overall retraction or shrinkage of the TM's collagen framework, leading to a generalized opening of the intra-trabecular spaces (Wise and Witter, 1979; Wise, 1981). Although attractive in its simplicity, it is more likely that, in addition to mechanical alterations, ALT also triggers multiple changes at both the cellular and subcellular levels (Van Buskirk et al, 1984; Van Buskirk, 1989).

Other researchers have since demonstrated that the TM, instead, appears to undergo a multitude of functional changes subsequent to such lasering. The rate of TM cell division in both human and non-human species (cat, monkey) was observed to be enhanced by this treatment, for example (Blysma et al, 1988; Acott et al, 1989; Blysma et al, 1989; Dueker et al, 1990; Acott et al, 1992; Kimpel et al, 1992; Bradley et al, 1996). Furthermore, TM cells in the juxtacanalicular regions have been shown to exhibit increased expression of the metalloproteinase stromelysin following laser trabeculoplasty (Parshley et al, 1996).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a diverse family of enzymes that play important roles in the remodeling of both normal and diseased tissues. At least 20 different MMPs have been identified to date, of which 17 are known to be expressed in humans (Woessner, 1998). The MMPs are often divided into broad categories based on their substrate specificities, such as the collagenases (which degrade mostly collagen) and the gelatinases (which degrade mostly gelatin). Other types of MMPs, such as the stromelysins, have the ability to degrade such extracellular matrix components as proteoglycans, fibronectin, and laminin, in addition to gelatin and collagen (Matrisian, 1992). Table 1 gives an overview of many of the most commonly-known MMPs, as well as the their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

With the exception of a few subtypes, MMPs are secreted as pro-enzymes which must be activated (cleaved) before possessing the ability to degrade substrate materials and before they can be bound by TIMPs (Welgus, 1991). Activation of MMPs is generally via the action of other active proteinases, such as kallikrein, plasmin, cathepsin-G, elastase, and trypsin, among others, as well as in the test tube by organomercurials (Okada et al, 1989; Nagase et al, 1990). Once active, MMP-mediated ECM degradation is thought to play key roles in a wide variety of both normal cellular events such as proliferation and migration during development and of pathophysiological processes such as cancer and rheumatoid arthritis (Matrisian, 1992).

Table 1: Key MMPs and TIMPs

Designation	Common name(s)	Common Substrate(s)*
MMP-1	Collagenase-1; Interstitial Collagenase	Fibrillar collagen (I, II, III, VII, VIII, X), gelatin, proteoglycans, MMP-2, MMP-9
MMP-2	Gelatinase A; Type IV Collagenase	Denatured collagen, collagen (IV, V, VII, X), aggrecan, fibronectin, laminin, elastin, MMP-1, MMP-9
MMP-3	Stromelysin-1; Transin	Proteoglycans, collagen (III, V, IX, X, XI; procollagen), elastin, aggrecan, gelatin, fibronectin, laminin, MMP-1, MMP-2/TIMP-2 complex, MMP-7, MMP-8, MMP-9, MMP-13
MMP-7	Matrilysin; PUMP	Collagen IV, gelatin, aggrecan, fibronectin, laminin, elastin, MMP-1, MMP-2, MMP-9, MMP-9/TIMP-1 complex
MMP-8	Collagenase-2; Neutrophil collagenase	Collagen (I, II, III, V, VII, VIII, X), gelatin, aggregan, fibronectin
MMP-9	Gelatinase B	Collagen (IV, V, VII, X, XIV), gelatin, elastin, aggrecan, fibronectin
MMP-10	Stromelysin-2	Proteoglycans, gelatin, fibronectin, laminin, collagen (III, IV, V, IX), MMP-1, MMP-8
MMP-11	Stromelysin-3	Laminin, fibronectin, gelatin, collagen

(contd. next page)

Table 1 (contd.): Key MMPs and TIMPs

MMP-12	Metalloelastase; Macrophage elastase	Elastin, fibronectin, laminin
MMP-13	Collagenase-3	Collagen (I, II, III, IV, IX, X, XIV), gelatin, aggrecan, MMP-9
MMP-14	MT-MMP-1	Collagen (I, II, III), gelatin, elastin, fibronectin, laminin, proteoglycans, MMP-2, MMP-13
MMP-15	MT-MMP-2	Fibronectin, laminin, MMP-2
MMP-16	MT-MMP-3	Collagen III, gelatin, fibronectin, MMP-2
MMP-17	MT-MMP-4	Unknown
MMP-18	Metalloelastase	Unknown (elastin?)
MMP-19	Metalloelastase	Gelatin, (elastin?)
MMP-20	Enamelysin	Amelogenin
TIMP-1	Human collagenase inhibitor; Fibroblast elongation factor	Inhibits "all" active MMPs
TIMP-2		Inhibits MMP-1, MMP-2
TIMP-3		
TIMP-4		

*NOTE: List includes only most commonly known substrates and is not proposed to be comprehensive.

Matrix metalloproteinases and IOP regulation

Remodeling of the TM's ECM material is believed to occur via the action of secreted degradative enzymes such as the MMKs. Supporting this view are the findings that a variety of MMP subtypes are expressed by TM tissues: MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), and MMP-9 (gelatinase B) have been shown to be produced by bovine, porcine, and human TM (Alexander et al, 1991 and 1998; Parshley et al, 1996; Samples et al, 1993; Magnino et al, 1999). MMPs have also been detected in measurable amounts in AH collected from human patients undergoing cataract surgery (Ando et al, 1993). Since excessive accumulation of ECM may cause ocular hypertension, agents that can enhance MMP-mediated turnover and maintenance of the TM's ECM may therefore offer a novel means to decrease the elevated IOP manifested by many glaucoma patients.

TM ECM is commonly composed of collagens (Types I, III, IV, V, VI, VIII), proteoglycans, fibronectin, laminin, and elastin. Vitronectin may also be present (Yue, 1996). Ex vivo laser trabeculoplasty of anterior segments in organ culture has been shown to stimulate the expression of stromelysin by TM cells in the juxtacanalicular region (Parshley et al, 1996). Stromelysins are therefore of particular interest in the eye because their broad substrate preferences encompass most of the ECM found in the aqueous outflow pathway. In particular, stromelysin-1 (MMP-3) is known to degrade a very wide variety of ECM materials, including laminin, fibronectin, collagen IV, collagen IX,

proteoglycans, and type I procollagen, among others (Welgus, 1991; see also Table 1). In addition, activated MMP-3 itself can, in turn, activate various other members of the MMP family, including MMP-1 (interstitial collagenase) which can become "super-activated" in the presence of active MMP-3 (Murphy, 1987).

Activation of both MMP-3 and MMP-1 in the uveoscleral tissues has been proposed as being one means by which the outflow-enhancing effect of prostaglandins is achieved (Lindsey et al, 1996; Weinreb et al, 1997). Further evidence that MMPs may play a role in maintaining aqueous outflow enhancement was shown by Bradley et al (1998) who reported that purified MMPs increased outflow rates in perfused human organ cultured eyes within 1-3 days of perfusion onset. Outflow-enhancing effects of the MMPs could be effectively blocked by synthetic MMP inhibitors, as well as by TIMPs.

Phagocytosis by trabecular meshwork cells

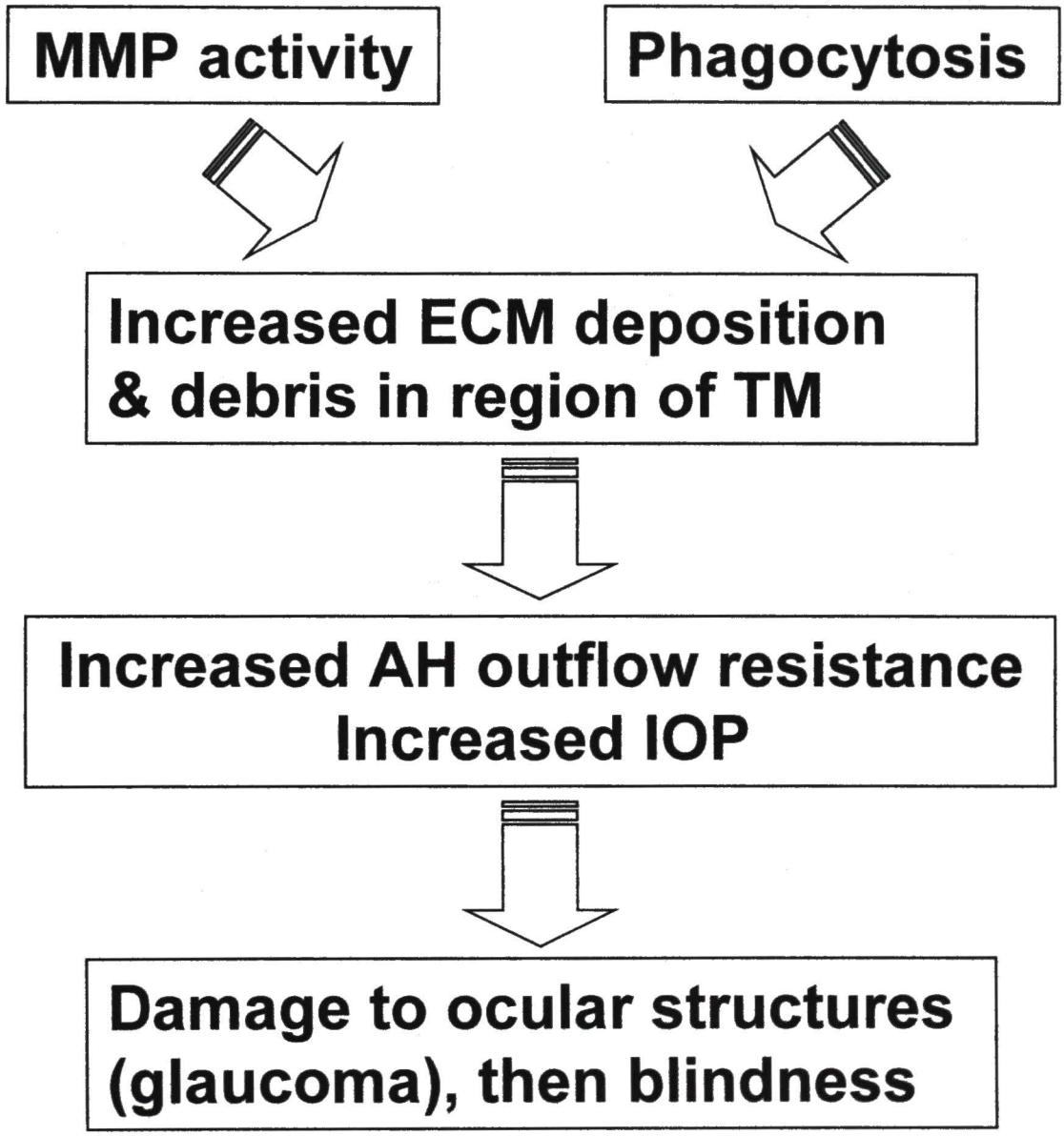
ECM components which have been degraded by MMPs or other means must subsequently be removed, either via washout due to normal aqueous outflow or by phagocytosis, a well-defined process by which many cell types, particularly those of the immune system, "ingest" foreign substances (Greenberg and Silverstein, 1993). Trabecular meshwork cells are actively phagocytic: the endothelial cells of the human TM have long been known to possess the ability to phagocytose degraded ECM materials, in addition to erythrocytes and cellular debris such as sloughed pigment granules (Grierson and Lee, 1973; Tripathi and Tripathi, 1982; Polansky et al, 1984). A loss, or decrease in, TM phagocytic

activity could therefore also account for at least a portion of the accumulated material noted in the juxtacanalicular regions of some glaucoma patients.

In fact, a decrease in TM phagocytosis following glucocorticoid administration has been speculated as contributing to steroid-induced glaucoma (Shirato et al, 1988), based on the known ability of steroids to suppress phagocytic activity in TM (Matsumoto and Johnson, 1997a). Conversely, platelet-derived growth factor (PDGF), which activates a receptor tyrosine kinase, has been shown to stimulate human TM phagocytosis (Tamura and Iwamoto, 1989). Thus, there is evidence to indicate that endogenous substances also regulate the phagocytic activity of human TM, although, to date, little is actually known of the intracellular signaling mechanisms involved.

It is unknown at this time whether upregulation of MMP levels is the only action of IL-1 on the TM. In particular, it is not known whether IL-1 would affect TM cell phagocytic action. Phagocytosis represents yet another TM cell function critical to ECM clearance and, hence, regulation of outflow facility. Elucidation of IL-1's effect(s) on TM phagocytosis may therefore shed further light on the IOP-lowering abilities of this agent. A hypothesized relationship between MMPs, phagocytosis, ECM and IOP is shown in Figure 2.

Figure 2


MMP activity
Phagocytosis
Increased ECM deposition & debris in region of TM
Increased AH outflow resistance Increased IOP
Damage to ocular structures (glaucoma), then blindness

Overview of Interleukin-1

Interleukins belong to a heterogeneous group of molecules known as cytokines; other examples of cytokines include the interferons and tumor necrosis factor α (TNFα). Interleukin-1α has long been known to be involved in the activation of various inflammatory processes: its release by antigen-presenting cells leads to secretion of the growth factor interleukin-2 by helper T cells, stimulating their proliferation (Alberts et al, 1989a). IL-1 also is reported in the literature under a variety of other names, such as lymphocyte-activating factor, endogenous pyrogen, monocytic cell factor, and leukocyte endogenous mediator (Dawson, 1991; Dunn, 1991). Despite its initially-identified association with inflammatory cells, it is now known that IL-1 can actually be produced by a wide variety of other cell types, including smooth muscle cells, endothelial cells, skin keratinocytes, astrocytes, microglia, corneal epithelium, kidney mesangial cells, and fibroblasts, among others (Dawson, 1991; Opdenakker et al, 1998).

Three members of the IL-1 family have been identified to date: IL-1α, IL-1β, and IL-1RA. Of these, IL-1RA has been determined to be an endogenous antagonist for IL-1 receptors; conversely, both human IL-1α and IL-1β act as receptor agonists. The mature forms of IL-1α, IL-1β are produced by proteolysis of their respective precursor molecules, proIL-1α and proIL-1β; such proteolytic action reduces the 31 kDa precursors to structures of approximately 17 kDa (O'Neill, 1995; Bankers-Fulbright et al, 1996).

IL-1α and IL-1β generally produce similar functional consequences, despite having only approximately 18-26% amino acid sequence homology (Gray et al, 1989). However, since the IL-1α form predominates in cellular membranes, it is believed responsible for more of the paracrine effects of IL-1, whereas IL-1β -- which is chiefly found in plasma – is likely responsible for the cytokine's endocrine-like effects. Interestingly, proIL-1α can also bind IL-1 receptors and elicit an agonistic response, an ability not shared by proIL-1β, which to date has not been shown to bind IL-1 receptors (Dawson, 1991).

At least two mammalian cell surface receptors are known to exist for IL-1, the IL-1RI (type I IL-1R) and IL-1RII (type II IL-1R) receptors. The type I and type II receptors share an overall basic structural similarity to each another, including a single transmembrane region and the presence of three immunoglobulin-like regions that are requisite for binding of the receptors to IL-1. However, the two receptors possess a low level of amino acid sequence homology, reaching only approximately 28% in their extracellular domains. Furthermore, the cytoplasmic portion of the IL-1RII receptor is much shorter (29 amino acids) compared to that for IL-1RI (213 amino acids) (Sims et al, 1988; Kuno et al, 1993; Kuno and Matsushima, 1994; Bankers-Fulbright et al, 1996).

Other studies have demonstrated that it is the IL-1RI receptor that is responsible for transducing the intracellular signaling mechanisms associated with IL-1 binding (McKean et al, 1993; Sims et al, 1993; Oldenburg et al, 1995). Such mechanisms include a wide-ranging collection of complex enzymatic

pathways, many of which "crosstalk" with one another. Based on the published information, these pathways can be summarized by the diagrams depicted in Figure 3. From these diagrams it can be seen that IL-1-activated signaling pathways converge towards NFκB-regulation protein expression, Phospholipase A_2-activated lipid transmitter production (the arachidonic acid cascade), and/or Activator Protein-1-associated transcription.

Figure 3: Known Interleukin-1 mediated intracellular signaling pathways

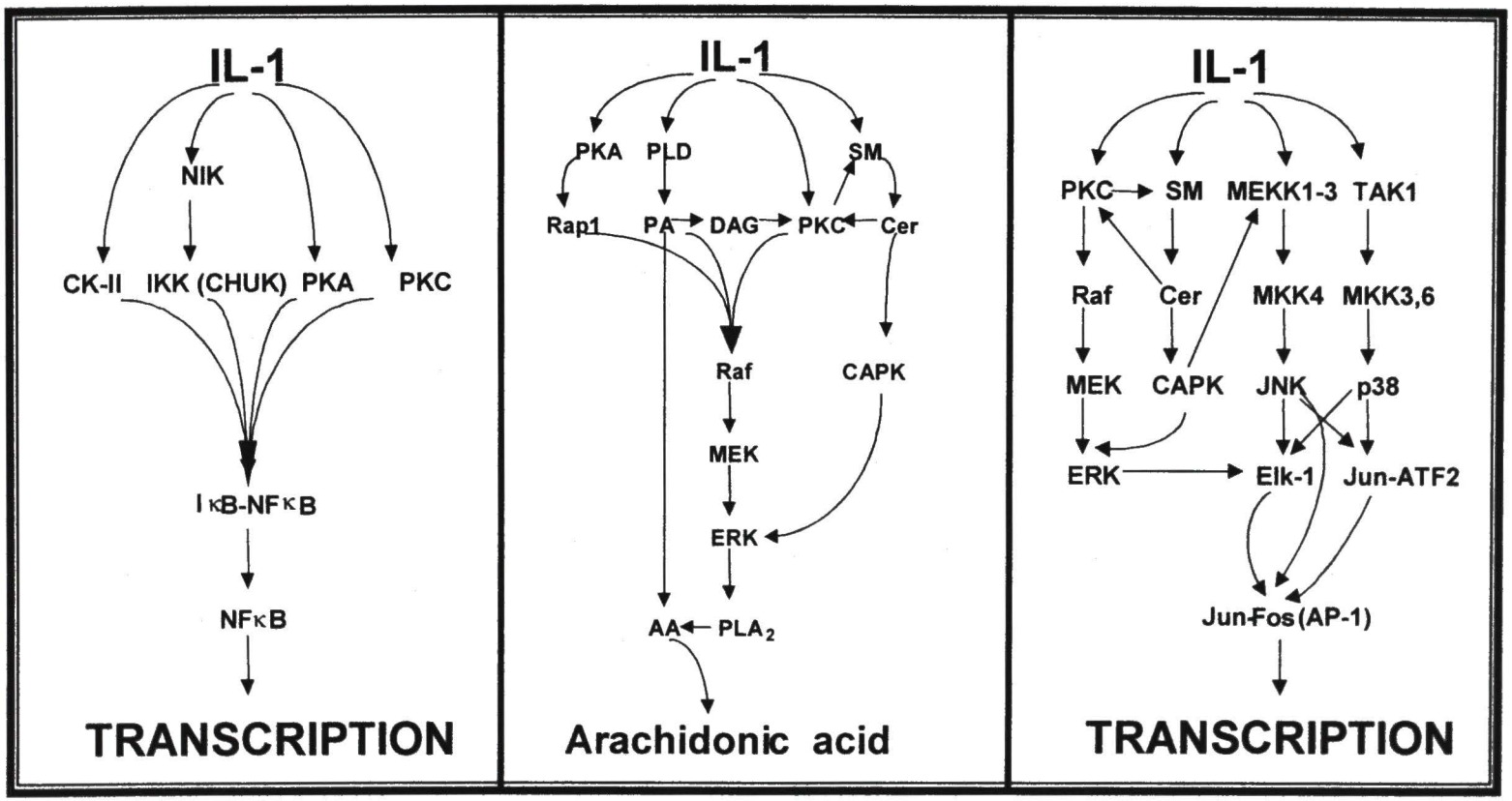


Figure 3: This diagram depicts potential signaling pathways by which IL-1α may exert its effects in target tissues after binding to its target receptors. Abbreviations used: **AA** (arachidonic acid), **AP-1** (activator protein 1), **ATF2** (activating transcription factor 2), **CAPK** (ceramide-activated protein kinase), **Cer** (ceramide), **CK-II** (casein kinase II), **DAG** (diacylglycerol), **Elk-1** (Eph-like kinase), **ERK** (extracellular signal-related kinase), **IκB** (inhibitor of nuclear factor κB), **IKK** (IκB-kinase; also known as conserved helix-loop-helix ubiquitous kinase, or "CHUK"), **MAP** (mitogen-activated protein), **MEK** (MAP/ERK kinase), **MEKK** (MEK kinase), **MKK** (MAP kinase kinase), **NF**κB (nuclear factor κB), **NIK** (NFκB-inducing kinase), **p38** (p38 kinase; also called $p38^{MAPK}$ or HOG-1), **PA** (phosphatidic acid), **PKA** (protein kinase A), **PKC** (protein kinase C), **PLA_2** (phospholipase A_2), **PLD** (phospholipase D), **Raf** (a protooncogene), **Rap1** (GTP binding protein of the Ras superfamily), **SM** (sphingomyelinase), **TAK1** (transforming growth factor-β-activated kinase 1. (Bankers-Fulbright et al, 1996; Bursten and Harris, 1994; Cano and Mahadevan, 1995; Daum et al, 1994; Eder, 1997; English, 1996; Graves et al, 1997; Janosch et al, 1996; Kuno and Matsushima, 1994; Lee et al, 1997; Malanin et al, 1997; Modur et al, 1996; O'Neill, 1995; Peplow, 1997; Ridley et al, 1997; Teski, 1996; Vossler et al, 1997; Welsh, 1996; Withers, 1997; Zhong et al, 1997)

Interleukin-1 and TM cell function

MMP production or activation can be induced by endogenous agents such as cytokines and growth factors in a wide variety of tissues (Borden and Heller, 1997), including those of the eye (see Clark, 1998, for review). In our own hands, IL-1α has been shown in preliminary studies to be the most efficacious activator of MMP-3 production in cultured human TM cells (Magnino et al 1999; Shade, unpublished observations). Based on this, it could therefore be hypothesized that this cytokine would increase aqueous outflow facility, due to its ability to stimulate TM MMP production. In support of this hypothesis, an in vivo study conducted by Kee and Seo (1997) demonstrated increased outflow facility following a single intracameral injection of IL-1α into the eyes of albino rats; increases were dose-dependent, with a maximal 37% increase in facility at 50 units IL-1α, the highest quantity tested.

More recently, it has also been reported that in vitro perfusion of human organ culture eyes with IL-1α similarly resulted in increased outflow facility (Bradley et al, 1998). The same research group had earlier shown that levels of both the MMP-3 subtype and interleukin-1α were enhanced following laser trabeculoplasty of enucleated human donor eyes (Parshley et al, 1996). Although it has not been proven that the rise in MMP-3 levels are – at least in part – attributable to a lasering-induced release of IL-1α or other cytokines/growth factors, it remains an attractive theory.

IL-1α has been shown to have divergent effects on phagocytic activity in other tissues, apparently depending upon the species and cell type being studied. For example, IL-1α was actually shown to inhibit phagocytosis of fibrillar collagen by rabbit periosteal fibroblasts (van der Zee et al, 1995), yet was without effect on the phagocytic activity of periosteal fibroblasts derived from mice (Everts et al, 1990). On the other hand, IL-1 has been implicated in the induction of human polymorphonuclear cell phagocytic activity by lipid A-activated monocytes, based on the ability of an anti-IL-1 antibody to decrease the inductive effect (Jirillo et al, 1992). IL-1α has also been observed to enhance the phagocytosis of both latex beads and *E. coli* by cultured human decidual stromal cells (Ruiz et al, 1997). At present, the effect(s) of IL-1α on TM cell phagocytosis is unknown.

As stated previously, IL-1α is an endogenous substance that has been shown to efficaciously reduce IOP. Despite its potential to increase aqueous outflow (and, hence, lower IOP), IL-1α is also a known mediator of cellular inflammatory mechanisms, and therefore is not suitable as a therapeutic agent. However, if the mechanism(s) and signaling pathways by which IL-1α enhance outflow can be determined, it may be possible to develop alternative means to mimic the outflow-enhancing effects of IL-1α and lasering (e.g. increased MMP-3 levels) while avoiding or minimizing side effects.

Experimental design

Prior to inauguration of the studies involving interleukin-1, it was deemed important that the human TM cell cultures to be used in these experiments be surveyed for certain basic intracellular signaling responses. The aim was to assure that the cell cultures generally responded in a manner similar to that published by other researchers for TM cells and/or tissues. Accordingly, selected cell lines were assayed for the activity of adenylyl cyclase, guanylyl cyclase, and phospholipase C enzymes, as well as for intracellular calcium mobilization.

The objectives of the interleukin research were accomplished in two phases: Phase One utilized established cultures of human trabecular meshwork (TM) cells in order to investigate the signaling pathways involved in IL-1α-stimulated production of matrix metalloproteinases (MMP) and the potential regulation of phagocytosis by IL-1α. Phase Two employed a perfused organ (human donor eyes) culture model in order to confirm the role of the ascertained pathway(s) in the alteration of aqueous outflow, using an appropriate compound that was identified via cell culture response. An outline of these objectives is shown on the following page.

Outline of the research

A. Basic characterization of TM cell cultures (effects of various pharmacological agents on the following second messenger systems: intracellular calcium mobilization, phospholipase C activity, adenylyl cyclase activity, guanylyl cyclase activity).

B. TM MMP production.

1. Effect of IL-1α on production of proMMP-1, -2, -3, and –9 and TIMP-1 and –2.
2. Effect of IL-1α on production of proMMP-3 by TM cells from non-glaucomatous vs glaucomatous donors.
3. Determination of signaling pathway(s) involved in stimulation of proMMP-3 production by IL-1α.
4. Evaluation of effect of small-molecule activators (of signaling pathway(s) identified in step 3) on TM proMMP-3 production.

C. TM phagocytic response.

1. Validation of phagocytosis assay.
2. Effect of IL-1α on TM phagocytic activity.
3. Determination if effect of IL-1α is via similar signaling mechanism(s) as that for MMP production.

D. Validate signaling pathway(s) identified above as important modulator(s) of aqueous outflow and IOP.

CHAPTER 2

METHODS

Cell culture

Stock cultures of human TM (HTM) were maintained at 5% CO_2 and 37° C in a medium consisting of Dulbecco's modified Eagle's medium (DMEM) with Glutamax I (Gibco/Life Technologies), supplemented with 10% fetal bovine serum (Hyclone) and 50 μg/ml gentamicin (Gibco/Life Technologies). Cells were passaged either via Cytodex 3 beads (Sigma) or by a brief (5-10 min.) enzymatic dissociation from the culture substrates with 0.05% trypsin/0.53 mM EDTA solution (Gibco/Life Technologies). Cell stocks were routinely bead-passed and underwent trypsinization only for the purpose of setting up coverslips or multiwell plates for assay. Other cell lines used in these studies were cultured in a similar manner.

Multi-well tissue culture plates (Costar) of cell monolayers were used for most studies; plates were prepared via trypsinization and 1:2 or 1:3 dilution of stock cultures with culture medium. Twenty-four well clusters were used for MMP, TIMP, phagocytosis, cyclic GMP, phospholipase A_2 and phospholipase C assays; 48 well clusters were used for cyclic AMP assays. For calcium studies, trypsinized stock cultures of cells were instead seeded onto sterile #0 glass

coverslips (Biophysica Technologies) placed in the wells of six-well culture plates (Costar) prior to use.

Cell Treatment

For all assays save the calcium experiments, cell monolayers at an estimated 80-90% confluency were washed twice with serum- and antibiotic-free culture medium. 0.5 ml of serum- and antibiotic-free medium was then added per well and the plate(s) returned to the cell culture incubator (5% CO_2 and 37° C) for twenty-four hours (washes and subsequent incubation were necessary in order to minimize serum factors which may interfere with the assays). On the following day, medium in the wells was exchanged for serum- and antibiotic-free medium containing the test agents of choice; final volume per well varied depending upon the assay type, as described in the following sections.

For calcium studies, cells on coverslips were allowed to grow for 4-7 days; when cells reached 30-50% confluency on the coverslips, the cells were rinsed twice with serum- and antibiotic-free DMEM medium, then returned to the cell culture incubator. The following day, the coverslips with cells were mounted in a chamber (Medical Systems Corp., Greenvale, NY) and the chamber was filled with 2 ml "loading buffer" (Mason et al, 1990), pH 7.2. The loading buffer was comprised of 125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 10 mM D-glucose, 0.1% bovine serum albumin (BSA, Fraction V), and 5 μM Fura 2-acetoxy-methylester (Fura 2-AM; Molecular

Probes, Inc., Eugene, OR). Cells were incubated in this solution for one hour at room temperature, followed by washing and final replacement with 2 ml loading from which Fura-2AM and albumin were omitted, then assayed in the presence and absence of test agents as detailed in the section entitled "Intracellular Calcium Mobilization".

ELISA Assays for MMP and TIMP

Treated cell monolayers were returned to the cell culture incubator (5% CO_2 and 37° C) for twenty-four hours after which secreted pro-MMP and TIMP was evaluated in the cell supernatants, using commercially-available sandwich-type ELISA kits. Briefly, aliquots of cell supernatant or reference standards (supplied with kits) were added to wells of 96 well plates precoated with antibodies to proMMPs or TIMPs, then incubated as specified before washing to remove unbound components. Successive incubations followed with biotinylated antibodies to proMMPs or TIMPs (to bind to the "captured" proMMPs or TIMPs from the cell supernatants) and then streptavidin peroxidase conjugates, with additional washes in between. Visualization of pro-MMP or TIMP content was then accomplished by incubation with TMB substrate (3,3',5,5'-tetramethylbenzidine) until sufficient color developed, as determined visually (peroxidase-catalyzed oxidation of TMB leads to blue color production); TMB incubations were not allowed to proceed past 30 minutes, to prevent assay oversaturation. Reactions were stopped by addition of phosphoric acid and resultant (yellow) color read immediately at 450 nm at ambient temperature in a

microplate reader (Dynatech, Model MR5000). Concentration of pro-MMP's or TIMP's in the culture supernatants could then be determined by interpolation from the standard curve included in each assay. Sources of the ELISA kits are as below:

		SOURCE	MINIMUM SENSITIVITY
pro-MMP	1	Oncogene Research Products	1.7 ng/ml
	2	The Binding Site	6.3 ng/ml
	3	The Binding Site	2.1 ng/ml
	9	Oncogene Research Products	0.6 ng/ml
TIMP	1	Oncogene Research Products	1.25 ng/ml
	2	Oncogene Research Products	3 ng/ml

Neutral Red Uptake

After removal of supernatants for MMP assay, monolayers were washed with serum- and antibiotic-free culture media, after which 0.5 ml of serum- and antibiotic-free media was added per well and the plate(s) returned to the cell culture incubator (5% CO_2 and 37° C) for approximately 15 minutes to allow re-equilibration. 50 µl of pre-warmed 3.33 mg/ml neutral red dye solution (Gibco/Life Technologies) was then added per well and the plate(s) were returned to the incubator for two hours, at which time the monolayers were again washed with serum- and antibiotic-free culture media. Final washes were aspirated as completely as possible, then monolayers were allowed to air dry at room temperature before addition of 0.5 ml cold solubilization buffer (1% glacial

acetic acid, 50% anhydrous ethanol) per well. Twenty minutes later aliquots of the colored solutions were transferred to individual wells of a 96 well microtiter plate and the absorbance of the samples read at 570 nm at ambient temperature in a microplate reader (Dynatech, Model MR5000); a decrease in optical density was interpreted as loss of cell viability.

Phagocytosis

Following pretreatment of the TM monolayers with test agents for 15 min, TM phagocytic activity was then estimated using the Vybrant™ Phagocytosis Assay Kit (Molecular Probes) with some modifications to the standard kit protocol. "Bioparticles", consisting of a sonicated (5 min), 1 mg/ml suspension in serum-free DMEM/F12 of fluorescein-labelled and inactivated *E. coli* bacteria, were added to the wells containing the treated monolayers, followed by incubation overnight (20 hr) at 37° C and 5% CO_2. After this time, the monolayers were washed to remove free bioparticles and the monolayers were exposed briefly (approximately one minute) to Trypan blue, in order to quench fluorescence of the remaining extracellular (i.e. nonphagocytosed) bioparticles. Relative fluorescence of the bioparticles within (i.e. presumably phagocytosed) the cells of the monolayers could then be ascertained using an Fmax™ fluorescence microplate reader (Molecular Devices) using "top-down" mode and a standard 485/538 nm (excitation/emission) wavelength filter set. An increase in phagocytic activity would be expected to correlate with an increased fluorescence level, as compared to untreated controls.

Perfused human organ culture models

Two different models were utilized: "constant pressure" and "constant flow" (see Figure 4). Both models utilized human donor eyes (obtained and used $\leq$ 24 hrs post-mortem) from authorized eye banks. The constant pressure model was used for "normal" (not diagnosed with glaucoma at time of death) eyes and the constant flow model used for eyes from glaucomatous donors, based on prior observations that viability of glaucomatous eyes was better-maintained using the constant flow technique.

Eyes were prepared for perfusion generally following methods described by Johnson and Tschumper (1987) and Clark et al (1995) by first carefully removing extraocular tissues, followed by bisection of the globes and removal of the iris, lens, vitreous, zonules. In some experiments the majority of the ciliary body was also removed. The conjunctiva was lightly scored approximately 3 mm posterior to the limbus with an ophthalmic surgical knife (Alcon) in order to sever the episcleral veins, thereby permitting fluid efflux. The anterior segments were then mounted in specially-designed Plexiglass chambers (BioEngineering Department, University of Texas Southwestern Medical School, Dallas) followed by perfusion at either a constant pressure (10-12 mm Hg) or a constant flow rate (2μL/minute). Perfusion was with sterile DMEM media containing antibiotics (penicillin/streptomycin) and allowed to equilibrate until a stable outflow rate (constant pressure model) or pressure (constant flow model) was achieved,

usually less than four days. Perfused eyes were maintained in a humidified incubator at 37° C with 5% CO_2 and ambient air.

Following stabilization, eye pairs were perfused with and without the test agents and observed for changes. For the constant pressure model, changes in flow rate of the perfusate were determined by weighing the fluid reservoir at predetermined periods and weight changes converted to volume per unit time (e.g. μL/min) in order to determine perfusion rates in the presence and absence of the test agents. For the constant flow model, a custom-made pressure transducer (BioEngineering Department, University of Texas Southwestern Medical School, Dallas) was connected to a cannula of the perfusion chamber and changes in IOP were recorded automatically via a computerized system (Datataker DT600, Science Electronics, Dayton, OH).

Eyes which did not stabilize within four days of onset of perfusion, or which did not exhibit flow rate ranges between 1.5-8 μL/min (constant pressure studies) or pressure ranges between 5-40 mm Hg (constant flow studies), were rejected. An estimated approximately 50% of eyes received were rejected for these reasons. Furthermore, since tissue viability is of paramount, the viability of the anterior segments was evaluated at termination of each study. The perfused tissues were fixed and morphology, in particular that of the TM, was assessed by both light and electron microscopy. Studies were deemed invalid if unacceptable findings such as excessive TM cell loss, denudation of TM beams, an excess of cellular debris in the TM region, loss of Schlemm's canal endothelial cells or

breaks in its lining, and/or collapse of the canal itself were present (Clark et al, 1995; Pang et al, 2000). Based on these criteria, approximately 30% of the perfused tissues were judged unsuitable for inclusion in the results reported herein.

Figure 4

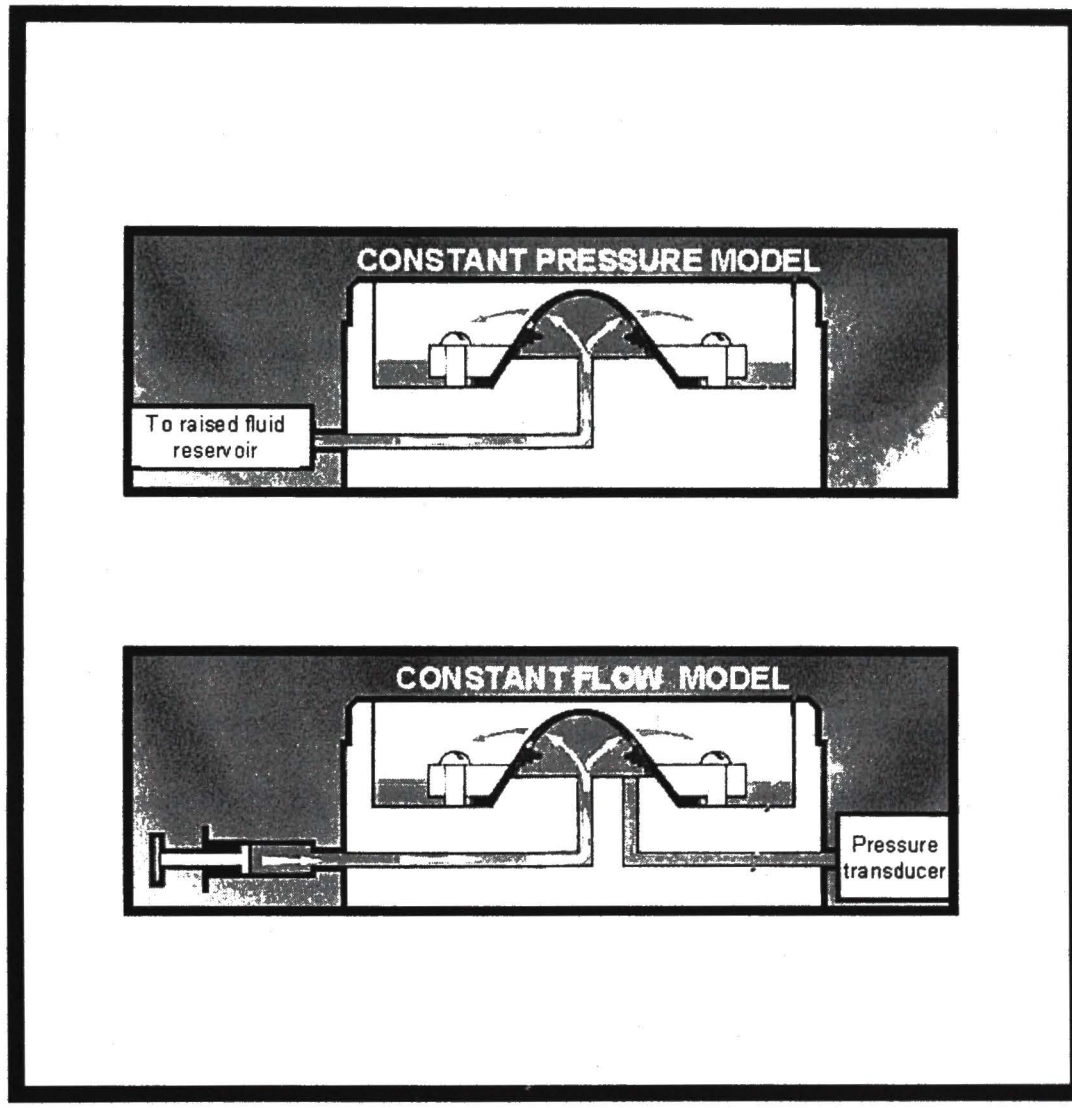


Figure 4: Comparison of the constant pressure and constant flow models used for perfusion studies.

Adenylyl cyclase

Adenylyl cyclase assays were conducted as previously described (Pang et al, 1994; Pang et al, 1996) using a serum-free 1:1 DMEM/F12 (Gibco/Life Technologies) mixture in which the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was included at a concentration of 1 mM, to prevent degradation of the cyclic nucleotides produced. Monolayers were incubated with agonists at room temperature for a period of 5 min with or without test agents. Assays were then terminated via aspiration of incubation media and immediate addition of 0.2 ml ice-cold 0.1 M acetic acid, followed five minutes later by neutralization with 0.3 ml ice-cold 0.1 M sodium acetate (pH 11.6). An 0.2 ml aliquot of each lysate was then removed per well and transferred to individual test tubes for assay of cyclic AMP content via commercially-available radioimmunoasay kits (Biomedical Technologies, Inc.). In those assays, cAMP present in the sample lysates competes with the corresponding radioiodinated (^{125}I) cyclic nucleotide for a primary antibody that is specific for cAMP. Precipitation with a secondary antibody and centrifugation then followed, in order to isolate bound cyclic nucleotides; resulting pellets were then analyzed for radioactive content in a Minaxi Autogamma 5000 counter (Packard). Quantification of cAMP in the sample lysates was achieved by comparison of sample radioactive content (cpm) with that of a cyclic nucleotide standard curve generated with each assay. Minimal detectable amount of cAMP in these assays was 50 fmole/0.2 ml lysate.

Guanylyl cyclase

Guanylyl cyclase assays were generally conducted as previously described (Pang et al, 1994; Pang et al, 1996; Haque et al, 1998). Incubations used a serum-free 1:1 DMEM/F12 (Gibco/Life Technologies) mixture in which the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was included at a concentration of 1 mM, to prevent degradation of the cyclic nucleotides produced. Monolayers were incubated at room temperature for a period of 10 min with or without test agent. Assays were subsequently terminated via aspiration of incubation media and immediate addition of 0.2 ml ice-cold 0.1 M acetic acid, followed five minutes later by neutralization with 0.3 ml ice-cold 0.1 M sodium acetate (pH 11.6). An 0.2 ml aliquot of each lysate was then removed per well and transferred to individual test tubes for assay of cyclic GMP content via commercially-available radioimmunoasay kits (Biomedical Technologies, Inc.). In those assays, cGMP present in the sample lysates competes with the corresponding radioiodinated (^{125}I) cyclic nucleotide for a primary antibody which is specific for cGMP. Precipitation with a secondary antibody and centrifugation follows in order to isolate bound cyclic nucleotides; resulting pellets were then analyzed for radioactive content in a Minaxi Autogamma 5000 counter (Packard). Quantification of cGMP in the sample lysates was achieved by comparison of sample radioactive content (cpm) with that of a cyclic nucleotide standard curve generated with each assay. Minimal detectable amount of cGMP in these assays was 10 fmole/0.2 ml lysate.

Phospholipase C activity

For these studies, phospholipase C assays were conducted as previously described (Pang et al, 1994; Shade et al, 1996) and enzymatic activity was inferred by measurement of total radioactive inositol phosphates' (IP_3 and its metabolites) accumulation in cell monolayers which had been allowed to incorporate myo-[2-^{3}H]-inositol (Amersham; 5 μCi in a volume of 0.5 ml serum-free DMEM/F12 media per well of 24-well culture plates) into membrane phospholipids for 24 hours prior to assay. On the day of assay, monolayers were rinsed extensively with serum-free media in order to remove unincorporated isotope, after which cells were exposed to test agents in the presence of 10 mM lithium chloride (LiCl). LiCl inhibits hydrolysis of inositol monophosphate, an IP_3 metabolite, into inositol and thus prevents inositol's reincorporation into membrane phospholipids.

After incubation for one hour at 37°C and 5% CO_2, the incubation media was removed and replaced with ice-cold 0.1 M formic acid, 1 ml per well, and the culture plate(s) incubated on ice for at least fifteen minutes, after which 0.9 ml of each resulting lysate was then applied to individual anion exchange columns (Dowex AG 1-X8 anion exchange resin in formate form; BioRad). After flow-through of the lysate, the columns were washed successively with purified water (10 ml) and 50 mM ammonium formate (8 ml), followed by elution of bound ^{3}H inositol phosphates with two separate volumes (2 ml each) of a 1.2 M ammonium formate/0.1 M formic acid solution.

Each 2 ml eluate was collected separately into individual glass scintillation vials (Kimble). Scintillation cocktail (Ecolume; ICN Biomedicals) was then added to the eluate in each scintillation vial, followed by analysis for radioactive tritium content in a scintillation counter (Packard TriCarb). Total cpm was determined by adding that for the two eluates obtained from each sample column. An increase or decrease in total cpm, as compared to that for vehicle controls, was interpreted as indicative of alterations of PLC activity.

Intracellular calcium mobilization

For these studies, ratio fluorometry of cells pre-loaded in a 5 μM solution of the cell-permeant, calcium-sensitive fluorescent dye Fura 2-acetoxymethylester (Fura 2-AM; Molecular Probes) were utilized to follow the effects of test agents on receptor-mediated changes in intracellular calcium as previously described (Pang et al, 1996; Shade et al, 1996). Briefly, test agents were added as aliquots from stocks of 100-fold concentration greater than the desired final concentration; where indicated, antagonists were added 15-20 seconds prior to carbachol. Test agents were removed between doses by washing with at least two chamber volumes of assay buffer. Intracellular calcium concentrations were allowed to return to baseline prior to addition of the next test agent concentration.

Intracellular esterases convert Fura 2-AM to Fura 2, which possesses an excitation peak near 380 nm when not bound to calcium vs approximately 340 nm when bound to calcium (maximal emission wavelength peak at approximately

510 nm). The ratio of fluorescence intensities generated by the two excitation wavelengths (340/380) thus corresponds to changes in intracellular calcium content; this ratio may be converted to estimations of calcium concentration.

Fluorescence of single cells, in the presence and absence of test agents, were recorded at room temperature using a Deltascan 4000 ratio fluorescence system with dual monochromator illuminator and microscope photometer with photon-counting photomultiplier detector (Photon Technology International) coupled to a Nikon Diaphot microscope.

Minimal and maximal potential calcium concentrations were determined by replacing the assay buffer with a calcium-free chelation buffer containing 2 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) followed by addition of 10 μM of the calcium ionophore ionomycin. When no further decrease in fluorescence at 340 nm was evident, i.e. "minimal" concentration had been achieved, the maximal value was then be determined by monitoring fluorescence after addition of excess (10 mM) free calcium to the incubation buffer. The experimental intracellular calcium concentrations could then be extrapolated via the equation of Grynkiewicz (1985), i.e. $[Ca^{2+}] = K_D \times (F_{380free}/F_{380sat}) \times (R-R_{min})/(R_{max}-R)$, and an estimated K_D (dissociation constant of Fura 2 for calcium) value of 135 nm. Where '$F_{380free}$' is fluorescence intensity at zero calcium concentration, with excitation at 380 nm; 'F_{380sat}' is fluorescence intensity at saturating calcium concentration, with excitation at 380 nm; 'R' is ratio of fluorescence intensities at 340 and 380 nm excitation wavelengths; 'R_{min}' is

fluorescence intensity ratio of Fura 2 in the unbound (calcium-free) form; and 'R_{max}' is fluorescence intensity ratio of calcium-saturated Fura 2.

Statistical Analysis

Mean results for assays were evaluated via one-way ANOVA then with Dunnett's test or via the Students *t* test (two-tailed) and were considered statistically significant if $p < 0.05$ for treatment groups as compared to the appropriate controls.

CHAPTER 3

BASIC CHARACTERIZATION OF TM CELL CULTURES

Four well-known second messenger pathways were chosen for study in order to provide verification that the cultured HTM cells represented a valid model system, i.e. that normal biochemical pathways were intact and functional. The effects of selected modulators on intracellular calcium mobilization, inositol phosphates turnover, and cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) production were thus tested. Overall, results confirmed that the TM cellular responses were similar to that published by other laboratories for cultured TM cells and/or TM tissue; furthermore, these studies verified that no loss of these critical intracellular signaling pathways occured as a result of cell culture.

Intracellular calcium mobilization

Alterations in intracellular calcium mobilization were observed using the technique of ratio fluorometry combined with a calcium-sensitive fluorescent probe, Fura 2-AM. Carbachol hydrochloride, a nonspecific cholinergic receptor agonist, was chosen to demonstrate HTM cell responsiveness in this system. A typical HTM cell (HTM-16A) response to carbachol is depicted in Figure 5. The tracing in this figure illustrates the rapid rise in free intracellular calcium concentration in the presence of 100 μM carbachol (arrow). The rapid peak in

calcium levels dissipates quickly, followed by an extended "plateau" phase during which free intracellular calcium levels are still significantly higher than that recorded in the unstimulated cell; the plateau phase may persist for several minutes, up to 30 minutes or more (data not shown), without diminishing. Peak and plateau values were consistent, with HTM-16A cells demonstrating an average peak value of 406 $\pm$ 53 nM (mean $\pm$ SEM; n=9) and average plateau value of 192 $\pm$ 38 nM (mean $\pm$ SEM; n=7) in response to 100 μM carbachol.

The HTM response to carbachol, i.e. increased intracellular calcium mobilization, was dose-dependent, as shown in Figure 6. Increasing carbachol concentrations effected a corresponding increase in peak free intracellular calcium levels, with a maximum response elicited at $\geq$ 1 mM carbachol; estimated EC_{50} for carbachol in this particular experiment was 11 μM; overall, carbachol stimulated HTM-16A calcium mobilization with an average EC_{50} of 7.6 μM (mean $\pm$ SEM; n=7) . Peak "plateau" free intracellular calcium levels showed a similar trend in response to increasing carbachol concentrations.

Furthermore, carbachol's effects could be blocked by atropine, an antagonist which is specific for muscarinic cholinergic receptors, as well as by the muscarinic subtype-selective antagonists p-fHHSiD (M_3 subtype) and pirenzepine (M_1 subtype) . Figure 7 depicts typical dose response curves for effect of the three muscarinic antagonists on peak phase carbachol-induced (10 μM) intracellular calcium mobilization; effects were dose-dependent with IC_{50} values of 12 nM, 90 nM, and 2 μM for atropine, p-fHHSiD, and pirenzepine,

respectively. Cumulative data for each of the three antagonists was similar with the same rank order of potency (i.e. atropine > p-fHHSiD > pirenzeine) and is summarized in Table 2 (see Phospholipase C section).

Figure 5

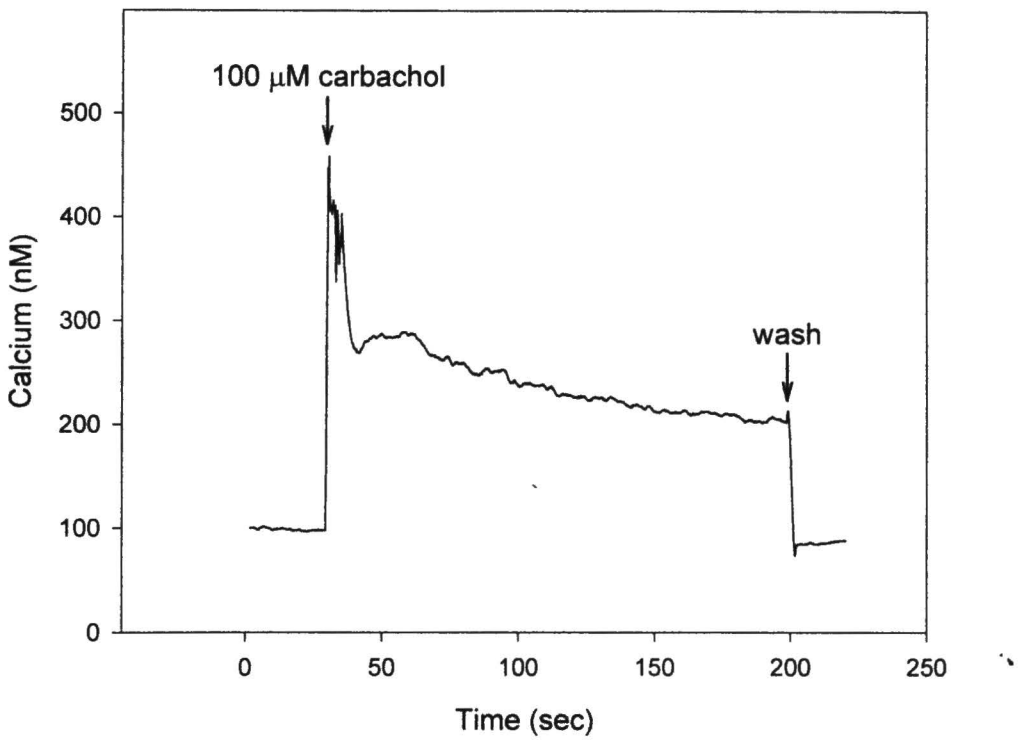


Figure 5: Effect of carbachol on TM intracellular calcium mobilization. Tracing depicts response of an individual HTM-16A cell following exposure to 100 μM carbachol. Similar results were obtained in 7 separate studies.

Figure 6

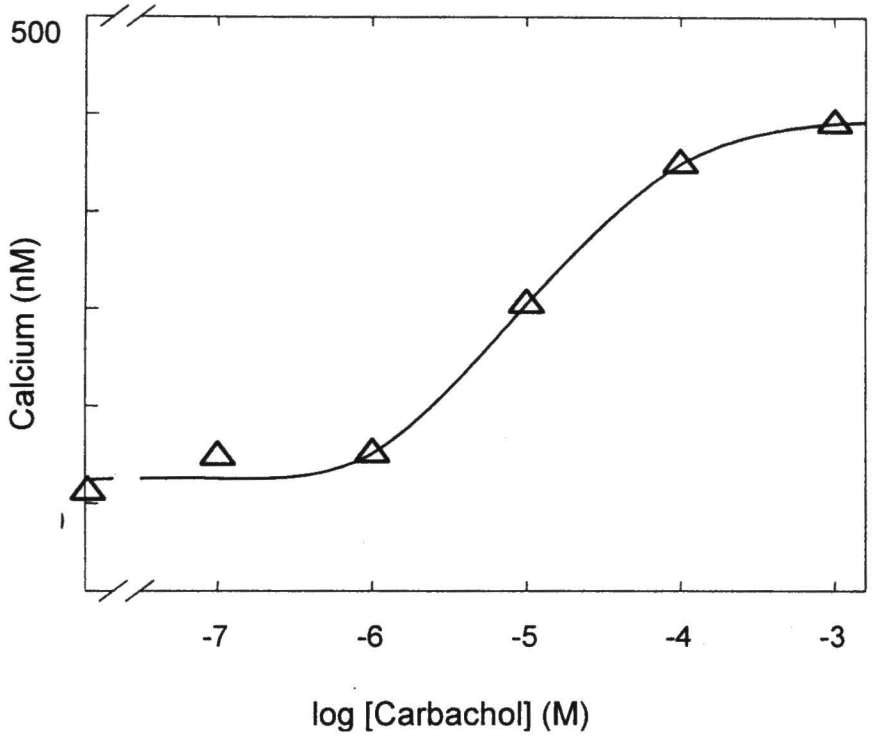


Figure 6: Dose-dependent effect of carbachol on TM intracellular calcium mobilization. Curve indicates response of an individual HTM-16A cell following exposure to increasing concentrations of carbachol. Similar results were obtained in 7 separate studies.

Figure 7

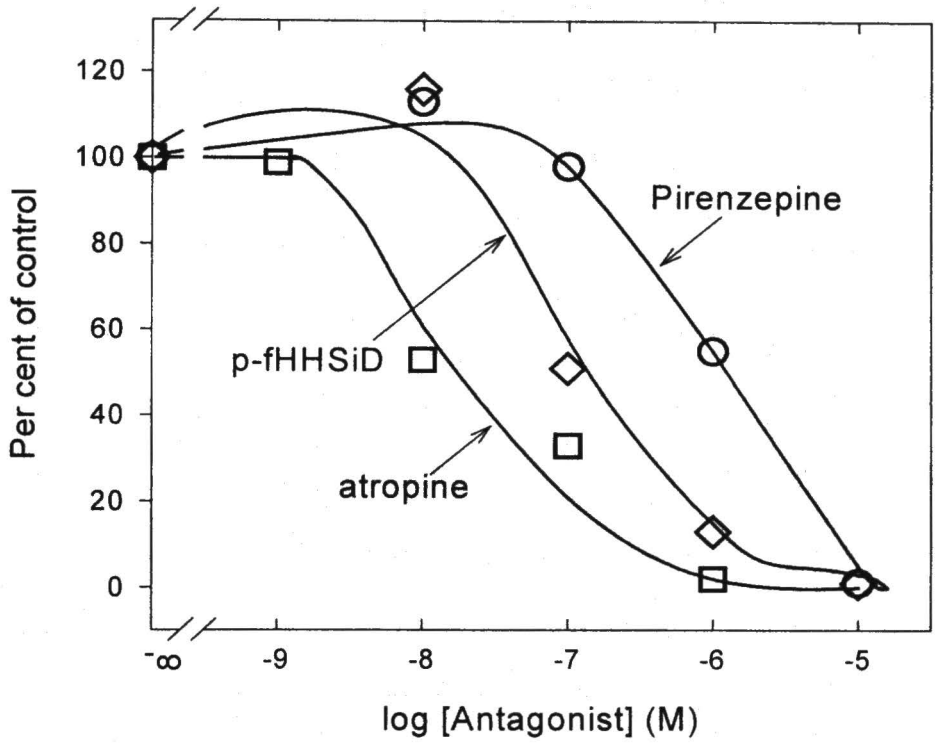


Figure 7: Dose-dependent effects of muscarinic receptor antagonists on carbachol-induced (10 μM) TM intracellular calcium mobilization. Curves indicate responses of individual HTM-16A cells following exposure to increasing concentrations of the antagonists. "100%" is defined as response to carbachol (10 μM) in the absence of antagonist, whereas "0%" is defined as that of the unstimulated (resting) calcium level for the individual cells. Similar results were obtained in 2-3 separate studies, performed in duplicate.

Phospholipase C activity

In these studies, an increase in the accumulation of radioactive inositol phosphates was interpreted as being indicative of an increase in PLC enzyme activity (see Methods section). The effect of carbachol (100 μM) on TM phospholipase C activity was determined, as was the effect of other agonists of receptors known to mediate PLC activation such as prostaglandin $F_{2\alpha}$ (100 nM; FP receptor) and histamine (10 μM; H1 receptor). All agents were used at concentrations previously shown in our laboratory to enhance PLC activity in other cells/tissues. These data are summarized in Figure 8: all three agents significantly stimulated accumulation of radioactive inositol phosphates as compared to basal (vehicle) controls. In addition, similar to that seen for calcium mobilization, carbachol's effects were dose-dependent; a typical carbachol dose-response curve is shown in Figure 9: increasing concentrations of carbachol elicited a corresponding increase in accumulated radioactive inositol phosphates, up to a maximum stimulatory carbachol concentration of $\geq$ 300 μM. EC_{50} for this particular experiment was 11 μM; average EC_{50} for all experiments was 12.6 μM (log EC_{50} = -4.9 $\pm$ 0.4, mean $\pm$ SEM; n=3).

Also similar to calcium mobilization studies, the effects of carbachol on PLC activity could be blocked by atropine and by the muscarinic subtype-selective antagonists p-fHHSiD (M_3 subtype) and pirenzepine (M_1 subtype) . Figure 10 depicts typical dose response curves for effect of the three muscarinic

antagonists, expressed as per cent of the maximal carbachol response (100 μM carbachol = 100%). Effects were again dose-dependent with IC_{50} values in the depicted experiments of 2.65 nM, 78 nM, and 805 nM for atropine, p-fHHSiD, and pirenzepine, respectively. Cumulative data for each of the three antagonists was similar to that seen for intracellular calcium mobilization, with the same rank order of potency (i.e. atropine > p-fHHSiD > pirenzeine), suggesting involvement of the M_3 muscarinic receptor subtype in both mechanisms; these data are summarized in Table 2.

Figure 8

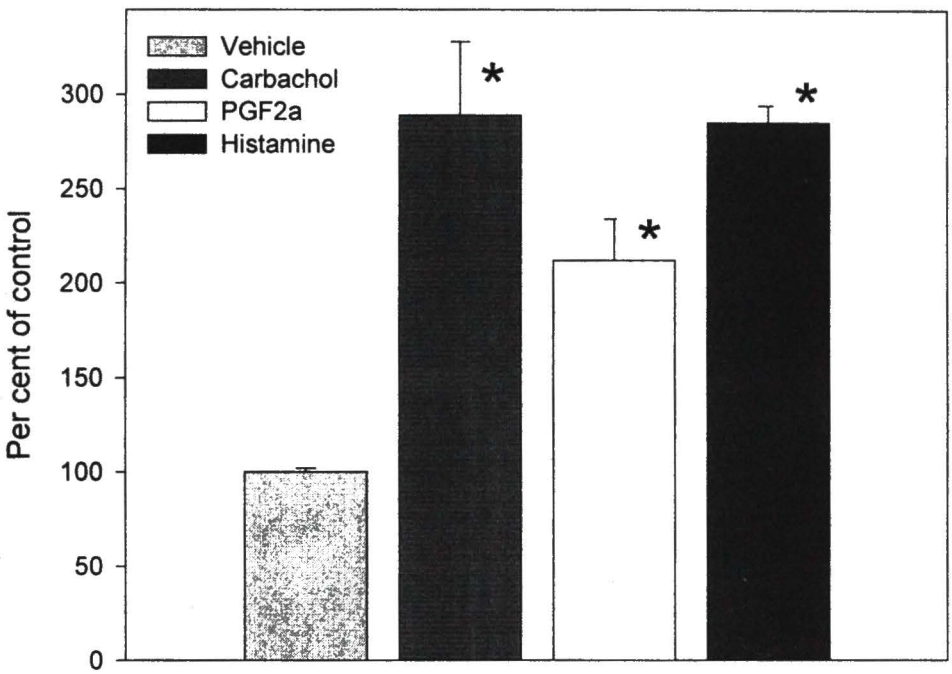


Figure 8: Effect of carbachol (100 μM; n=12), prostaglandin $F_{2\alpha}$ (100 nM; n=8), and histamine (10 μM; n=9) on IP production in HTM-16A cells (results are mean ± SEM; average untreated controls define 100%; n=12; * denotes p values < 0.05 as compared to vehicle controls).

Figure 9

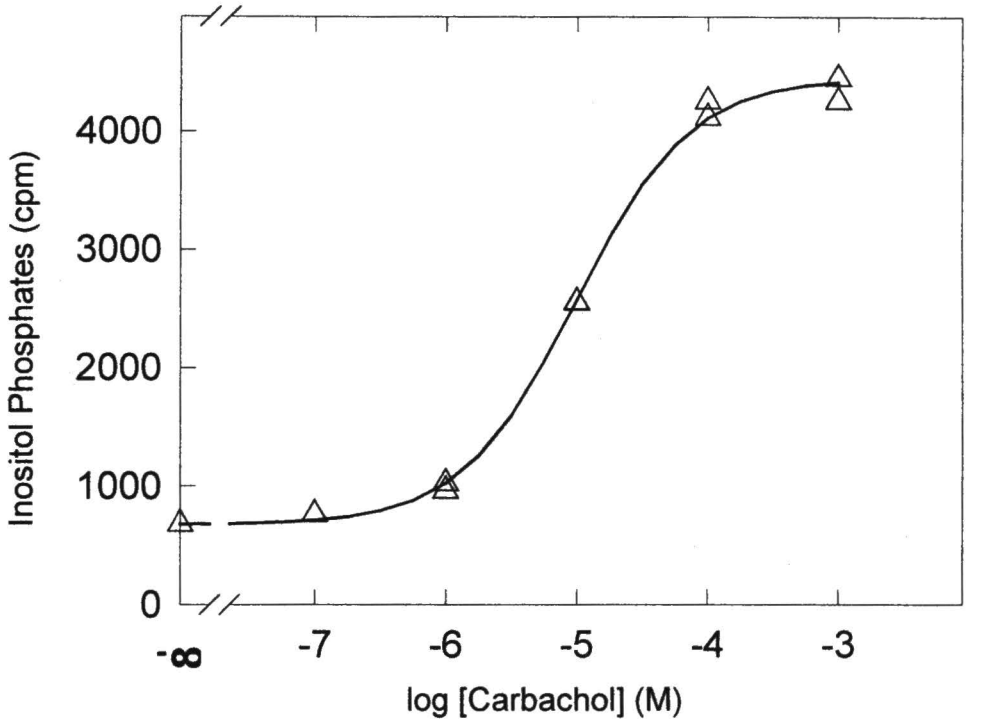


Figure 9: Dose-dependent effect of carbachol on inositol phosphates' turnover in HTM-16A cells (symbols represent individual wells in a single assay, performed in duplicate). Similar results were obtained in 3 separate studies.

Figure 10

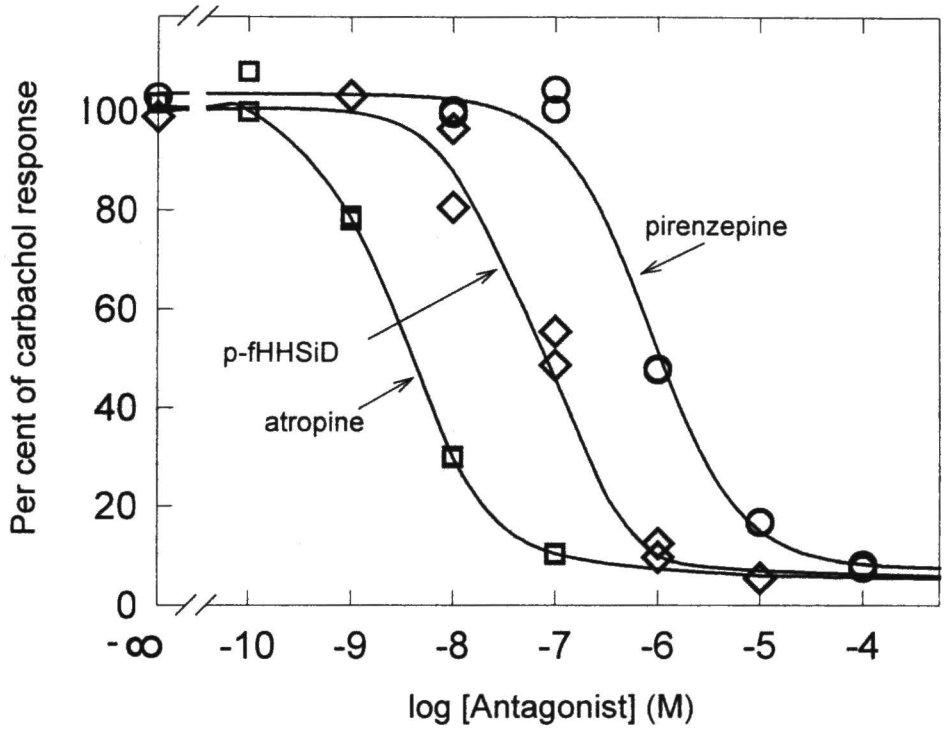


Figure 10: Dose-dependent effects of muscarinic receptor antagonists on carbachol-induced (10 μM) HTM-16A inositol phosphates' turnover (symbols represent individual wells in a single assay, performed in duplicate). Similar results were obtained in 2-3 separate studies.

Table 2

Summary of effects of muscarinic receptor antagonists on carbachol (100 μM)-stimulated phosphoinositide production and calcium mobilization in HTM cells

Antagonist	pK_i* for inhibition of phosphoinositide production	pK_i** for inhibition of intracellular calcium mobilization
Atropine	9.53, 9.61 (n=2)	9.07 ± 0.23 (n=8)
p-fHHSiD	7.97 ± 0.06 (n=3)	8.08 ± 0.14 (n=6)
Pirenzepine	7.00 ± 0.06 (n=3)	6.43 ± 0.28 (n=4)

*Expressed as mean ± SEM of (n) experiments, each performed in duplicate

**Expressed as mean ± SEM of (n) individual cells tested

Adenylyl cyclase activity

Activation of the enzyme adenylyl cyclase leads to increased levels of the second messenger cyclic adenosine monophosphate (cAMP); alterations in the cellular production of cAMP may thus used to predict changes in adenylyl cyclase activity.

To determine whether the HTM cell cultures possessed intact adenylyl cyclase signaling pathways, monolayers were challenged with the direct cyclase activator forskolin (10 μM). Two other agents (prostaglandin E_2 and isoproterenol, each at 1μM) known to be agonists at receptors linked to stimulation of adenylyl cyclase activity were also tested. Results of these experiments are summarized in Figure 11. In the presence of phosphodiesterase inhibitors (in order to prevent rapid degradation of accumulated cAMP) and at the concentrations tested, each agent dramatically increased production of cyclic AMP, as compared to basal (vehicle) controls. Such increases ranged from approximately $\geq$ 20 fold (forskolin, prostaglandin E_2) to approximately 45 fold (isoproterenol) when determined in the presence of the phosphodiesterase inhibitor IBMX (1 mM).

Figure 11

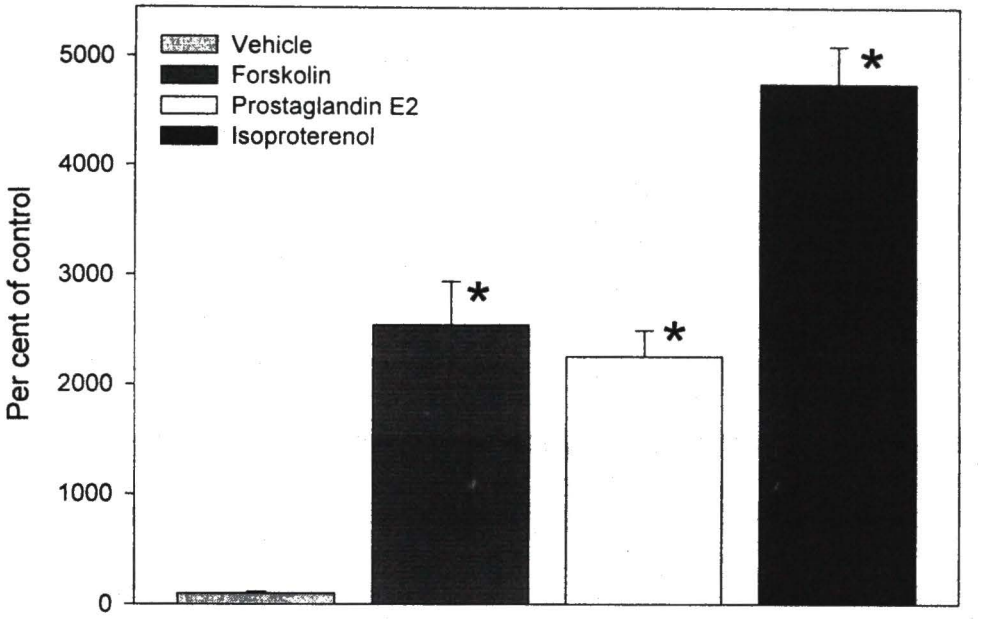


Figure 11: Effects of forskolin (10 μM; n=15), prostaglandin E_2 (1 μM; n=12), and isoproterenol (1 μM; n=12), on production of cyclic AMP by HTM-16A cells (results are mean ± SEM; average untreated controls define 100%; n=21; * denotes p values < 0.05 as compared to vehicle controls).

Guanylyl cyclase activity

Activation of the enzyme guanylyl cyclase leads to increased levels of yet another second messenger molecule: cyclic guanosine monophosphate (cGMP); similar to that for adenylyl cyclase, alterations in the cellular production of the associated second messenger (cGMP) may thus used to predict changes in guanylyl cyclase activity.

In order to determine whether the HTM cell cultures possessed intact guanylyl cyclase signaling pathways, monolayers were treated with sodium nitroprusside (SNP; 1 mM) an activator of the soluble form of the guanylyl cyclase enzyme. Results of these experiments are summarized in Figure 12. At the concentration tested, SNP significantly increased production of cyclic GMP, as compared to basal (vehicle) controls, increasing intracellular cGMP levels approximately 2.5 fold when determined in the presence of the phosphodiesterase inhibitor IBMX (1 mM).

Figure 12

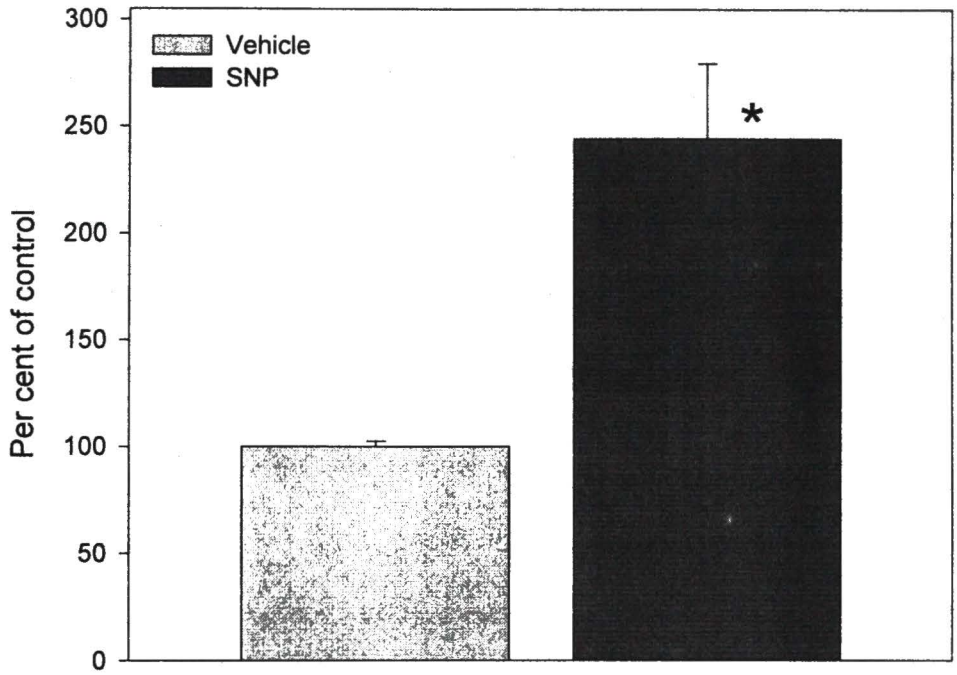


Figure 12: Effects of SNP (1 mM; n=9) on production of cyclic GMP by TM cells (results are mean ± SEM; average untreated controls define 100%; n=9; * denotes p value < 0.05 as compared to vehicle controls).

Verification of TM cell origin

TM cell lines used in these studies were previously identified as having been derived from trabecular meshwork based on immunocytochemical confirmation of the expression of key markers (e.g. collagen, laminin, fibronectin) as well as by microscopic observation of typical TM cell morphology, according to methods established previously (Steely et al, 1992; Clark et al, 1994). All lines used in these studies were derived from male donors, with one exception (GTM-85B); those lines mentioned in the text as being from "glaucomatous" donors were all derived from confirmed POAG patients at time of death.

CHAPTER 4

MATRIX METALLOPROTEINASE ASSAY RESULTS

Dose-response relationship and time course of IL-1α-activated proMMP-3 expression

For these experiments, supernatants from vehicle or IL-1α-treated HTM-35D cell monolayers were tested for presence of the secreted MMP enzymes and/or their inhibitors. Initial experiments focused on determining an optimal incubation time for cellular response to IL-1α, in addition to determining an optimal concentration of IL-1α to be used in subsequent assays. Incubation of the HTM cells with test agents occurs under serum-free conditions in order to reduce potential additive or inhibitory effects of serum factors. Thus, because prior experience with the HTM cells had shown that up to a twenty-four hour serum-free period was not detrimental to the cells (as determined by neutral red uptake), dose-response curves were generated for effect of a 24 hr exposure to IL-1α on proMMP-3 (stromelysin) production by HTM-35D cells. Effect on MMP-3 secretion was chosen for these initial studies because it is the enzyme expected to be most likely involved in HTM ECM degradation.

A typical dose-response curve for a twenty-four hour exposure to IL-1α is shown in Figure 13. ProMMP-3 levels rose steadily with increasing concentrations of IL-1α, with the maximal response occuring at $\geq$ 25 ng/ml of the

cytokine; effects at 25 ng/ml were somewhat more variable, likely due to a somewhat diminished cell viability at this concentration, as determined by neutral red uptake. EC_{50} for the depicted experiment was estimated at 484 pg/ml. Based on the dose-response studies, a concentration of 5 ng/ml IL-1α (i.e. approximately 10-fold that of the EC_{50} value) was selected for determining the time course of IL-1α's effect on proMMP-3 levels; effect on proMMP-1 levels was also included, for comparison. These data are depicted in Figure 14: both proMMP-3 and proMMP-1 levels were significantly ($p < 0.05$) elevated as early as 8 hours post-dosing with IL-1α as compared to vehicle-treated controls. Levels of both enzymes were also significantly increased in supernatants from IL-1α treated cells (as compared to vehicle-treated) at 24 and 48 hours post-dosing, however the rate of production of the MMPs appeared to diminish somewhat by 48 hours despite no apparent effect on cell viability. Cells were also challenged with the cytokine for a period of 72 hours (data not shown): however the viability of both the IL-1α treated and the vehicle-treated monolayers was reduced (as determined by neutral red uptake) at this time point, thus these data were excluded from consideration.

Figure 13

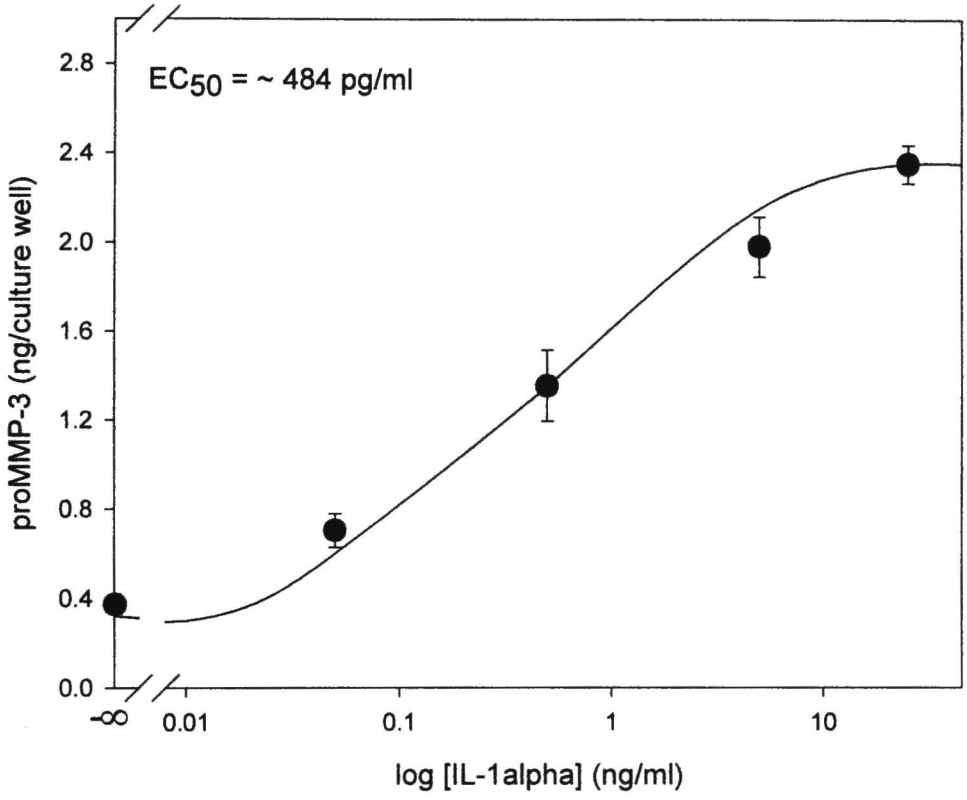


Figure 13: Typical dose-dependent effect of IL-1α on accumulation of proMMP-3 in HTM-35D cell supernatants (mean ± SEM; n=3).

Figure 14

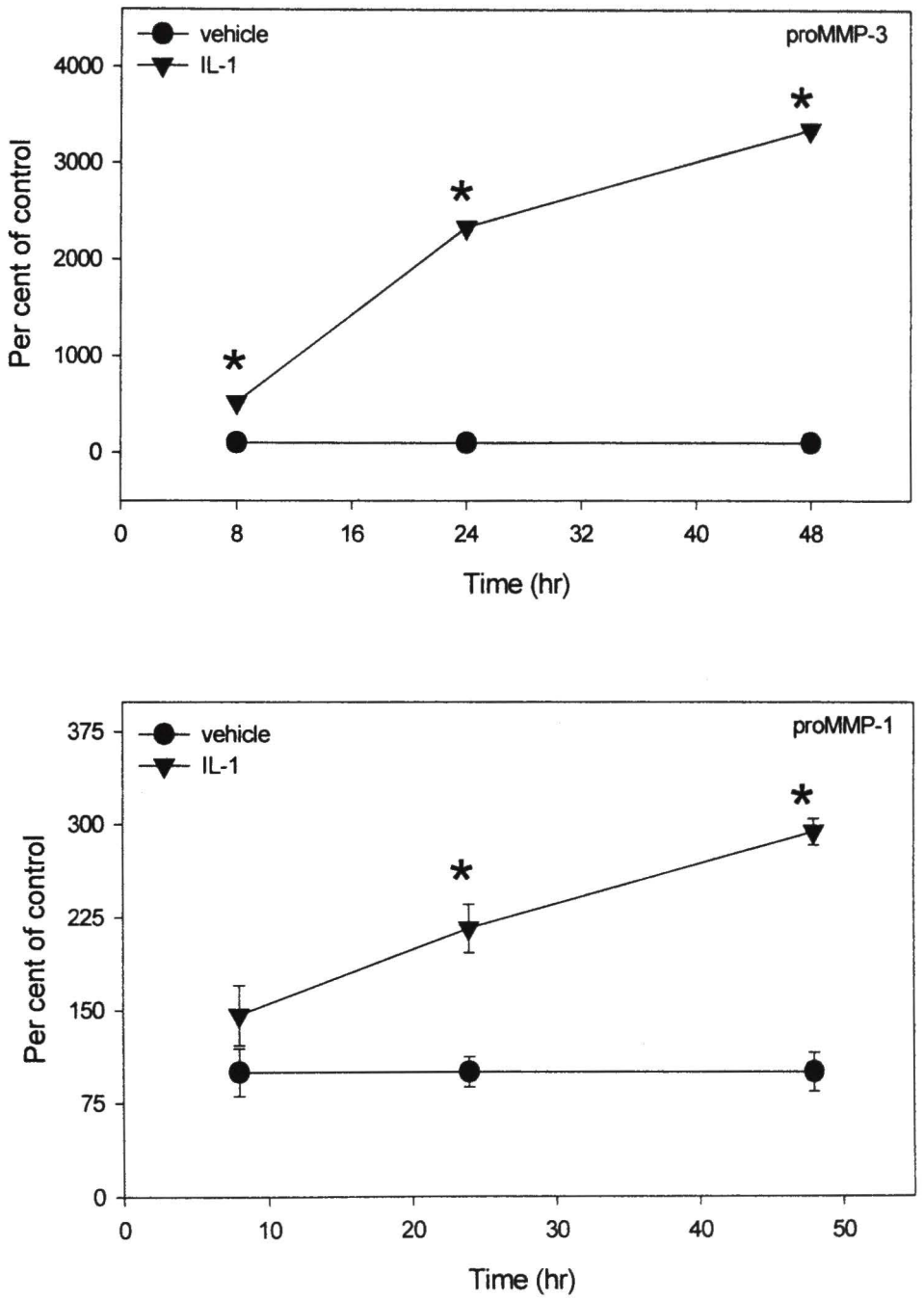


Figure 14: Time course of effect of IL-1α (5 ng/ml) on accumulation of proMMP-3 and proMMP-1 in HTM-35D cell supernatants (mean ± SEM; n=3; * denotes $p < 0.05$ as compared to vehicle controls).

Effect of IL-1α on expression of various MMPs and TIMPs

Because of their faster growth rate and readily-available supply, HTM-35D cells were further utilized to evaluate the effect of a twenty-four exposure to IL-1α (25 ng/ml and 5 ng/ml) on production of the pro-enzyme forms of MMP-1, MMP-2, MMP-3, and MMP-9, as well as that of TIMP-1 and TIMP-2. TPA (phorbol 12-myristate 13-acetate), a well-known non-specific activator of MMP production was included at the same concentrations in each experiment, for comparison. Data from these experiments is listed in Tables 3, 4, and 5.

From these data, it appears that IL-1α is primarily a highly efficacious stimulator of proMMP-3 production in the HTM-35D cells. Levels of the pro-enzyme forms of MMP-1, MMP-9, and TIMP-1 were also enhanced, however to a much lesser extent. proMMP-2 and TIMP-2 production did not appear to be affected. Similarly, the phorbol ester TPA increased levels of proMMP-1, proMMP-3, proMMP-9, and TIMP-1, but was without effect on production of proMMP-2 and TIMP-2; when tested under these conditions, proMMP-1 levels (rather than proMMP-3) were most significantly increased by TPA in the HTM-35D cells.

Table 3

Effect of IL-1α and TPA on proMMP-1 and proMMP-3

production by HTM-35D cells

Test Agent	Per cent of Control*	
	proMMP-1	proMMP-3
Vehicle	100 ± 3 (n=31)	100 ± 2 (n=172)
25 ng/ml IL-1α	267 ± 11 (n=8)*	2179 ± 57 (n=8)*
5 ng/ml IL-1α	143 ± 10 (n=31)*	1844 ± 90 (n=158)*
25 ng/ml TPA	1894 ± 61 (n=8)*	880 ± 22 (n=8)*
5 ng/ml TPA	989 ± 33 (n=11)*	610 ± 21 (n=10)*

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Table 4

Effect of IL-1α and TPA on proMMP-2 and proMMP-9

production by HTM-35D cells

Test Agent	Per cent of Control*	
	proMMP-2	proMMP-9
Vehicle	100 ± 4 (n=8)	100 ± 3 (n=11)
25 ng/ml IL-1α	101 ± 4 (n=8)	324 ± 27 (n=11)*
5 ng/ml IL-1α	79 ± 3 (n=8)	254 ± 22 (n=11)*
25 ng/ml TPA	93 ± 5 (n=8)	685 ± 84 (n=11)*
5 ng/ml TPA	91 ± 9 (n=8)	581 ± 47 (n=11)*

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Table 5

Effect of IL-1α and TPA on TIMP-1 and TIMP-2 production by HTM-35D cells

Test Agent	Per cent of Control*	
	TIMP-1	TIMP-2
Vehicle	100 ± 2 (n=14)	100 ± 3 (n=8)
25 ng/ml IL-1α	258 ± 17 (n=14)*	99 ± 7 (n=8)
5 ng/ml IL-1α	241 ± 18 (n=14)*	86 ± 4 (n=8)
25 ng/ml TPA	359 ± 29 (n=14)*	86 ± 6 (n=8)
5 ng/ml TPA	371 ± 28 (n=14)*	65 ± 3 (n=8)*

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Effect of IL-1α on proMMP-3 expression in TM cells from non-glaucomatous and glaucomatous donors

Five established non-glaucomatous HTM cell lines and five established glaucomatous HTM cell lines, from donors of various ages, were incubated with and without 5 ng/ml IL-1α, after which changes in secreted proMMP-3 levels were assessed by ELISA of the supernatants from the treated cell monolayers.

Table 6 lists the responses seen for each of the TM lines tested. All cell lines tested responded vigorously to a 24 hr challenge with 5 ng/ml IL-1α; response levels ranged from a low of approximately ten fold increase over basal (HTM-79 cells) to a nearly 40 fold increase (GTM-81C cells). Although response levels varied, no correlation could be noted for level of response with regard to donor age or to whether donors were "normal" or "glaucomatous" in these studies.

Table 6

Effect of IL-1α on proMMP-3 production by TM cells

CELL LINE	DONOR AGE	TEST AGENT(S)	
		Vehicle	5 ng/ml IL-1α
(Non-glaucomatous)			
HTM-35D	6 mos.	100 ± 1 (n=198)	1878 ± 81 (n=198)*
HTM-16A	18 yrs.	100 ± 4 (n=24)	1610 ± 112 (n=24)*
HTM-75C	85 yrs.	100 ± 5 (n=12)	2578 ± 319 (n=12)*
HTM-79	58 yrs.	100 ± 2 (n=12)	983 ± 130 (n=12)*
TM 332/344	47 yrs.	100 ± 4 (n=12)	1348 ± 110 (n=12)*
(Glaucomatous)			
GTM-23D	67 yrs.	100 ± 6 (n=12)	1659 ± 137 (n=12)*
GTM-76D	76 yrs.	100 ± 4 (n=12)	1205 ± 196 (n=12)*
GTM-81C	88 yrs.	100 ± 6 (n=12)	3827 ± 324 (n=12)*
GTM-83C	75 yrs.	100 ± 5 (n=12)	1582 ± 167 (n=12)*
GTM-85B	85 yrs.	100 ± 8 (n=12)	1363 ± 145 (n=12)*

Values are mean ± SEM, expressed as per cent of vehicle control

*$p < 0.05$ vs. respective vehicle controls.

Pharmacological determination of signaling pathway(s) involved in IL-1α-activated proMMP-3 expression

TM cells were incubated 24 hours with IL-1α (5 ng/ml) and with or without inhibitors of critical components (enzymes) of the known signaling pathways (see Figure 3). Resulting changes in secreted MMP-3 were then assessed by ELISA in HTM cell supernatants. Inhibitors used included such agents as NF-κB SN50 (a cell-permeable peptide which inhibits translocation of active NF-κB complexes into the nucleus; Abate and Schroder, 1998), indomethacin (cyclooxygenase inhibitor which, at high concentrations, can also inhibit lipoxygenases, therefore useful for determination of arachidonic acid cascade involvement; Maclouf et al, 1977; Shen and Winter, 1977), and SR11302 (a retinoid compound reported to inhibit AP-1 activation; Fanjul et al, 1994). Finally, inhibitors of other enzymes, known to be involved in signaling events "upstream" to the three mechanisms listed, were also tested against 5 ng/ml IL-1α in order to further delineate the participation (or lack of participation) of these pathways. Each inhibitor was initially tested at concentrations approximately 10 fold greater than their published IC_{50} or at the highest allowable concentration that was not toxic to the cells during the treatment periods (as determined by neutral red uptake). Incubations with all inhibitors were concurrent to incubation with IL-1α.

Table 7 lists those enzyme inhibitors that exhibited less than 10% suppression of IL-1α-induced proMMP-3 production by HTM-35D cells. In particular, inhibitors of the NF-κB pathway, as well as inhibitors of enzymes

participating in the generation of arachidonic acid, were without effect in this system, as were inhibitors of several upstream enzymes. For example, inhibitors of the enzymes casein kinase II, phospholipase A2, lipoxygenase, cyclooxygenase, sphingomyelinase, and certain subtypes of protein kinase C were without significant effect.

Conversely, coincubation with inhibitors of MEK, p38 MAPK, and PKCμ enzymes significantly suppressed IL-1α-induced accumulation of proMMP-3 in HTM-35D cell supernatants, as did the AP-1 sequestor SR11302, indicating a role for these enzymes and for the AP-1 transcription factor in MMP-3 production by HTM cells. Results of single, high concentrations of these agents are listed in Table 8. The effects of three of these agents (Gö6976, SB202190, and SR11302) were further explored and shown to be dose-dependent; typical dose-response curves are depicted in Figures 15, 16, and 17.

Based on these results, the AP-1 pathway was identified as being the key mechanism for proMMP-3 production by HTM-35D cells. Therefore, in order to verify the predominance of AP-1, the effect of a single, high dose (1 μM) SR11302 on IL-1α-induced proMMP-3 production was compared using the same five HTM lines from non-glaucomatous donors and five HTM lines from glaucomatous donors which had previously been tested for response to IL-1α. Results of these assays are summarized in Table 9; in each case, the agent SR11302 significantly inhibited the ability of IL-1α (5 ng/ml) to promote proMMP-3 production by the various cell lines.

Effect of IL-1α on MMPs in other ocular cells

Finally, it has been demonstrated by other researchers that many types of MMPs are also present in other ocular cells/tissues, such as MMP-1, -2, -7, and -9 in cornea (Berman, 1993; Fini et al, 1992), MMP-2 in sclera (Rada and Brenza, 1995), MMP-1, -2, -3, and –9 in ciliary muscle (Weinreb et al, 1997), and MMP-1, -2, and –3 in optic nerve head (Yan et al, 2000). Therefore, a brief survey of other available ocular cell lines was conducted in order to determine if (1) such cells also respond to IL-1α by an increased production of MMP-3, and (2) if such increases are also mediated via the AP-1 pathway. Results of these limited studies are listed in Table 10. These results confirm that tissues such as the lamina cribosa (LC), ciliary muscle (HCM), and fibroblasts from the corneal-scleral region (HCSF) also exhibit a robust increase in proMMP-3 production following a 24 hour exposure to IL-1α (5 ng/ml). However, these increases may be independent of AP-1, based on the observed lack of response to SR11302.

Table 7

Enzyme inhibitors exhibiting <10% suppression of HTM proMMP-3 production

ENZYME CATEGORY	INHIBITOR(S) TESTED*	CONCENTRATION USED
Casein kinase II	5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB)	10 μM
NFκB	Diethyldithiocarbamate (DDC)	100 μM
	NFκB SN50	15-20 μM
Phospholipase	AACOCF3	10 μM
	12-epi-scalaradial	1 μM
	Indomethacin	10 μM
Lipoxygenase	Curcumin	0.5 mM
Cyclooxygenase	Indomethacin	10 μM
	Curcumin	0.5 mM
Sphingomyelinas	Fumonisin B1	300 nM
PKC α, β, γ, δ, ζ	Bisindolylmaleimide 1 (Bis 1)	100 nM
α, β, γ, δ, ε	Gö 6983	500 nM

*vs. stimulation of HTM-35D cells with 5 ng/ml IL-1α for 24 hrs; mean ± SEM

Table 8

Enzyme inhibitors exhibiting >10% suppression of

HTM proMMP-3 production

ENZYME CATEGORY	INHIBITOR TESTED	CONCENTRATION USED	% INHIBITION OF proMMP-3 PRODUCTION*
MEK	PD098,059	100 μM	30 ± 13 (n=8)**
p38 MAPK	SB202190	100 nM	66 ± 3 (n=20)***
	SB203580	5 μM	78 ± 4 (n=6)***
PKCα, β, μ	Gö 6976	200 nM	64 ± 4 (n=20)***
Jun-Fos (AP-1)	SR11302	1 μM	94 ± 5 (n=13)***

*vs. stimulation of HTM-35D cells with 5 ng/ml IL-1α for 24 hrs; mean ± SEM.

**$p < 0.1$ or ***$p < 0.05$ vs IL-1α-treated controls.

Figure 15

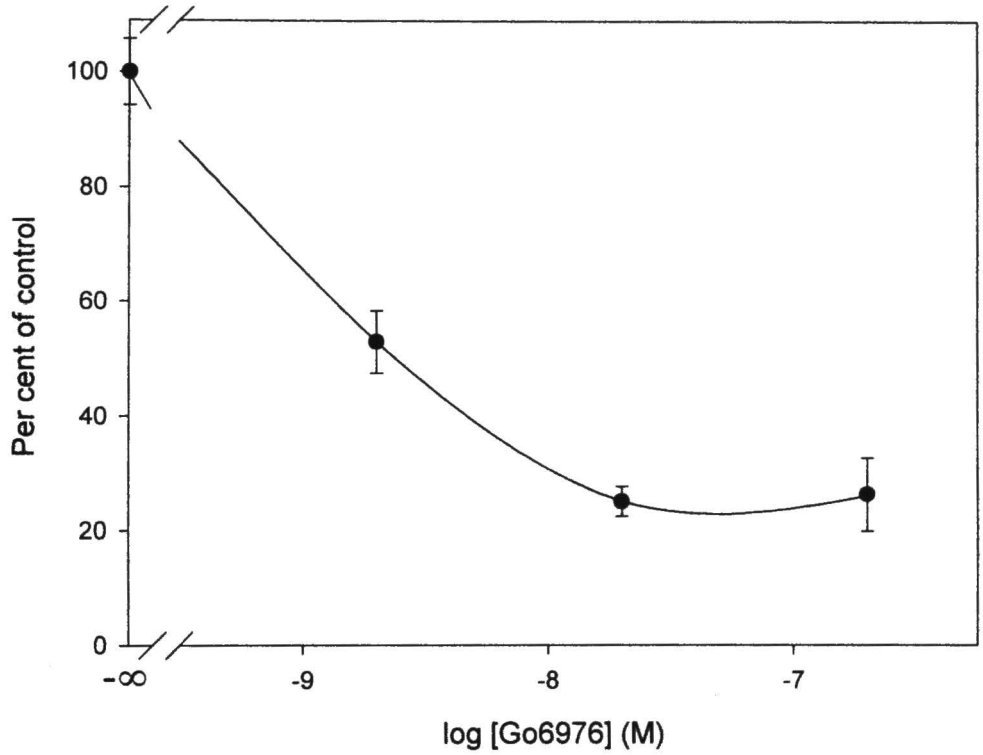


Figure 15: Effect of the PKC inhibitor Gö6976 on IL-1α-induced HTM-35D proMMP-3 production.

Figure 16

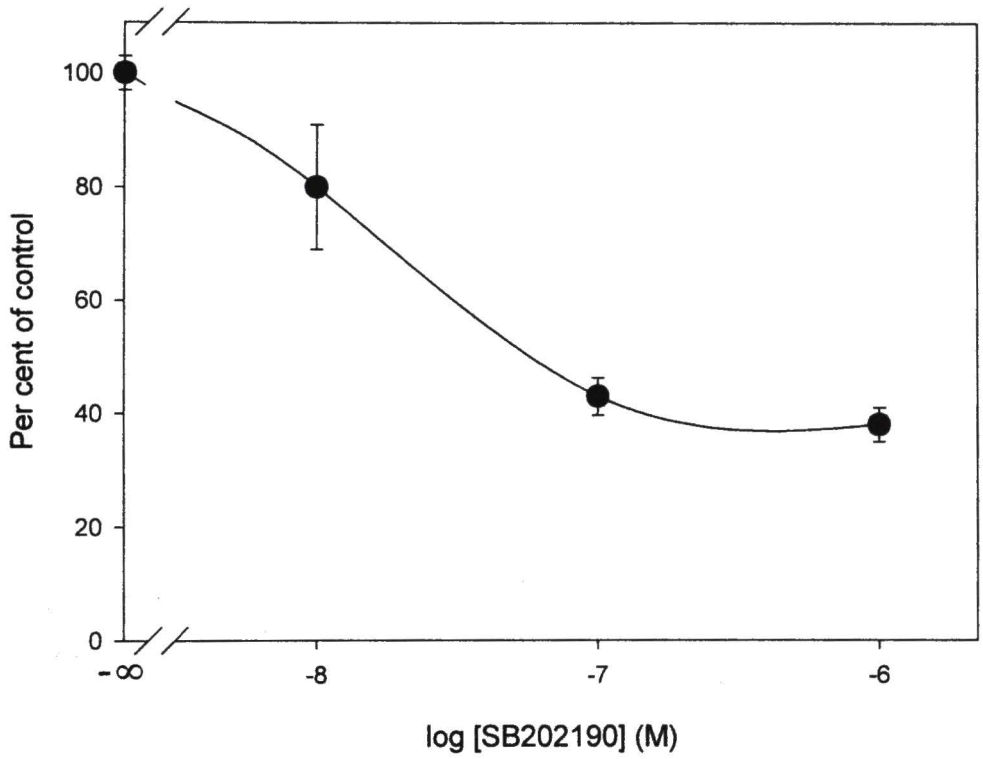


Figure 16: Effect of the p38 inhibitor SB202190 on IL-1α-induced HTM-35D proMMP-3 production.

Figure 17

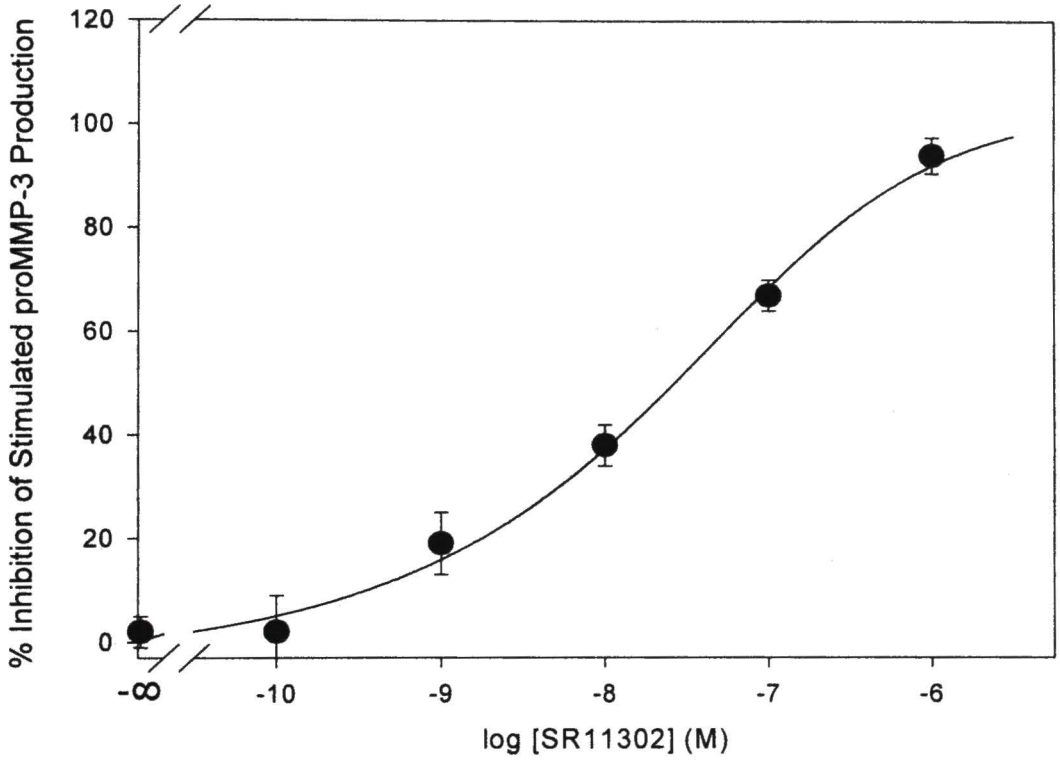


Figure 17: Effect of the AP-1 sequestor SR11302 on IL-1α-induced HTM-35D proMMP-3 production.

Table 9

Effect of SR11302 on proMMP-3 production by HTM cells

CELL LINE	DONOR AGE	TEST AGENT(S)		
		Vehicle	5 ng/ml IL-1α	5 ng/ml IL-1α + 1 μM SR11302
(Non-glaucomatous)				
HTM-35D	6 mos.	100 ± 5 (n=13)	1737 ± 279 (n=13)*	322 ± 62 (n=13)**
HTM-16A	18 yrs.	100 ± 8 (n=12)	1873 ± 139 (n=12)*	165 ± 19 (n=12)**
HTM-75C	85 yrs.	100 ± 5 (n=12)	2578 ± 319 (n=12)*	1118 ± 248 (n=12)**
HTM-79	58 yrs.	100 ± 2 (n=12)	983 ± 130 (n=12)*	259 ± 34 (n=12)**
TM 332/344	47 yrs.	100 ± 4 (n=12)	1348 ± 110 (n=12)*	231 ± 35 (n=12)**
(Glaucomatous)				
GTM-23D	67 yrs.	100 ± 6 (n=12)	1659 ± 137 (n=12)*	707 ± 93 (n=12)**
GTM-76D	76 yrs.	100 ± 4 (n=12)	1205 ± 196 (n=12)*	534 ± 67 (n=12)**
GTM-81C	88 yrs.	100 ± 6 (n=12)	3827 ± 324 (n=12)*	1332 ± 180 (n=12)**
GTM-83C	75 yrs.	100 ± 5 (n=12)	1582 ± 167 (n=12)*	617 ± 96 (n=12)**
GTM-85B	85 yrs.	100 ± 8 (n=12)	1363 ± 145 (n=12)*	209 ± 45 (n=12)**

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

**$p < 0.05$ vs. respective IL-1α-treated controls .

Table 10

Effect of SR11302 on proMMP-3 production by other ocular cell types

CELL LINE	DONOR AGE	TEST AGENT(S)		
		Vehicle	5 ng/ml IL-1α	5 ng/ml IL-1α + 1 μM SR11302
(Non-glaucomatous)				
LC	82	100 ± 5 (n=9)	2873 ± 434 (n=9)*	2839 ± 381 (n=9)
HCSF	82	100 ± 2 (n=9)	1859 ± 226 (n=9)*	1792 ± 232 (n=9)
HCM	67	100 ± 7 (n=9)	2376 ± 430 (n=9)*	1825 ± 382 (n=9)
(Glaucomatous)				
LC	82	100 ± 8 (n=9)	2210 ± 121 (n=9)*	2459 ± 233 (n=9)

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Effects of AP-1 activators on proMMP-3 expression

Few agents are mentioned in the literature as being "AP-1 activators"; however, the compounds β-naphthoflavone, 3-methylcholanthrene, and tert-butylhydroquinone (Ainbinder, 1997), have been reported as possessing that ability. Accordingly, each of these agents was tested for potential ability to stimulate proMMP-3 production by HTM cells. Each agent was tested at a concentration (10 μM) reported to increase AP-1 levels in the same ten HTM cell lines (five from non-glaucomatous donors, five from glaucomatous donors) that were used for evaluation of IL-1α. Agents were tested in parallel with IL-1α (5 ng/ml) as an internal standard of comparison. Results from these assays are given in Tables 11 (β-naphthoflavone), 12 (3-methylcholanthrene), and 13 (t-BHQ).

In contrast, contrary to the MMP-3 stimulation by IL-1α, the "AP-1 activators" did not universally stimulate proMMP-3 production in the various HTM cell lines, nor was the response to these agents as robust as that seen for IL-1α. Only three (HTM-35D, HTM-16A, and TM332/344) out of five "non-glaucomatous" lines responded to β-naphthoflavone and to 3-methylcholanthrene; none of the five "glaucomatous" lines responded to either of these agents. Response to t-BHQ was somewhat more consistent, in that four out of the five "non-glaucomatous" lines responded (see Table 13) and two (GTM-81C and GTM-85B) out of five "glaucomatous" lines responded. The

response to t-BHQ was dose-dependent over a range of 0 to 100 μM in HTM-35D cells (Figure 18).

Table 11

Effect of β-naphthoflavone (β-NF) on proMMP-3 production by HTM cells

CELL LINE	DONOR AGE	TEST AGENT(S)		
		Vehicle	5 ng/ml IL-1α	10 μM β-NF
(Non-glaucomatous)				
HTM-35D	6 mos.	100 ± 9 (n=14)	1883 ± 134 (n=12)*	241 ± 28 (n=14)*
HTM-16A	18 yrs.	100 ± 4 (n=15)	1338 ± 155 (n=15)*	164 ± 11 (n=15)*
HTM-75C	85 yrs.	100 ± 5 (n=12)	2578 ± 379 (n=12)*	86 ± 5 (n=12)
HTM-79	58 yrs.	100 ± 1 (n=12)	983 ± 130 (n=12)*	107 ± 6 (n=12)
TM332/344	47 yrs.	100 ± 4 (n=12)	1348 ± 100 (n=12)*	179 ± 11 (n=12)*
(Glaucomatous)				
GTM-23D	67 yrs.	100 ± 6 (n=12)	1659 ± 137 (n=12)*	176 ± 57 (n=12)
GTM-76D	76 yrs.	100 ± 4 (n=12)	1205 ± 196 (n=12)*	93 ± 11 (n=12)
GTM-81C	88 yrs.	100 ± 6 (n=12)	3827 ± 324 (n=12)*	93 ± 4 (n=12)
GTM-83C	75 yrs.	100 ± 5 (n=12)	1582 ± 167 (n=12)*	88 ± 5 (n=12)
GTM-85B	85 yrs.	100 ± 8 (n=12)	1363 ± 145 (n=12)*	108 ± 9 (n=12)

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Table 12

Effect of 3-methylcholanthrene (3-MC) on proMMP-3 production by HTM cells

CELL LINE	DONOR AGE	TEST AGENT(S)		
		Vehicle	5 ng/ml IL-1α	10 μM 3-MC
(Non-glaucomatous)				
HTM-35D	6 mos.	102 ± 9 (n=13)	2506 ± 257 (n=13)*	271 ± 21 (n=13)*
HTM-16A	18 yrs.	100 ± 6 (n=11)	1826 ± 176 (n=11)*	136 ± 11 (n=11)*
HTM-75C	85 yrs.	100 ± 5 (n=12)	2578 ± 379 (n=12)*	87 ± 4 (n=12)
HTM-79	58 yrs.	100 ± 1 (n=12)	983 ± 130 (n=12)*	100 ± 3 (n=12)
TM332/344	47 yrs.	100 ± 4 (n=12)	1348 ± 110 (n=12)*	160 ± 5 (n=12)*
(Glaucomatous)				
GTM-23D	67 yrs.	100 ± 6 (n=12)	1659 ± 137 (n=12)*	115 ± 23 (n=12)
GTM-76D	76 yrs.	100 ± 4 (n=12)	1205 ± 196 (n=12)*	89 ± 9 (n=12)
GTM-81C	88 yrs.	100 ± 6 (n=12)	3827 ± 324 (n=12)*	99 ± 7 (n=12)
GTM-83C	75 yrs.	100 ± 5 (n=12)	1582 ± 167 (n=12)*	90 ± 5 (n=12)
GTM-85B	85 yrs.	100 ± 8 (n=12)	1363 ± 145 (n=12)*	77 ± 7 (n=12)

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Table 13

Effect of tert-butylhydroquinone (t-BHQ) on proMMP-3 production by HTM cells.

CELL LINE	DONOR AGE	TEST AGENT(S)		
		Vehicle	5 ng/ml IL-1α	10 μM t-BHQ
(Non-glaucomatous)				
HTM-35D	6 mos.	100 ± 3 (n=30)	1706 ± 122 (n=30)*	211 ± 17 (n=33)*
HTM-16A	18 yrs.	100 ± 6 (n=14)	2944 ± 356 (n=14)*	227 ± 26 (n=14)*
HTM-75C	85 yrs.	100 ± 5 (n=12)	2578 ± 379 (n=12)*	124 ± 11 (n=12)
HTM-79	58 yrs.	100 ± 1 (n=12)	983 ± 130 (n=12)*	169 ± 10 (n=12)*
TM332/344	47 yrs.	100 ± 4 (n=12)	1348 ± 110 (n=12)*	204 ± 7 (n=12)*
(Glaucomatous)				
GTM-23D	67 yrs.	100 ± 6 (n=12)	1659 ± 137 (n=12)*	77 ± 10 (n=12)
GTM-76D	76 yrs.	100 ± 4 (n=12)	1205 ± 196 (n=12)*	90 ± 12 (n=12)
GTM-81C	88 yrs.	100 ± 6 (n=12)	3827 ± 324 (n=12)*	333 ± 53 (n=12)*
GTM-83C	75 yrs.	100 ± 5 (n=12)	1582 ± 167 (n=12)*	164 ± 37 (n=12)
GTM-85B	85 yrs.	100 ± 8 (n=12)	1363 ± 145 (n=12)*	130 ± 9 (n=12)*

Values are mean ± SEM, expressed as per cent of vehicle control.

*$p < 0.05$ vs. respective vehicle controls.

Figure 18

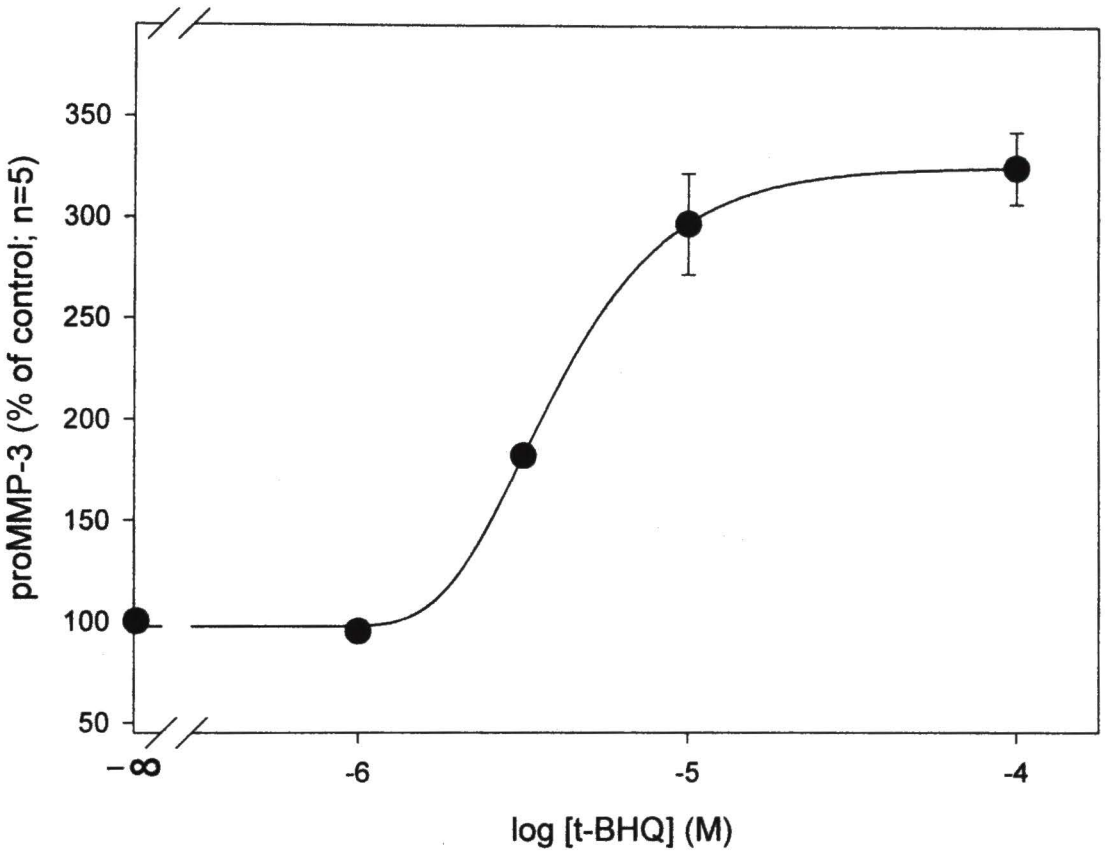


Figure 18: Effect of t-BHQ on HTM-35D proMMP-3 production.

CHAPTER 5

PHAGOCYTOSIS ASSAY RESULTS

Effect of PDGF and TPA on TM phagocytosis

The ability of a known phagocytosis-inducing agent (platelet-derived growth factor; PDGF) to enhance uptake of foreign substances was tested using human TM cell cultures. This step was important in order to establish that the cells were capable of phagocytic activity similar to that reported by other laboratories. For these experiments, PDGF-treated HTM cell monolayers were tested using a commercially available kit that employs fluoresceinated bioparticles (*E. coli*) as a means to track cellular phagocytic activity. These bioparticles had previously been shown to be readily taken up by cultured TM cells, as shown in the electron micrograph depicted in Figure 19 (McNatt et al, unpublished observations). In addition to PDGF, another known stimulator of phagocytosis, phorbol ester (TPA), was tested to further validate cellular responses.

The HTM-16A cell line was used for these phagocytosis studies because it more consistently and readily phagocytosed the bioparticles, as compared to other HTM lines tested (including the HTM-35D line which was primarily used for MMP studies; data not shown). PDGF-BB was used at a high concentration (50 ng/ml) in order to assure potential for response, based on a published report

which demonstrated dose-dependent phagocytic activity by TM cells exposed to 5-40 ng/ml PDGF (Tamura et al, 1989). HTM-16A cells treated for a period of 20 hours with 50 ng/ml PDGF-BB responded with an approximate 2.5 fold increase in phagocytic activity, compared to unstimulated cells (Figure 20). Additionally, cells treated during the same 20 hour period with 5 ng/ml TPA also responded with an increase in bioparticle uptake, to a greater degree ($\geq$ 3 fold) than that seen for PDGF-BB. The effect of TPA on phagocytic actvity was apparently mediated at least in part by protein kinase C (PKC), since the PKC inhibitor Bisindolylmaleimide I (Bis I; 100 nM) partially blocked the effect of TPA when incubated concurrently with the phorbol ester (Figure 21).

Figure 19

Figure 19: Electron micrograph of HTM cells. Phagocytosed bioparticles appear as electron dense regions (arrows) within the cells.

Figure 20

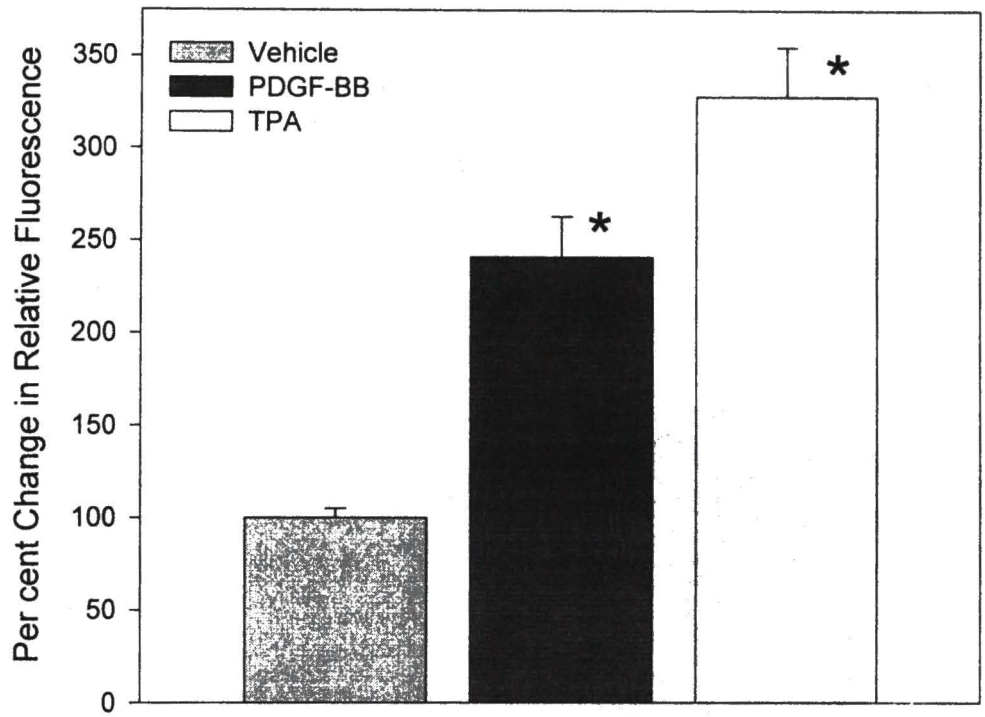


Figure 20: Effect of PDGF-BB (50 ng/ml) and TPA (5 ng/ml) on uptake of fluorescent bioparticles by HTM cells (mean $\pm$ SEM; n = 20; average value for vehicle-treated controls defines 100%; * denotes $p < 0.05$ as compared to vehicle controls).

Figure 21

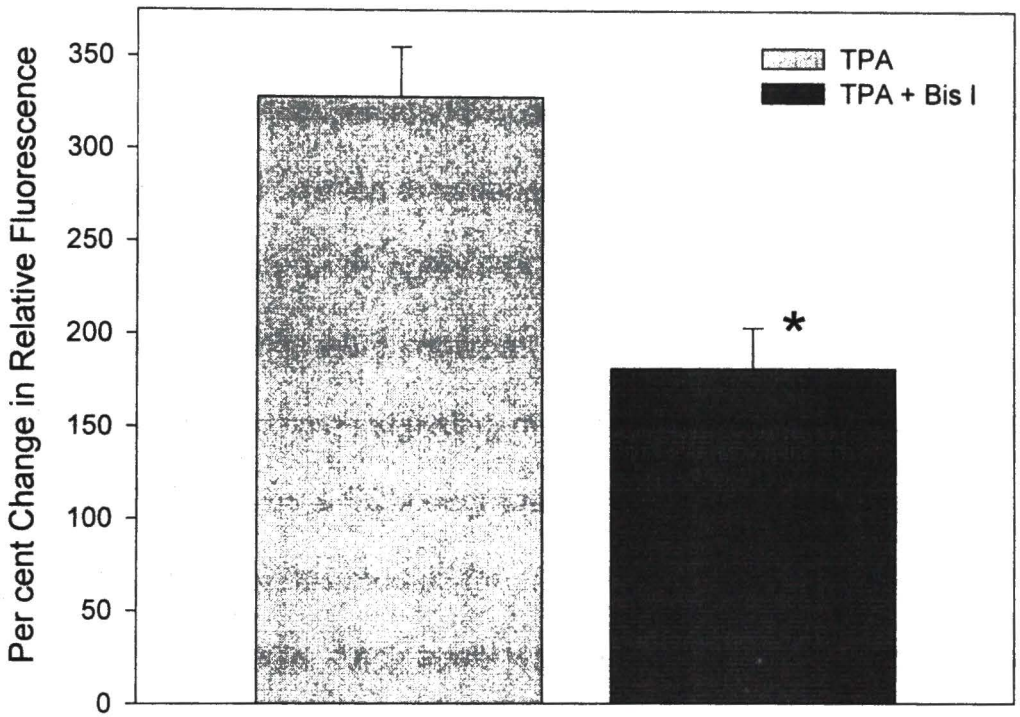


Figure 21: Ability of the non-specific PKC inhibitor Bis I (100 nM) to block TPA (5 ng/ml) –induced uptake of fluorescent bioparticles by HTM cells (mean ± SEM; n = 12; average value for vehicle-treated controls defines 100%; * denotes $p < 0.05$ as compared to TPA-treated controls).

Effect of IL-1α on TM phagocytosis

There are no published reports of the effect of IL-1α on TM phagocytosis, therefore it is unknown whether a portion of IL-1α's outflow-increasing effects may also be due to alterations in TM phagocytic capability. For these experiments, TM phagocytosis in response to IL-1α was assessed using the same assay format (fluoresceinated bioparticles). A 20-hour challenge of HTM-16A cells with 5 ng/ml IL-1α led to increased phagocytic activity at a level comparable to that seen for 50 ng/ml PDGF-BB (Figure 22).

These experiments were important in order to establish whether IL-1α-treated TM cells use common signaling mechanism(s) for the two activities of interest, i.e. phagocytosis & ECM degradation via MMP production. Two inhibitors were tested in this model: the NF-κB SN50 inhibitor peptide and the AP-1 sequestor SR11302. Each agent was tested at concentrations similar to that used for their evaluation in the MMP assays (20 μM NF-κB SN50, 1 μM SR11302) vs 5 ng/ml IL-1α; as with MMP assays, inhibitors were added concurrently to IL-1α when dosing the cells.

Results of these studies are summarized in Figure 23. Similar to that seen for IL-1α-stimulated proMMP-3 production, HTM-16A phagocytic activity due to IL-1α appears to be mediated, at least in part, by AP-1. Coincubation of the cells with both SR11302 and IL-1α led to a reduction to approximately 1.5 fold in relative fluorescence over unstimulated controls from a high level of

approximately 2.5 fold for IL-1α alone. Interestingly, coincubation of the HTM-16A cells with both IL-1α and NF-κB SN50 led to an increase in relative fluorescence as compared to that for IL-1α alone; such effect was not due to autofluorescence of the peptide inhibitor, based on testing with the peptide alone in cell- and bioparticle-free wells (data not shown). Furthermore, the inhibitor peptide itself was capable of stimulating phagocytic activity, however such effects were weak and variable (data not shown).

Finally, since AP-1 appeared to be a common signaling pathway utilized by IL-1α for both MMP production and phagocytic activity in HTM cells, the AP-1 activator agent t-BHQ was tested in the phagocytosis model. At a concentration of 10 μM, t-BHQ stimulated HTM-16A phagocytic activity by an average of approximately 1.75 fold compared to unstimulated controls (Figure 24).

Figure 22

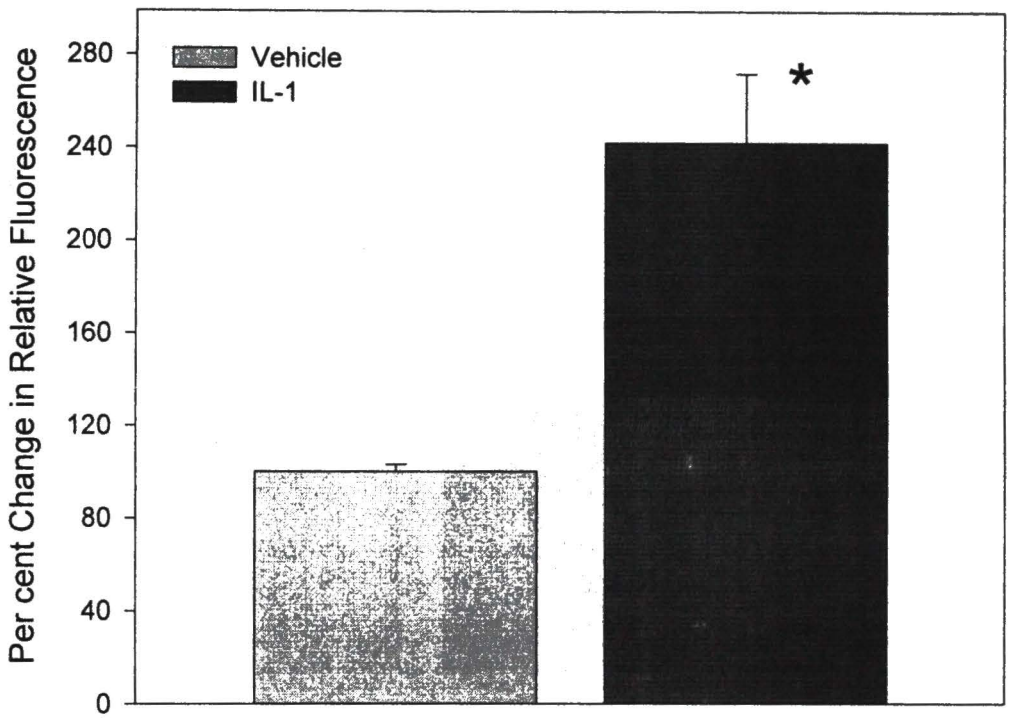


Figure 22: Effect of IL-1α (5 ng/mL) on uptake of fluorescent bioparticles by HTM cells (mean ± SEM; n = 16; average value for vehicle-treated controls defines 100%; * denotes $p < 0.05$ as compared to vehicle controls).

Figure 23

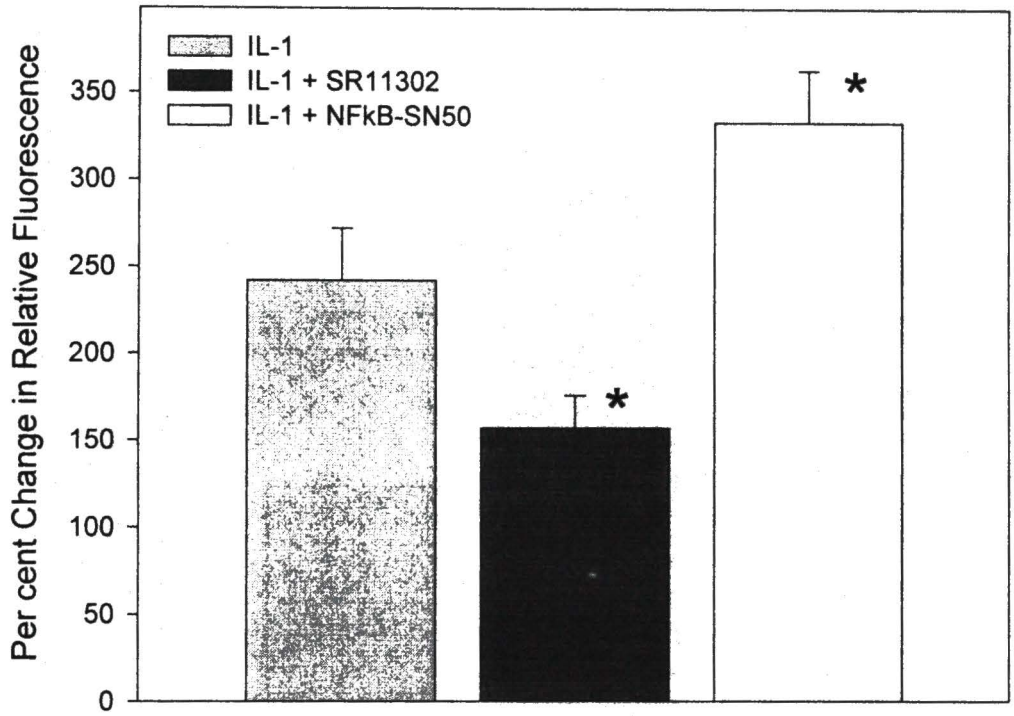


Figure 23: Ability of the AP-1 sequestor SR11302 (1 μM) and inhibitor peptide NFkB-SN50 (20 μM) to block IL-1α (5 ng/ml) –induced uptake of fluorescent bioparticles by HTM cells (mean ± SEM; n = 9; average value for vehicle-treated controls defines 100%; * denotes $p < 0.05$ as compared to IL-1α-treated controls).

Figure 24

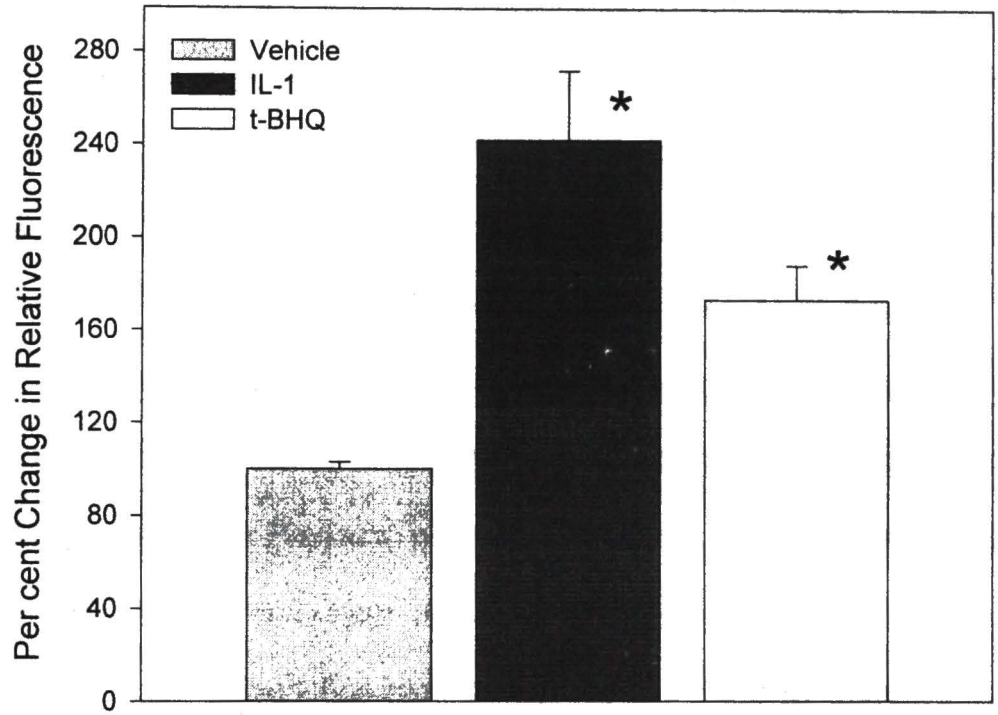


Figure 24: Effect of IL-1α (5 ng/ml) and t-BHQ (10 μM) on uptake of fluorescent bioparticles by HTM cells (mean ± SEM; n = 12; average value for vehicle-treated controls defines 100%; * denotes $p < 0.05$ as compared to vehicle controls).

CHAPTER 6

PERFUSED ORGAN CULTURE RESULTS

Effect of t-BHQ on outflow facility in non-glaucomatous eyes

Perfused ocular anterior segments were utilized in order to investigate the effect of AP-1 activation on outflow facility from human donor eyes. Study data were considered to be valid for analysis only if the perfused tissues were determined to be still viable at termination of the studies (see Methods section for assay details). t-BHQ was chosen as the test agent because it gave the most consistent results, with respect to proMMP-3 production, in the assays utilizing HTM cell cultures. IL-1α itself was not tested because, as stated previously, its effect on outflow had already been demonstrated in a similar model (Bradley et al, 1998). Additionally, the labor-intensiveness of the perfusion model precluded testing of no more than a minimal number of agents.

An overall measurable increase in outflow facility was detected in nearly all viable anterior segments challenged with t-BHQ (10 μM). Figures 25 and 26 demonstrate this effect: Figure 25 shows the time course of change in flow rate from six paired anterior segments from "normal" (no record of diagnosed glaucoma) human donor eyes. In all but one of the pairs, perfusion with t-BHQ resulted in a measurable increase in outflow facility as compared to vehicle-treated fellow eyes and also prevented a trend towards a slow, steady decrease

in outflow which was almost invariably demonstrated by the vehicle-treated controls. A composite of these data is depicted in Figure 26. It can be seen that the average flow rate in t-BHQ-treated eyes was significantly different ($p < 0.05$) from vehicle-treated at 8, 24, 72, and 96 hrs post-treatment. At 96 hrs post-treatment, perfusion of the eyes was terminated and the perfused segments were fixed for microscopic examination of TM morphology.

Effect of t-BHQ on proMMP-3 content in perfusates

In order to demonstrate a potential causal link between the observed increase in outflow facility in the perfusion model and an increase in MMP activity, aliquots of perfusion medium were collected from the anterior segments treated with vehicle or with vehicle plus t-BHQ (10 μM). The aliquots were then analyzed for proMMP-3 content in the same manner as that used for analysis of cell culture supernatants, i.e. ELISA. After adjusting for differences in flow rate, the amount of proMMP-3 produced could then be calculated and expressed as ng/day. Results of these analyses are shown Figure 27. proMMP-3 levels appear to increase steadily within the first 48 hours of t-BHQ treatment, followed by a steady-state elevation up until at least 96 hours post-initial exposure to t-BHQ. These increases contrast with a relatively unchanging level of proMMP-3 seen in segments exposed solely to vehicle.

Effect of SR11302 on t-BHQ-mediated outflow

Furthermore, similar to that seen for proMMP-3 production and for phagocytic activity, the increase in flow rate generated by t-BHQ appears to be attributable to activation of AP-1, since concurrent incubation of the perfused segments with t-BHQ (10 μM) and the AP-1 sequestor SR11302 (100 nM) prevented the increase in outflow facility seen with t-BHQ alone (Figure 28). Suppression by SR11302 of the t-BHQ-mediated outflow increase was apparent as early as 24 hr post-treatment and was essentially 100% effective by 72 hr post-treatment. The suppressive effect persisted at least up until 96 hr, at which point the perfusion of the eyes was terminated and the segments were fixed for microscopic examination of TM morphology.

Effect of t-BHQ on IOP of glaucomatous eyes

Finally, in order to determine whether AP-1 activation could effectively increase outflow facility in POAG patients, t-BHQ was also tested in perfused glaucomatous human anterior segments. Unfortunately, glaucomatous eyes possessed an extremely low flow rate ($\leq$ 1 μL/min) and greatly-reduced viability in the constant pressure model, presumably due to their impaired outflow pathways. Therefore, glaucomatous eyes were evaluated using the constant flow model of perfusion and changes in IOP, rather than flow rate, were monitored. A summary of the results generated by these studies is depicted in Figure 29. Perfusion of the glaucomatous anterior segments with t-BHQ (10 μM) produced a measurable decrease in IOP over time, up to at least 72 hr post-

treatment with t-BHQ, as compared to untreated controls. These data suggest that, similar to that seen with non-glaucomatous anterior segments, t-BHQ – and, thus AP-1 activation – improves the outflow of aqueous humor in perfused human eyes.

Figure 25

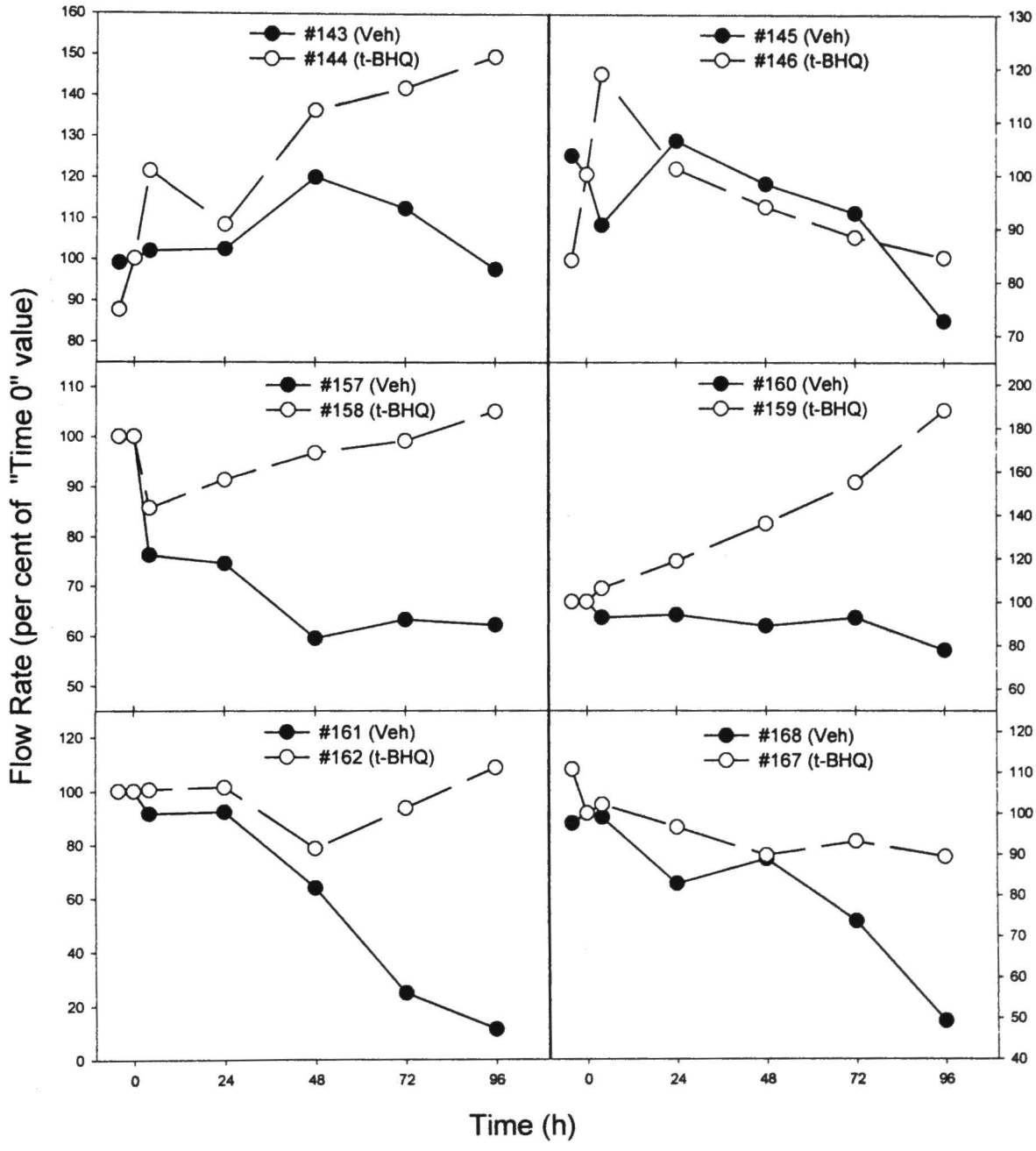


Figure 25: Effect of t-BHQ (10 μM) on outflow facility in individual pairs of perfused human anterior segments; flow rate at T = 0 defines "100%".

Figure 26

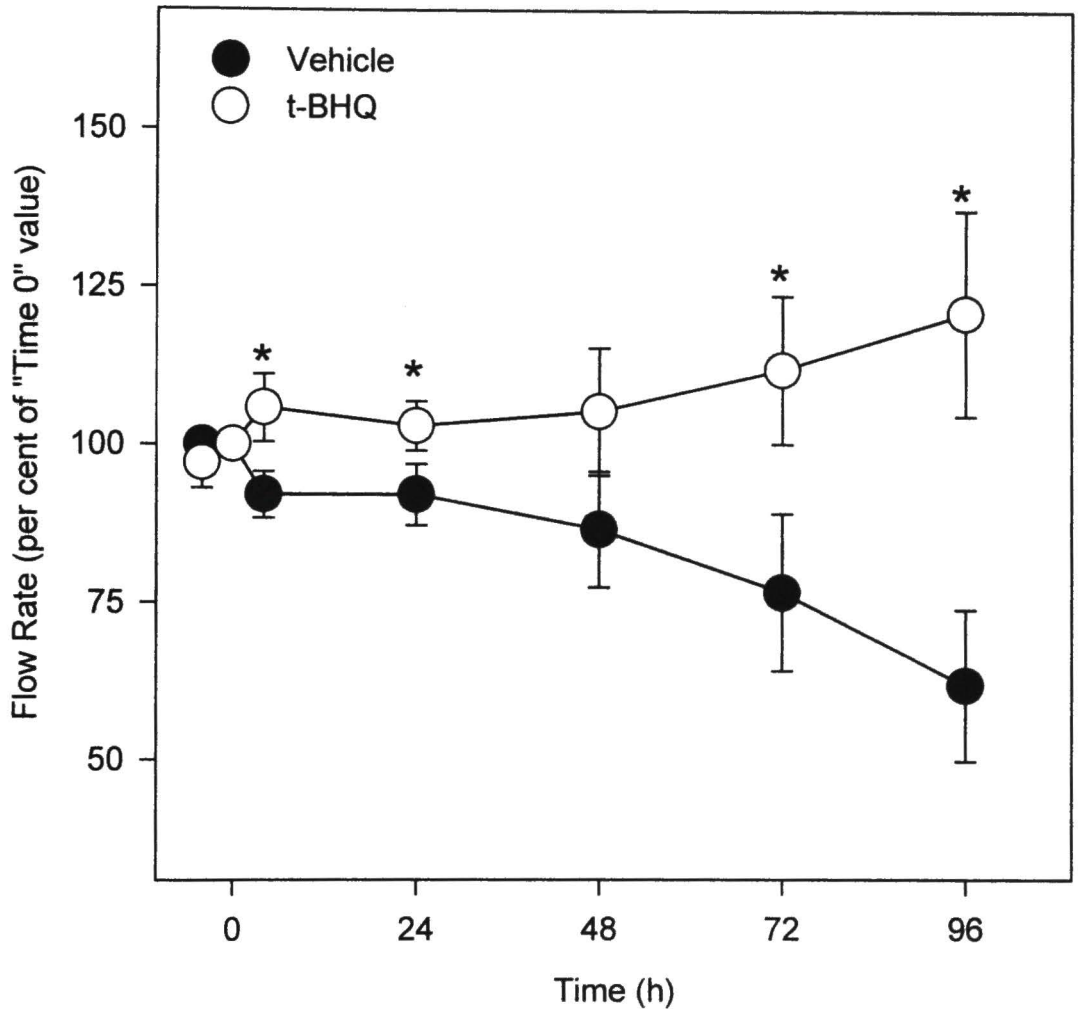


Figure 26: Average per cent effect of t-BHQ (10 μM) on outflow facility in perfused human anterior segments; flow rate at T = 0 defines "100%" (mean ± SEM of 6 paired eyes; * denotes p values < 0.05 as compared to vehicle controls).

Figure 27

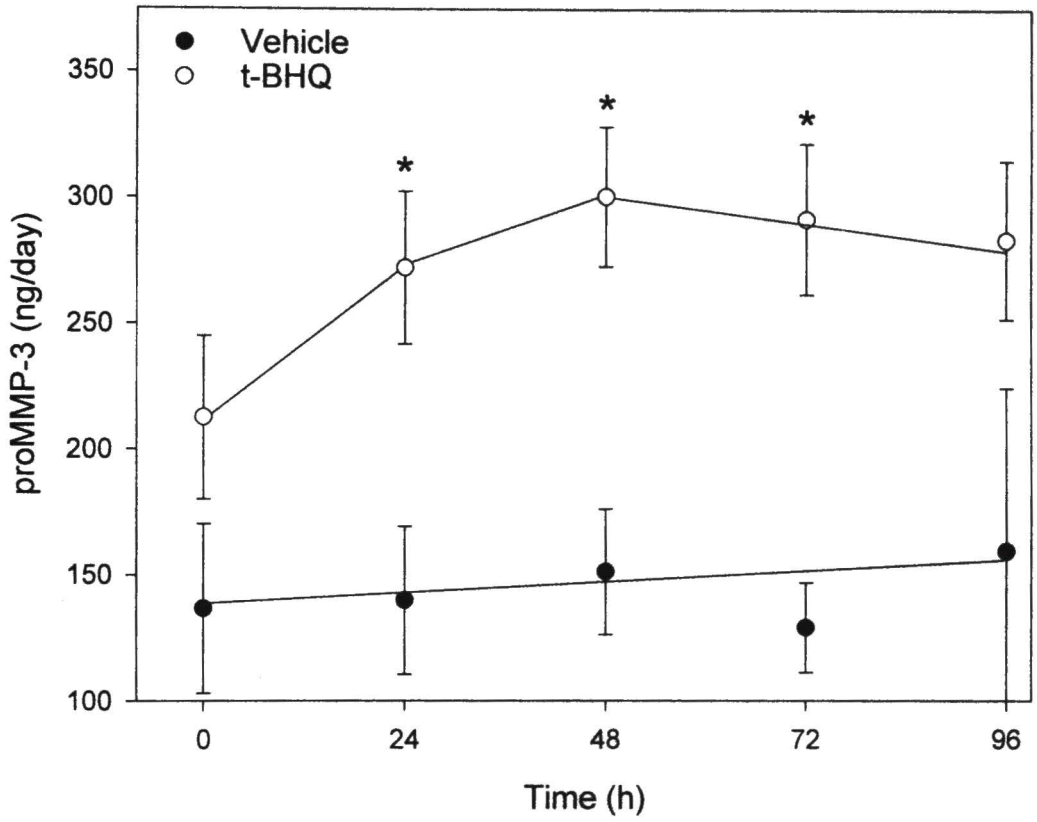


Figure 27: Time course of the effect of t-BHQ (10 μM) on proMMP-3 production in perfused human anterior segments (n = 4; * denotes p values < 0.05 as compared to vehicle controls).

Figure 28

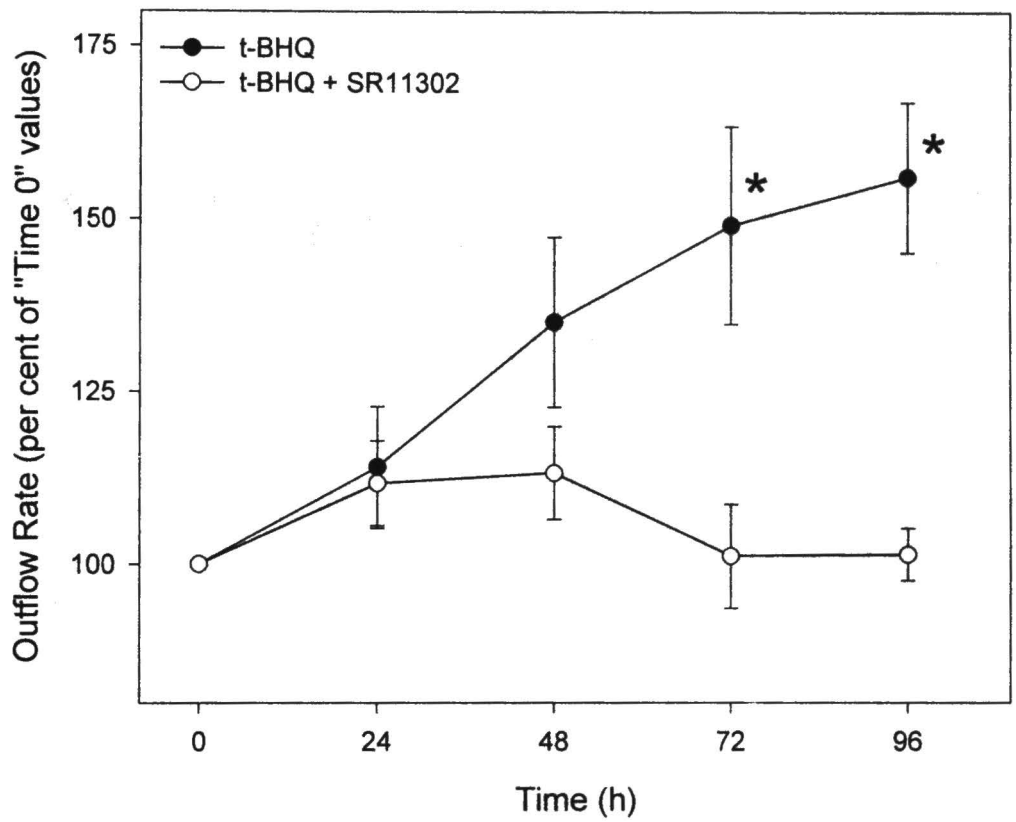


Figure 28: Time course of the ability of SR11302 (100 nM) to block t-BHQ (10 μM) –mediated increase in outflow facility from perfused human anterior segments. (Flow rate at T = 0 defines "100%"; * denotes $p < 0.05$ vs t-BHQ alone).

Figure 29

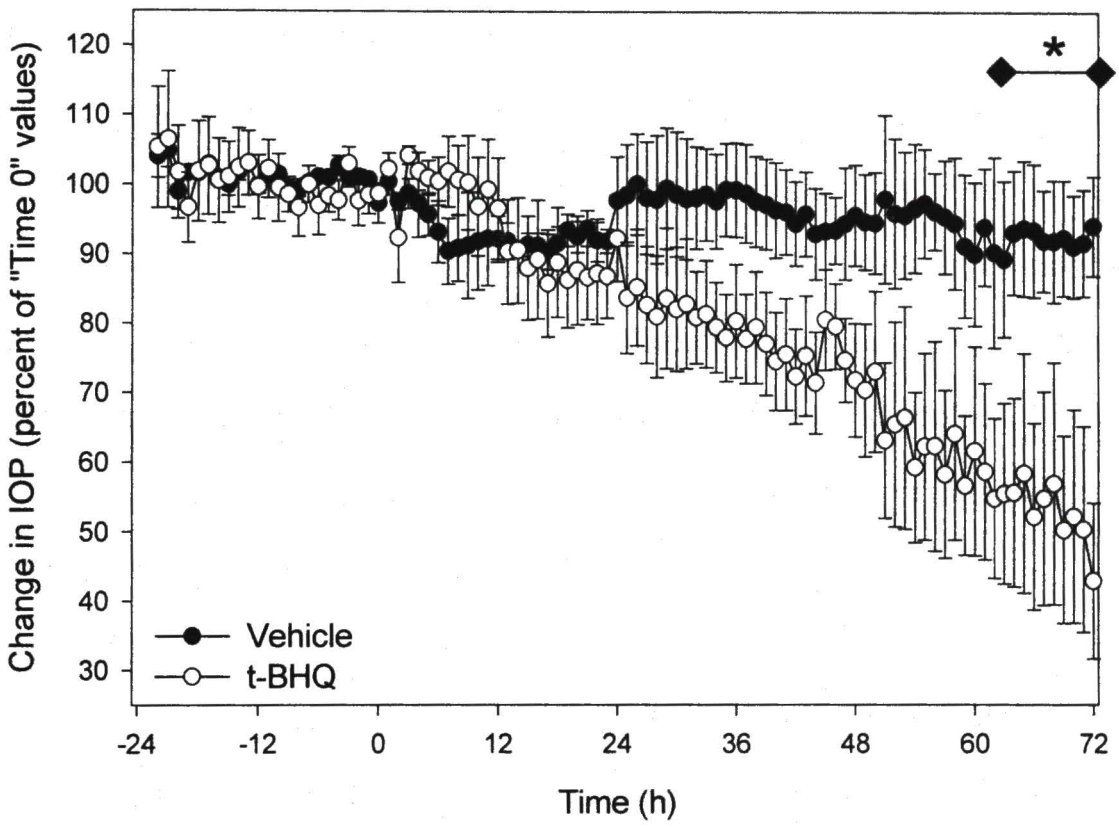


Figure 29: Effect of t-BHQ (10 μM) on intraocular pressure in perfused human glaucomatous anterior segments. IOP at T = 0, i.e. initiation of drug treatment, defines 100% (n = 3 for vehicle-treated and n = 4 for t-BHQ-treated tissues; * denotes $p < 0.05$ as compared to average vehicle controls).

CHAPTER 7

DISCUSSION

Basic characterization of TM cell cultures

The TM cell cultures used in these studies were carefully isolated from explants of the TM regions of post-mortem human donor eyes. Prior to their use, they were extensively characterized as TM based on morphology, cytoskeletal element expression, and extracellular matrix composition as previously described (Steely et al, 1992; Wilson et al, 1993; Clark et al, 1994). In addition, I further characterized these cells based on various second messenger signaling pathways.

Cyclase activity

Key second messenger signaling pathways appeared to be intact in the cultured TM cells used in these studies, as demonstrated by their similar responses to stimuli as compared to that published by other researchers for both TM cells and tissues. For example, the cultured TM cells responded vigorously to known stimulators of adenylyl cyclase activity. The direct activator forskolin, as well as the EP receptor agonist prostaglandin E_2 and the beta adrenergic receptor agonist isoproterenol each stimulated the production of cyclic AMP by the TM cell cultures. Responses were similar to that reported by other laboratories for human TM tissue (Busch et al, 1993; Friedman et al, 1999),

indicating functional adenylyl cyclase activity in these cells. Furthermore, the nitrovasodilator SNP stimulated production of cyclic GMP by the cells, indicating the presence of a functional soluble guanylyl cyclase; a similar effect of SNP has been reported by this laboratory for SV-40 transformed human TM cells (Pang et al, 1994).

Inositol phosphates' production

Phospholipase C activity was also readily detectable in these cells, based on cellular inositol phosphates' production in response to carbachol. Effects were dose-dependent and in good agreement with data previously published by this laboratory (Shade et al, 1996) and that of others' (WoldeMussie et al, 1990; Friedman et al, 1990). Additionally, an increase in IP production in the TM cells was noted when histamine was used as a stimulant, confirming a similar result reported by Friedman et al (1990) in primary human TM as well as results reported previously by this laboratory for an SV-40 transformed human TM cell line (Pang et al, 1994). TM cells also responded to stimulation with prostaglandin $F_{2\alpha}$ with an increase in IP production, similar to that seen with the same SV-40 transformed cells.

Intracellular calcium mobilization

Accumulation of the product inositol trisphosphate leads to release of calcium from intracellular stores (Alberts et al, 1989b). Thus an increase in TM PLC activity would be expected to be accompanied by a rise in free intracellular calcium levels. Measurable increases in human TM intracellular calcium levels

have been noted in response to a wide variety of agents, such as endothelin (Tao et al, 1998), prostaglandin $F_{2\alpha}$ (Anthony et al, 1998), natriuretic peptides (Pang et al, 1996), and histamine (WoldeMussie and Ruiz, 1992). Furthermore, a recent report by Steinhausen et al (2000) confirms results published previously by this laboratory (Shade et al, 1996) in that the muscarinic agonist carbachol was shown to increase calcium in human TM cells. Similar to those results, incubation with carbachol produced a dose-dependent increase in free intracellular calcium content in the current studies. Carbachol's effects on both PLC activity and intracellular calcium mobilization were likely mediated via a muscarinic receptor of the M_3 subtype, based on an antagonist potency profile of atropine > p-fHHSiD > pirenzepine.

These findings indicate that crucial signaling mechanisms remained functional in the TM cell cultures. Taken together with the confirmation of the presence of key extracellular matrix and cytoskeletal elements, it was concluded that results derived from these cells are consistent with those of other laboratories and that the responses noted are therefore likely representative of TM tissue in general.

Matrix metalloproteinase production

MMP subtype expression by TM cells

Measurable basal levels of the pro-enzyme forms of MMP-1, MMP-2, MMP-3, and MMP-9, as well as of the inhibitors TIMP-1 and TIMP-2, were detected in the cultured cells. A twenty-four hour exposure of the cells to IL-1α

resulted in significant upregulation of the expression of proMMP-1, proMMP-3, proMMP-9, and TIMP-1, but not of proMMP-2, similar to that reported by Samples et al (1993); expression of TIMP-2 was unchanged. The lack of upregulation of MMP-2 and TIMP-2 is not surprising since the expression of both are known to be generally constitutive (Birkedal-Hansen et al, 1993; Borden and Heller, 1997) and therefore levels would be expected to remain constant for each.

Effects of IL-1α on production of MMP-1 and MMP-3 were time-dependent, with measurable increases occuring as early as 8 hours post-dosing of the TM cells, a time-frame consistent with that noted for other cell types (Borden and Heller, 1997). Response to IL-1α was consistently robust at 24 hours post-dosing; this is in good agreement with published data that demonstrated that measurable increases in outflow occured between 12 and 24 hours in perfused human anterior segments (Bradley et al, 1998). IL-1α effects on TM MMP-3 production were also dose-dependent, again corroborating results reported by other researchers (Samples et al, 1993; Magnino et al, 1999).

IL-1α stimulates MMP response in both "normal" and glaucomatous TM

IL-1α appeared to stimulate the production of proMMP-3 equally well in TM cells derived from both normal (patients not known to have glaucoma prior to death) and "glaucomatous" (known POAG patients at time of death) donors. Furthermore, there did not appear to be a distinct correlation between donor age and level of proMMP-3 production in response to IL-1α. Both findings are novel

and have not been previously reported in the literature. These data are thus important because they indicate that, regardless of age, the trabecular tissue of glaucoma patients may possess the capacity to respond to MMP-activators such as IL-1α in a manner equivalent to that of non-diseased tissue.

IL-1α signaling mechanisms linked to TM MMP-3 production

As stated previously, side effects issues with IL-1α effectively prohibits its usage as a therapeutic agent: such side effects can include pro-inflammatory responses, fever induction, destruction of cartilage matrix/bone resorption, thrombohemorrhagic lesions, hypercalcemia, exacerbation of autoimmune arthritis, central nervous system dysfunction, and muscle-wasting, among others (Dawson, 1991; Dunn, 1991). Furthermore, topical ocular delivery of large molecules such as IL-1α is impractical due to corneal impenetrability. Thus an additional series of studies were conducted in order to determine the signaling IL-1α on pathway(s) utilized by TM cells for upregulation of proMMP-3 levels in response to the cytokine.

Binding of interleukin-1 to its receptor leads to activation of multiple signaling pathways (Figure 3). These pathways appear to culminate in at least three main end-points: (1) activation of NF-κB-mediated gene transcription, (2) initiation of the arachidonic acid cascade, and (3) activation of AP-1-mediated gene transcription. It is thus possible that at least a portion of the side effects associated with IL-1α may be mediated by pathways not related to IL-1α's ability to stimulate TM MMP-3 production. Elucidation of the pathways linked to IL-1α-

mediated MMP-3 production may therefore allow for the identification of means to produce an IOP-lowering effect with minimal side effects. Hence, pharmacological tools were used in order to pinpoint the critical signaling mechanisms involved in the MMP-3 effect of IL-1α.

Regulators of key signaling pathway enzymes were thus evaluated for ability to affect IL-1α-mediate proMMP-3 production by the TM cells. Enzyme inhibitors were tested at 10-fold above their published IC50s, unless demonstrated by neutral red uptake to be toxic to the cells at that concentration. In the event of observed toxicity, the inhibitor concentrations were then reduced to non-toxic levels, but were never less than equivalent to 1X of their IC50 values. Furthermore, redundant inhibitors (including inhibitors with overlapping specificities) were used for key pathway enzymes in order to reduce potential errors which might otherwise result if only single agents were tested.

Lack of arachidonic acid cascade and NF-κB involvement

Results using inhibitors of key pathway enzymes did not reveal a central role for either the arachidonic acid/phosphlipase A_2-mediated lipid cascades or NF-κB-mediated transcription mechanisms. For example, the phospholipase A_2 enzyme inhibitors AACOCF3, 12-epi-scalaradial, and indomethacin were all without measurable effect on IL-1α-induced proMMP-3 production by the TM cells. Also without effect were inhibitors of lipoxygenase (curcumin) and cyclooxygenase (indomethacin, curcumin), sphingomyelinase (fumonisin B1), and certain isoforms of protein kinase C (see Table 7).

NF-κB also did not appear to play a role in IL-1α-induced proMMP-3 production by the TM cells under the assay conditions employed for these studies. Neither DRB (inhibitor of casein kinase II) nor NF-κB SN50 (inhibitor of activated NF-κB nuclear translocation) prevented the rise in proMMP-3 production by TM cells stimulated with IL-1α. The lack of NF-κB involvement is somewhat surprising since it is known that the human MMP-3 gene possesses an NF-κB binding site in its promoter regions (Quinones et al, 1989 and 1994). However, it has been shown in other cell types that an autoregulatory feedback loop exists between NF-κB and its inactivator IκBα in which IL-1α stimulation of NF-κB nuclear translocation leads to a rapid (within one hour) resynthesis of IκBα and the subsequent inactivation of NF-κB itself (Han et al, 1999). Thus it is possible that the potential effects of NF-κB on TM proMMP-3 production may have been effectively negated during the 24-hour time period utilized for MMP studies.

While these data indicate that neither NFκB or the arachidonic acid cascade appear to be essential in IL-1α-induced TM proMMP-3 production, they cannot answer whether either pathway might, instead, exert a modulatory effect not detected under the assay conditions used. For example, future studies might therefore focus on the time course of inhibition in order to determine if NF-κB may instead play an early role in IL-1α-induced enzymatic activities. Similar

studies could also be conducted for components of the arachidonic acid cascade.

Key role of AP-1

ProMMP-3 production by HTM-35D cells was effectively decreased by inhibitors of three key enzymes in the signaling cascades leading to AP-1 formation: PKC, p38 MAPK, and MEK. PKC involvement may be isoform-specific: inhibition of conventional (cPKC; calcium-dependent) PKCs α, β, γ, and of novel (nPKC; calcium-independent) PKCs δ, and ε by Bis I did not significantly affect TM cell response (Gekeler et al, 1996). Nor was response significantly affected by Gö 6983 which inhibits not only the cPKCs α, β, and γ, but also the nPKCs δ and ε and the atypical (aPKC; diacylglycerol-independent) PKC ζ. Conversely, however, IL-1α-induced proMMP-3 levels were significantly suppressed by Gö 6976, an agent which inhibits the cPKCs α and β, but also reportedly inhibits the novel protein kinase Cμ-subtype (PKD) with high affinity (IC_{50} = 20 nM) as compared to the much lower affinity of Gö 6983 for PKCμ (IC_{50} = 20 μM), perhaps pointing to a key role for PKCμ in this response (Gschwendt et al, 1996).

IL-1α-induced proMMP-3 levels were also significantly suppressed by the p38 MAPK inhibitors SB202190 and S203580, as well as blocked to a lesser extent by the MEK inhibitor PD098,059. Furthermore, sequestration of AP-1 by 1 μM SR11302 effectively blocked over 90% of the IL-1α-mediated increase in

proMMP-3 levels by HTM-35D cells; effect of SR11302 appeared to be dose-dependent in these cells.

The data thus clearly supports the involvement of AP-1 in the stimulation of TM proMMP-3 production by IL-1α; this agrees well with the known presence of two AP-1 transcription factor binding sites on the human proMMP-3 gene (Benbow and Brinckerhoff, 1997). Identification of the AP-1 pathway as the critical mechanism is therefore significant in that it may offer the potential for minimization of side effects related to either the NF-κB or arachidonic acid cascade pathways. It may also allow for the discovery of small-molecule AP-1 pathway activators useful for glaucoma therapy since it appears likely that the AP-1 pathway is important even in diseased tissue, based on the ability of SR11302 to block proMMP-3 production induced by IL-1α in both "normal" and "glaucomatous" TM cell lines.

TM response is variable

Similar to that seen for IL-1α response, the degree of blockade by SR11302 was variable, however the variability did not appear to be related to either donor age or history of glaucoma. The reason for the differences in MMP response among the various TM cell lines is currently unknown. One potential explanation may lie in the heterogeneity of the trabecular meshwork tissue itself. Although TM cells in culture have been reported by others to be comprised of a "single morphological cell type" (Alvarado et al, 1982; Polansky et al, 1984), it is well-known that the TM tissue in vivo is comprised of at least three distinct

regions: uveal, corneoscleral, and juxtacanalicular (cribiform) layers (Shield, 1992b).

The cells derived from the these different layers have been demonstrated by others to differ in both their proliferative capacity as well as in their expression of the heat shock-like protein αB-crystallin when maintained in cell culture (Lutjen-Drecoll, 1999). While it is unknown at this time whether TM cells from different meshwork regions possess other functional differences, it is possible that such differences may exist. Thus, one potential explanation for the variability in proMMP-3 production in the current experiments may be that the cell lines utilized were derived from different regions of the meshwork. Another potential explanation for the variability in TM response may lie within the realm of genetics, i.e. there may be inherited differences in the promoter regions of MMP genes which could affect their transcriptional regulation by AP-1. It would be interesting, therefore, to conduct further studies in order determine if different patient populations and/or different TM tissue regions vary in their ability to produce MMPs in response to stimuli such as cytokines, growth factors, or other stimuli, including the "AP-1 activators" described herein.

Variability in the response of other ocular tissues

The central involvement of the AP-1 pathway in the IL-1α MMP effect was not duplicated by the other human ocular cell lines tested. Response was also variable when comparing effects of SR11302 on IL-1α-induced proMMP-3 production by cell lines derived from various ocular tissues other than TM. In

those studies, only the response of human ciliary muscle cells appeared to be slightly, albeit not significantly, reduced by SR11302. In contrast, IL-1α-mediated proMMP-3 production by cultured human lamina cribosa (LC) cells (both "normal" and "glaucomatous") and of fibroblasts derived from the human corneal-scleral margin was not affected by co-incubation with SR11302. Based on this limited survey, it would thus seem that there is the possibility of variable ocular tissue responses (in addition to heterogeneous TM responses) within a given patient population. Precedence for such heterogeneic responses has been noted in non-ocular tissues and has been attributed to concurrent occupation of additional promoter sites such as PEA3 sites, as well as to the composition of the AP-1 complex itself, in that AP-1 dimers composed of Fos and Jun or of Jun and Jun appear to have enhanced potential for activation (Benbow and Brinckerhoff, 1997).

The variability of response between different ocular tissues could be used to advantage in the discovery and design of glaucoma therapeutic agents. For example, it may be possible to produce enhancement of MMP-3 levels in the TM, and possibly ciliary muscle, yet avoid upregulation of the enzyme in the optic nerve head (ONH) and LC region: at least one research group has speculated that increased MMP activity in the ONH region may be responsible for the tissue degeneration which leads to cupping of the optic nerve head in glaucoma patients (Yan et al, 2000). The same group has published limited data evaluating expression of MMP-1, MMP-2, and MMP-3 in astroglial cells of the

optic nerve heads of post-mortem human eyes which showed, interestingly, that while MMP-2 and MMP-3 levels were increased in both POAG and normal pressure glaucoma optic nerve heads, the level of MMP-1 was actually decreased in POAG eyes and relatively unchanged in normal pressure glaucoma eyes.

Potential role of IOP-induced mechanical stress

The significance of the decrease in MMP-1 levels within the optic nerve head of POAG patents is unknown at this time, however a similar suppression of MMP-1 levels has been reported to occur in human vascular smooth muscle cells in response to low-level mechanical stresses (Yang et al, 1998). It is possible that a similar effect may occur in optic nerve heads subjected to increased IOP. Conversely, elevated IOP may actually increase levels of certain MMPs, based on recent work which showed that MMP-2 production was enhanced in mechanically-stretched TM cell cultures (Bradley et al, 2000). Mechanical strain due to elevated IOP may also explain the increased expression of MMP-2 and MMP-3 in the aforementioned POAG patients.

Although the exact mechanisms are unknown, protein kinase B appears to be involved in the elevation of TM MMP-2 levels by stretch (Bradley et al, 2000). Furthermore, it has been shown that the stretching of human cardiac fibroblasts in vitro leads to increased expression of tissue plasminogen activator (Tyagi et al, 1998). Tissue plasminogen activator converts urokinase to plasmin, which, in turn, activates the pro-enzyme forms of many MMPs (Matrisian, 1992).

Dexamethasone has been shown to suppress both TM plasminogen and MMP levels in vitro, leading to speculation that an ensuing increase in ECM which is assumed to occur in vivo may be involved in steroid-induced glaucoma (Snyder et al, 1993). Elevated levels of plasminogen have been reported to be increased with age in human vitreous (Vaughan-Thomas et al, 2000), however, its levels did not appear to be correlated with any particular disease state in samples of human aqueous humor (Steinkamp et al, 1993).

Finally, another means by which IOP-related stretch may increase MMP levels could be via increased binding of AP-1 to its promoter sites followed by an increase in MMP gene transcription, an event known to occur in response to stretch in other tissues such as bladder smooth muscle cells (Park et al, 1999).

Identification of small-molecule "AP-1 activators"

Further exploration of the role of AP-1 in TM proMMP-3 production was conducted using agents which have been reported as possessing the ability to increase expression of AP-1 complexes. These agents, β-naphthoflavone, 3-methylcholanthrene, and t-BHQ, were shown to be stimulators of proMMP-3 production by some, but not all, of the same array of "normal" and "glaucomatous" TM cell lines used in the previous studies with IL-1α. t-BHQ was the most consistently efficacious in the "normal" TM cell lines, and was the only one of the three agents which stimulated proMMP-3 production in the glaucomatous TM lines tested; two out of five of the glaucomatous lines responded to this agent with an increase in proMMP-3 levels.

It is important to point out that these agents may also mediate other effects unrelated to AP-1 activation; such effects could conceivably be in direct opposition to their ability to induce MMP-3 production by the TM cells. Such effects may be one reason for their inability to stimulate production to the same degree as that seen with IL-1. However, although responses were weak and variable, the ability of each of the agents to stimulate TM proMMP-3 production further validates the importance of the AP-1 pathway in this process.

Additionally, the identification of non-cytokine compounds which also increased TM proMMP production is significant in that it lends support to the concept that development of small-molecule AP-1 "activating" agents is possible for use as anti-glaucoma agents. A caveat to this theory is that since the studies reported herein reflect only changes in the "pro", i.e. unactivated, forms of MMPs, it is possible that simply increasing levels of pro-enzyme transcription via AP-1 may not necessarily lead to a concomittant increase in levels of the active enzymes necessary to effect ECM remodeling. Furthermore, since AP-1 promoter sites are also present on the genes for TIMPs (Borden and Heller, 1997), any beneficial increase in MMPs due to AP-1 activation may be rapidly negated by the inhibitory effects of a similar increase in TIMP levels. However, only TM production of TIMP-1, and not TIMP-2, was affected by IL-1α (see Table 5), therefore it is possible that similar disparate effects on TIMPs may be noted with other AP-1 activators.

While it is true that TIMPs bind to and inhibit the activated form of MMP enzymes, they are not capable of inhibiting the process of activation of either the collagenase or stromelysin pro-enzymes. Therefore it is within the realm of possibility that such activation could take place and sufficient ECM digestion could occur before being terminated by the action of TIMPs (Matrisian, 1992). The ability of the AP-1 activator t-BHQ to increase outflow in the perfused human organ culture model, as discussed in the following section, would seem to support such a scenario.

Perfused human anterior segments

The importance of the AP-1 pathway in IOP regulation was validated using perfused ex vivo human anterior segments. Two separate models were utilized, a "constant flow" model in which tissues were perfused at a constant rate of fluid flow and a "constant pressure" model in which the hydrostatic pressure of the incoming perfusion fluid was kept at a constant level (see Methods section for details). Although the models vary in the basic premise regarding fluid delivery, others have previously reported no significant difference between each method's ability to maintain TM viability, providing flow rate is carefully monitored in the constant flow model (Johnson, 1996). However, in the current studies, viability of tissue from glaucomatous donors appeared to be better-maintained when perfused via constant flow. Because tissue viability at termination of perfusion is of obvious concern, all anterior segments were fixed at the end of each study, followed by light and electron microscopic evaluation of

the TM region's morphology (Pang et al, 2000). Data from tissues thus judged to be unhealthy were excluded from inclusion in the Results since it was not possible to determination if the tissues were non-viable at the outset of perfusion or if viability had, instead, declined during the course of assay.

t-BHQ improves outflow and lowers IOP in perfused ex vivo tissues

In these studies, the anterior segments were perfused with t-BHQ at a concentration (10 μM) which was shown to increase the production of proMMP-3 by the TM cell cultures. At this concentration, t-BHQ significantly ($p < 0.05$ compared to vehicle-treated tissues) increased the outflow rate from "normal" donor tissues as well as decreased the intraocular pressures in "glaucomatous" tissues. During the perfusion period, outflow in the vehicle-treated tissues consistently diminished with time. Although the exact reasons for this phenomenon are unknown, it is tempting to speculate (1) that the decrease in flow rate could be attributed to an increase in the accumulation of debris in the untreated anterior segments and (2) that no similar decrease was noted in the treated eyes due to t-BHQ-mediated improvement in the patency of the outflow tissues.

Correlation with MMP results

Effects on outflow were evident within 24 h and persisted for up to at least four days. These results correlated well with the time course of MMP-3 activation previously noted in the TM cell cultures. Taken together with the observed elevation of proMMP-3 in perfusates from t-BHQ-treated tissues, these

data are consistent with the hypothesis that at least a portion of the outflow effects may be due to an increase in MMP-3 production. The cellular source of the perfusate proMMP-3 remains undetermined. It may be possible, however, to rule out the corneal endothelium based on electron microscopic of fixed, post-perfusion tissues since no visually-detectable effects on corneal endothelium were noted in the t-BHQ-perfused tissues as compared to vehicle-treated controls. Such lack of effect(s) might not be unexpected based on the demonstrated lack of effect of IL-1 (which is hypothesized to be acting via similar signaling mechanisms) on MMP-2 and its minimal effects on MMP-9. Both MMP-2 and –9 are believed likely to play roles in corneal remodelling (Matsubara et al, 1991).

AP-1 involvement

t-BHQ-mediated increases in outflow were blocked by SR11302, clearly confirming the involvement of the AP-1 pathway. However, the actual contribution of MMP-3 to this effect remains unclear; clarification of such could be carried out via use of a specific inhibitor of MMP-3 such as CGS 27023A (MacPherson et al, 1997). Nevertheless, the ability of the AP-1 activator t-BHQ to increase outflow and perfusate proMMP-3 levels in the human organ culture model would seem to support the conclusion that AP-1 upregulates MMP-3 and that this increase in enzyme levels thereby contributes to the observed outflow enhancement.

Such responses are highly significant because this model is expected to allow prediction of efficacy in vivo and because currently there are no known animal models that could be used to adequately predict/evaluate the effects of MMP activation. There are drawbacks to this model, however. For example, many of the resident tissues are excised in order to prepare the anterior segments for mounting within the perfusion chambers. Potential contributions of these tissues thus cannot be taken into consideration. Furthermore, test agents are administered via direct tissue perfusion as oppposed to topical application to the cornea, the most widely-used means of delivery for ocular medications. Therefore, factors which would ordinarily affect true bioavailability (e.g. corneal permeability, local and systemic metabolism, absorption into the blood stream, etc.) cannot be predicted by this model.

Potential for novel glaucoma therapy

Regardless of these drawbacks, the observed ability of the AP-1 activator to decrease IOP of glaucomatous anterior segments indicates that administration of such compounds may also prove to be an effective means for lowering the IOP of human glaucoma patients. It also points the way toward the potential development of novel therapeutic targets that act on mechanisms upstream of AP-1 activation. For example, activators of p38 MAP kinases and of the PKCμ subtype may prove useful as IOP-lowering agents, pending the validation of the role of these enzymes in models such as the perfused ocular anterior segment model or in animal studies, should a suitable model become available.

The utility of IOP-lowering as glaucoma therapy has been called into question because the relevance of this effect in relation to protection of the RGCs is currently not clear. Recent research is being directed more towards the identification of agents that would provide direct protection to the RGCs/optic nerve (Caprioli, 1997; Neufeld, 1998). Neuroprotection-based research is heavily based on those mechanisms which are believed to contribute to glaucomatous neuronal degeneration: theories of such include loss of blood flow to the optic nerve head and/or interruption of the flow of neurotrophic factors due to RGC axonal injury (Quigley, 1995). There may also be a direct pressure-related component, as demonstrated by Agar et al (2000) who showed that increased hydrostatic pressure can induce apoptotic responses in neuronal cultures independent of other cellular insults. Because of this, there is also considerable interest in the development of agents which would not only have the capacity to decrease IOP but which would also be neuroprotective.

At least two currently-available commercial anti-glaucoma agents claim efficacy in both of these mechanisms (IOP-lowering and neuroprotection): brimonidine and betaxolol (Wheeler et al, 1999; Osborne et al, 1999). Although the mechanisms are unclear, at least a portion of the neuroprotective effects of each may possibly be due to increased expression of neurotrophic factors in the retina and/or optic nerve head. In this same vein, MMP-activating compounds may also prove to be neuroprotective, in addition to IOP-lowering, although the mechanism of neuroprotection would likely be due to mechanisms other than

upregulation of neurotrophic factors. For example, neural regeneration has been shown to be inhibited by a build-up of ECM materials, such as chondroitin sulfate proteoglycans, produced by reactive astrocytes both in vivo (McKeon et al, 1991) and in vitro (Canning et al, 1996).

Therefore, agents such as MMPs could prove neuroprotective since their actions would alleviate the ECM build-up and allow for new neuritic growth: an event which has already been shown to occur, for example, in both neonatal and adult peripheral nerves in vitro in response to pretreatment with MMP-2 and MMP-9 prior to injury (Ferguson and Muir, 2000). However, as stated previously, MMPs have been proposed as playing a role in optic nerve head cupping associated with glaucoma (Hernandez, 1999; Yan et al, 2000); therefore induction of retinal/optic nerve MMP levels would require a fine balance in order to maintain a neuroregenerative vs a neurodegenerative environment.

TM cell phagocytosis

One means by which degraded ECM can be cleared from the anterior chamber is by phagocytic actions of the TM. Initial studies verified that TM cells were capable of phagocytosing fluorescein-labelled bacteria ("bioparticles") and that this process occured in both unstimulated and stimulated (PDGF-BB) cells, confirming the work of Tamura et al (1989). The bioparticle assay was chosen because it offered several advantages over previously-used methodologies. For example, the fluorescein tag allows for greater assay sensitivity and eliminates the need for other detection means such as labeling with a radioactive isotope.

It also allows for evaluation of samples via automated microplate readers, increasing the throughput of the assay as compared to laborious counting of incorporated particles under a microscope or with a cell sorter. Use of trypan blue to quench fluorescence of extracellular unincorporated particles also eliminates the need for routine manual microscopic observation, making the assay more user-friendly.

New observations on regulation of TM phagocytosis

Using this assay, the protein kinase C activator TPA was also shown to promote TM phagocytosis. This represents a novel finding for TM cells, despite the existence of reports which indicate PKC activation promotes phagocytosis in other cell types. The response to TPA could be blocked by the PKC inhibitor Bis I, implicating the PKC pathway in this action. In this respect, the TM phagocytic response appears to be similar to that of other phagocytic cells, such as CNS microglia, which demonstrate increased levels of phagocytic activity following exposure to phorbol myristate acetate (Smith et al, 1998).

IL-1α was then also tested for effect on TM phagocytic response. Under the same conditions used for PDGF-BB and TPA, IL-1α also significantly stimulated the uptake of fluoresceinated bioparticles, an effect which could be partially blocked by co-treatment with SR11302 at the same concentration (1 μM) previously shown to significiantly suppress IL-1α-induced proMMP-3 production by TM cells. As with TPA, this is the first known report of the effect of this cytokine on TM cell phagocytosis, an action which appears (at least in part) to be

linked to AP-1 activation based on the effect of SR11302. Additional evidence for involvement of the AP-1 pathway was supplied by studies which investigated TM phagocytic response following treatment with the putative AP-1 activator t-BHQ which, at a concentration (10 μM) sufficient to stimulate proMMP-3 production, also stimulated bioparticle uptake by these cells.

Finally, the effect of IL-1α on TM phagocytosis may be negatively-regulated by the NF-κB pathway since, interestingly, response to IL-1α was actually enhanced by co-treatment with the NFκB inhibitor peptide NFκB-SN50. IL-1α is known to activate both NF-κB and AP-1-mediated pathways in many cell types (see Figure 3). Co -stimulation by IL-1α of the two pathways might therefore represent a protective mechanism for TM cells, preventing overactivation of the phagocytic response.

Consequences of TM phagocytic activity

The ability of both t-BHQ and IL-1α to enhance TM phagocytic response may thus contribute to their observed effects on outflow and IOP, i.e. the phagocytosis of degraded ECM and debris might be expected to aid in reduction of resistance to outflow. While the consequences of increased TM cell phagocytic activity would thus appear to be beneficial in terms of clearance of the aqueous outflow pathway, it has also been postulated that the over-stimulation of TM phagocytosis may actually be detrimental. For example, it has been demonstrated that bovine TM cells exhibit diminished surface contact and increased migration/TM cell loss following ingestion of latex microspheres in vitro

(Zhou et al, 1995; Zhou et al, 1999). TM cell migration and loss has also been noted following in vivo injection of adult cat eyes with zymosan particles (Johnson et al, 1989). However, no similar loss of TM cells or increased TM migration was detected following challenge of either normal or glaucomatous perfused human anterior segments with latex microspheres (Matsumoto and Johnson, 1997b). Future studies should shed further light on each of these effects: for example, it would be interesting to perfuse ex vivo human anterior segments with bioparticles in the presence or absence of IL-1α or an AP-1 activator, followed by microscopic evaluation for bioparticle ingestion by the TM tissues, as well as for evidence whether TM cell loss/migration has occured.

Summary

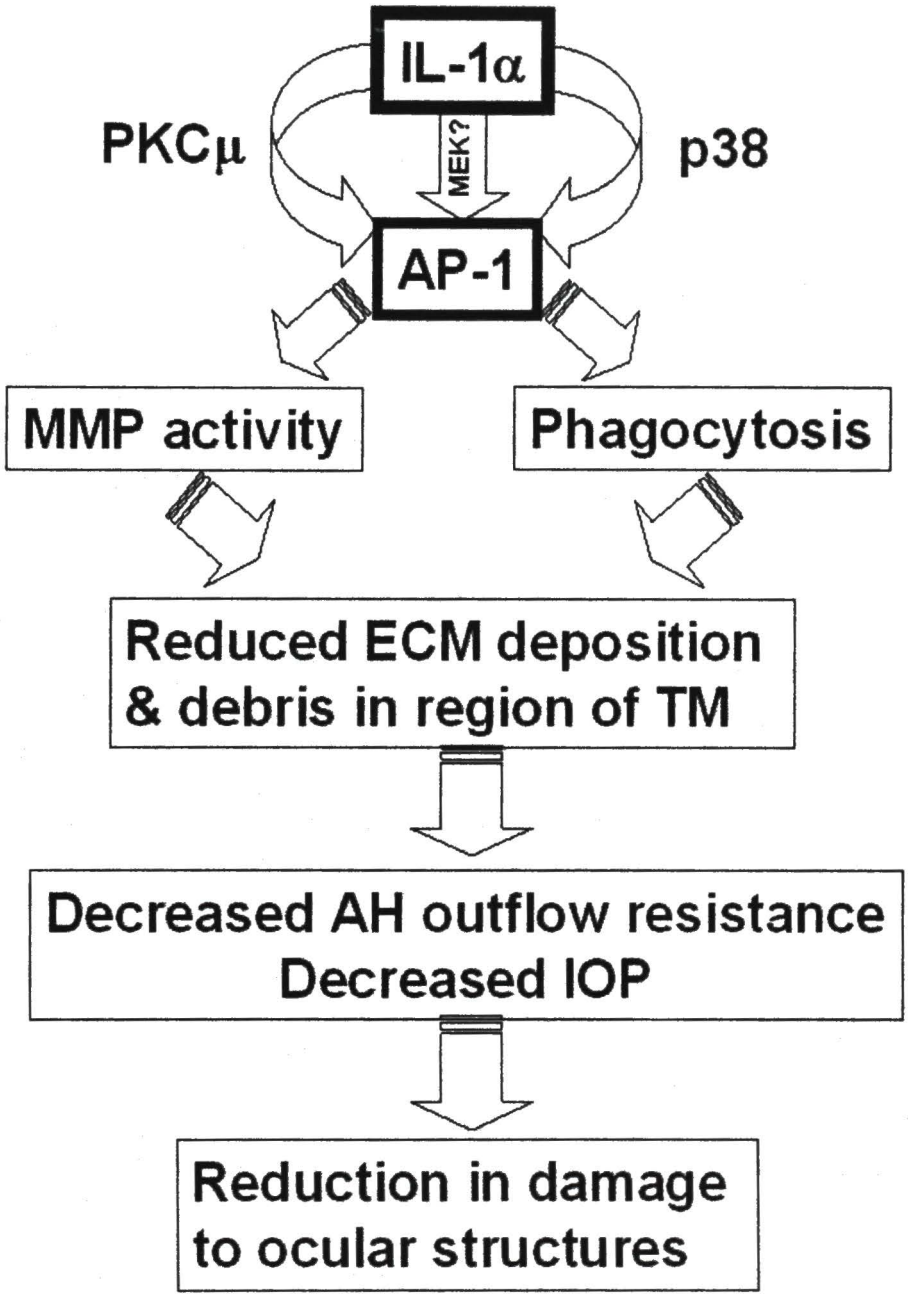
In summary, I have demonstrated that:

1. TM cell proMMP-3 levels are upregulated by IL-1α.
2. AP-1 activation is a critical pathway in the proMMP-3 upregulation.
3. Small-molecule AP-1 activators also enhance TM proMMP-3 levels.
4. Small-molecule AP-1 activators increase outflow/lower IOP in perfused human anterior segments (normal and glaucomatous).
5. Small-molecule AP-1 activators can enhance proMMP-3 levels in the perfusate of these same anterior segments, an effect blocked by SR11302.
6. TM cell phagocytic activity is increased by both IL-1α and small-molecule AP-1 activators; effects are blocked by SR11302.

The hypothesized cumulative effects of these actions are depicted in Figure 30. Signaling pathways associated with IL-1α ultimately lead to AP-1 activation (and, likely, yet undiscovered pathways) within ocular tissues such as the TM. Activation of AP-1, in turn, enhances TM MMP-3 production and phagocytic response by some as yet unknown means, perhaps via enhanced MMP gene transcription and/or enhanced transcription of cytoskeletal proteins critical to the phagocytic process.

In this scenario, the concerted activities of the MMPs (degradation of ECM) and phagocytosis (clearance of degraded ECM and debris) lead to reduction of aqueous outflow resistance, a decrease in intraocular pressure, and (hopefully) a decline in the rate of associated glaucomatous damage to the eye. Although this progression of events is, admittedly, partially speculative, the results of the studies described in this report are at the least consistent with the conclusions that AP-1 mediates both the MMP- and phagocytosis-enhancing effects of IL-1α on the TM, as well as appears to be involved in ex vivo IOP-lowering.

Figure 30


IL-1α
PKCμ
MEK?
p38
AP-1
MMP activity
Phagocytosis
Reduced ECM deposition & debris in region of TM
Decreased AH outflow resistance
Decreased IOP
Reduction in damage to ocular structures

REFERENCES

Abate A and Schroder H. (1998) Protease inhibitors protect macrophages from LPS-induced cytotoxicity: possible role for NF-kappaB. *Life Sci*, **62**:1081-1088.

Acott TS, Samples JR, Bradley JMB, Bacon DR, Blysma BS, Van Buskirk EM. (1989) Trabecular repopulation by anterior trabecular meshwork cells after laser trabeculoplasty. *Am J Ophthalmol*, **107**:1-6.

Acott TS. Trabecular extracellular matrix regulation. In "Pharmacology of Ophthalmology." Eds. Drance SM, Van Buskirk EM, Neufeld AH. Pp 125-157, 1992. Williams & Wilkins, Baltimore.

Agar A, Yip SS, Hill MA, Coroneo MT. (2000) Pressure related apoptosis in neuronal cell lines. *J Neurosci Res*, **60**:495-503.

Ainbinder E, Bergelson S, Pinku R, Daniel V. (1997) Regulatory mechanisms involved in activator-protein-1 (AP-1)-mediated activation of glutathione-*S*-transferase gene expression by chemical agents. *Eur J Biochem*, **243**:49-57.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. The immune system (chap. 18). In "Molecular Biology of the Cell", 2nd edition. Pp 1001-1057, 1989(a). Garland Publishing, Inc., New York.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Cell signaling (chap. 12). In "Molecular Biology of the Cell", 2nd edition. Pp 681-726, 1989(b). Garland Publishing, Inc., New York.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated kinase kinase in vitro and in vivo. *J Biol Chem*, **270**:27489-27494.

Alexander JP, Samples JR, Van Buskirk EM, Acott TS. (1991) Expression of matrix metalloproteinases and inhibitor by human trabecular meshwork. *Invest Ophthalmol Vis Sci*, **32**:172-180.

Alexander JP, Samples JR, Acott TX. (1998) Growth factor and cytokine modulation of trabecular meshwork matrix metalloproteinase and TIMP expression. *Curr Eye Res*, **17**:276-285.

Alvarado JA, Wood I, Polansky JR. (1982) Human trabecular cells. II. Growth pattern and ultrastructural characteristics. *Invest Ophthalmol Vis Sci*, **23**:464-478.

Ando H, Twining SS, Yue BYJT, Zhou X, Fini ME, Kaiya T, Higginbotham EJ, Sugar J. (1993) MMPs and proteinase inhibitors in the human aqueous humor. *Invest Ophthalmol Vis Sci*, **34**:3541-3548.

Anthony TL, Pierce KL, Stamer WD, Regan JW. (1998) Prostaglandin F2 alpha receptors in the human trabecular meshwork. *Invest Ophthalmol Vis Sci*, **39**:315-321.

Bankers-Fulbright JL, Kalli KR, McKean DJ. (1996) Interleukin-1 signal transduction. *Life Sci*, **59**:61-83.

Benbow U and Brinckerhoff CE. (1997) The AP-1 site and MMP gene regulation: What is all the fuss about? *Matrix Biol*, **15**:519-526.

Berman MB. (1993) Regulation of corneal fibroblast MMP-1 collagenase secretion by plasmin. *Cornea*, **12**:420-432.

Bill A. (1975) The drainage of aqueous humor. *Invest Ophthalmol Vis Sci*, **33**:2687-2692.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. (1993) Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med*, **4**:197-250.

Blysma SS, Samples JR, Acott TS, Van Buskirk EM. (1988) Trabecular cell division after argon laser trabeculoplasty. *Arch Ophthalmol*, **106**:544-547.

Blysma SS, Pirouzkar B, Acott TS, Van Buskirk EM, Samples JR. (1989) In-vivo cell division after argon laser trabeculoplasty. *Invest Ophthalmol Vis Sci*, **S30**:280.

Borden P and Heller RA. (1997) Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. *Crit Rev Euk Gene Expression*, **7**:159-178.

Bradley JMB, Parshley JR, Samples JR, Acott TS. (1996) Trabecular response to laser trabeculoplasty is mediated by a factor produced after treatment. *Invest Ophthalmol Vis Sci*, **37**:S151.

Bradley JMB, Vranka J, Colvis CM, Conger DM, Alexander JP, Fisk AS, Samples JR, Acott TS. (1998) Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. *Invest Ophthalmol Vis Sci*, **39**:2649-2658.

Bradley JMB, Kelley MJ, Acott TS. (2000) Involvement of the PKB signal transduction pathway in trabecular cell response to mechanical stretching. *Invest Ophthalmol Vis Sci*, **41**:S504.

Brubaker RF. (1991) Flow of aqueous humor in humans. *Invest Ophthalmol Vis Sci*, **32**:3145-316.

Bursten SL, Harris WE. (1994) Interleukin-1 stimulated phosphatidic acid-mediated phospholipase D activity in human mesangial cells. *Am J Physiol*, **266**:c1093-1104.

Busch MJ, Kobayashi K, Hoyng PF, Mittag TW. (1993) Adenylyl cyclase in human and bovine trabecular meshwork. *Invest Ophthalmol Vis Sci*, **34**:3028-3034.

Canning DR, Hoke A, Malemud CH, Silver J. (1996) A potent inhibitor of neurite outgrowth that predominates in the extracellular matrix of reactive astrocytes. *In J Dev Neurosci*, **14**:153-175.

Cano E, Mahadevan LC. (1995) Parallel signal processing among mammalian MAPKs. *Trends Biochem Sci*, **20**:117-122.

Caprioli J. (1997) Neuroprotection of the optic nerve in glaucoma. *Acta Ophthalmol Scand*, **75**:364-367.

Clark AF, Wilson K, McCartney MD, Miggans ST, Kunkle M, Howe W. (1994) Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*, **35**:281-294.

Clark AF, Wilson K, deKater AW, Allingham RR, McCartney MD. (1995) Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. *Invest Ophthalmol Vis Sci*, **36**:478-489.

Clark AF (1998) New discoveries on the roles of matrix metalloproteinases in ocular cell biology and pathology. *Invest Ophthalmol Vis Sci*, **39**:2514-2516.

Coleman AL (1999) Glaucoma. *The Lancet*, **354**:1803-1810.

Craig JE and Mackey DA. (1999) Glaucoma genetics: where are we? where will we go? *Curr Opin Ophthalmol*, **10**:126-134.

Daum G, Eisenmann-Tappe I, Fries H, Troppmair J, Rapp UR. (1994) The ins and outs of Raf kinases. *Trends Biochem Sci*, **19**:474-480.

Dawson MM. Interleukin-1 (chap. 4). In "Lymphokines and Interleukins." Pp 83-105, 1991. CRC Press, Inc., Boca Raton.

Dueker DK, Norberg M, Johnson DH, Tschumper RC, Feeney-Burns L. (1990) Stimulation of cell division by argon and Nd:YAG laser trabeculoplasty in cynomolgus monkeys. *Invest Ophthalmol Vis Sci*, **31**:115-124.

Dunn CJ. Cytokines as mediators of chronic inflammatory diseases (chap. 1). In "Cytokines and Inflammation." Ed. Kimball ES. Pp 1-33, 1991. CRC Press, Inc., Boca Raton.

Eder J. (1997) Tumour necrosis factor α and interleukin-1 signaling: do MAPKK kinases connect it all? *Trends Pharmacol Sci*, **18**:319-322.

English D. (1996) Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signaling. *Cell Signal*, **8**:341-347.

Everts V, Wolvius E, Saklatvala J, Beertsen W. (1990) Interleukin 1 increases the production of collagenase but does not influence the phagocytosis of collagen fibrils *Matrix*, **10**:388-393.

Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, Graupner G, Lu X-P, Pfahl M. (1994) A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature*, **372**:107-111.

Fini ME, Yue BY, Sugar J. (1992) Collagenolytic/gelatinolytic metalloproteinases in normal and keratoconus corneas. *Curr Eye Res*, **11**:849-862.

Forrester JV, Dick AD, McMenamin P, Lee WR. General and ocular pharmacology (chap. 6). In "The Eye. Basic Sciences in Practice." Pp 237-264, 1996. WB Saunders Company, Philadelphia.

Friedman Z, Bloom E, Crook RB, Polansky JR (1990) Inositol phosphate formation and prostaglandin production in cultured human trabecular meshwork (HTM) cells. *Invest Ophthalmol Vis Sci*, **31(suppl)**:247.

Friedman Z, Bloom E, Polansky JR. (1999) Adrenergic drug effects on cyclic AMP in cultured human trabecular meshwork cells. *Ophthalmic Res*, **31**:53-58.

Ferguson TA and Muir D. (2000) MMP-2 and MMP-9 increase the neurite-promoting potential of schwann cell basal laminae and are upregulated in degenerated nerve. *Mol Cell Neurosci*, **16**:157-167.

Gekeler V, Boer R, Uberall F, Ise W, Schubert C, Utz I, Hofmann J, Sanders KH, Schachtele C, Klemm K, Grunicke H. (1996) Effects of the selective bisindoylmaleimide protein kinase C inhibitor GF 109203X on p-glycoprotein-mediated multidrug resistance. *Br J Cancer*, **74**:897-905.

Graves LM, Bornfeldt KE, Krebs EG. Historical perspectives and new insights involving the MAP kinase cascades. In "Signal Transduction in Health and Disease. Advances in Second Messenger and Phosphoprotein Research." Eds.: Corbin J and Francis S. Vol. 31, pp 49-62, 1997. Lippincott-Raven Publishing, Philadelphia.

Greenberg S, Silverstein SC. Phagocytosis (Chapter 27). In "Fundamental Immunology." Ed.: Paul WE. Pp 941-964, 1993. Raven Press, Ltd., New York.

Grierson I, Lee WR. (1973) Erythrocyte phagocytosis in the human trabecular meshwork. *Br J Ophthalmol*, **57**:400-415.

Grierson I, Day J, Unger WG, Ahmed A. (1986) Phagocytosis of latex microspheres by bovine meshwork cells in culture. *Graefe's Arch Ophthal*, **224**:3440-3450.

Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ. (1996) Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase C isozymes. *FEBS Lett*, **392**:77-80.

Guyton AC. (1991) The eye: II. Receptor and neural function of the retina. (Chap. 59) In: *Textbook of Medical Physiology*. Ed.: Wonsiewicz MJ. 8th ed. Pp. 546-559. WB Saunders Company: Philadelphia, USA.

Han Y, Meng T, Murray NR, Fields AP, Brasier AR. (1999) Interleukin-1-induced nuclear factor-κB-IκBα autoregulatory feedback loop in hepatocytes. *J Biol Chem*, **274**:939-947.

Haque MSR, Pang I-H, Magnino PE, DeSantis L. (1998) Activation of phospholipase C and guanylyl cyclase by endothelins in human trabecular meshwork cells. *Curr Eye Res*, **17**:1110-1117.

Hart WM. (1992) Intraocular pressure. In: *Adler's Physiology of the Eye.* 9th ed. Pp. 248-267. Mosby-Yearbook Inc.: St. Louis, USA.

Hernandez MR. (2000) The optic nerve head in glaucoma: Role of astrocytes in tissue remodeling. *Progr Ret Eye Res*, **19**:297-321.

Janosch P, Schellerer M, Seitz T, Reim P, Eulitz M, Brielmeier M, Kolch W, Sedivy JM, Mischak H. (1996) Characterization of IκB kinases. IκB-α is not phosphorylated by Raf-1 or protein kinase C isozymes, but is a casein kinase II substrate. *J Biol Chem*, **271**:13868-13874.

Jirillo E, Decandia P, Ribaud MR, Cannuscio B, DeSimone C, Antonaci S. (1992) Enhancement of polymorphonuclear cell phagocytosis by lipid A-activated monocytes via cell-to-cell contact. A possible role for membrane-associated cytokines. *Immunopharmacol-Immunotoxicol*, **14**:343-354.

Johnson DH and Tschumper RC. (1987) Human trabecular meshwork organ culture: A new method. *Invest Ophthalmol Vis Sci*, **28**:945-953.

Johnson DH, Richardson TM, Epstein DL. (1989) Trabecular meshwork recovery after phagocytic challenge. *Curr Eye Res*, **8**:1121-1130.

Johnson DH. (1996) Human trabecular meshwork cell survival is dependent on perfusion rate. *Invest Ophthalmol Vis Sci*, **37**:1204-1208.

Kaufman PL, Wiedman T, Robinson JR. Cholinergics (chap. 4). In "Pharmacology of the Eye." Ed. Sears ML. Pp 149-191, 1984. Springer-Verlag, New York.

Kee C and Seo K. (1997) The effect of interleukin-1α on outflow facility in rat eyes. *J Glauc*, **6**:246-249.

Kimpel MW, Johnson DH. (1992) Factors influencing in vivo trabecular cell replication as determined by ^{3}H-thymidine labeling; an autoradiographic study in cats. *Curr Eye Res*, **11**:297-306.

Kuno K, Okamoto S, Hirose K, Murakami S, Matsushima K. (1993) Structure and function of the intracellular portion of the mouse interleukin 1 receptor (type I). *J Biol Chem*, **286**:13510-13518.

Kuno K and Matsushima K. (1994) The IL-1 receptor signaling pathway. *J Leukoc Biol*, **56**:542-547.

Lee WR and Grierson I. (1974) Relationships between intraocular pressure and the morphology of the outflow apparatus. *Trans Ophthalmol Soc UK*, **94**:430-449.

Lee FS, Hagler J, Chen ZJ, Maniatis T. (1997) Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*, **88**:213-222.

Lindsey JD, Kashiwagi K, Boyle D, Kashiwagi F, Firestein GS, Weinreb RN. (1996) Prostaglandins increase proMMP-1 and proMMP-3 secretion by human ciliary smooth muscle cells. *Curr Eye Res*, **15**:869-875.

Lu P C-S, Hongqing Y, Maeda M, Azar DT. (1999) Immunolocalization and gene expression of matrilysin during corneal wound healing. *Invest Ophthalmol Vis Sci*, **40**:20-27.

Lutjen-Drecoll E. (1999) Functional morphology of the trabecular meshwork in primate eyes. *Prog Ret Eye Res*, **18**:91-119.

Maclouf J, Sors H, Rigaud M. (1977) Recent aspects of prostaglandin biosynthesis: a review. *Biomedicine*, **26**:362-375.

MacPherson LJ, Bayburt EK, Capparelli MP, Carrol BJ, Goldstein R, Justice MR, Zhu L, Hu S, Melton RA, Fryer L, Goldberg RL, Doughty JR, Spirito S, Blancuzzi V, Wilson D, O'Byrne EM, Ganu V, Parker DT. (1997) Discovery of CGS 27023A, a non-peptidic, potent, and orally active stromelysin inhibitor that blocks cartilage degradation in rabbits. *J Med Chem*, **40**:2525-2532.

Magnino P, Pang I-H, Clark A.F. (1999) Characterization of matrix metalloproteinase expression in cultured human trabecular meshwork cells. *1999 Annual Meeting of the Association for Ocular Pharmacology and Therapeutics*. (Abstract)

Malanin NL, Boldin MP, Kovalenko AV, Wallach D. (1997) MAP3K-related kinase involved in NFκB induction by TNF, CD95, and IL-1. *Nature*, **385**:540-544.

Matrisian LM. (1992) The matrix-degrading metalloproteinases. *BioEssays*, **14**:455-463.

Matsubara M, Girard MT, Kublin CL, Cintron C, Fini ME. (1991) Diffrential roles for two gelatinolytic enzymes of the matrix metalloproteinase family in the remodelling cornea. *Dev Biol*, **147**:425-439.

Matsumoto Y and Johnson DH. (1997a) Dexamethasone decreases phagocytosis by human trabecular meshwork cells *in situ*. *Invest Ophthalmol Vis Sci*, **38**:1902-1907.

Matsumoto Y and Johnson DH. (1997b) Trabecular meshwork phagocytosis in glaucomatous eyes. *Ophthalmologica*, **211**:147-152.

McKean DJ, Podzorski RP, Bell MP, Nilson AE, Huntoon CH, Slack J, Dower SK, Sims JE. (1993) Murine helper T cell-2 lymphocytes express type I and type II IL-1 receptors, but only the type I receptor mediates costimulatory activity. *J Immunol*, **151**:3500-3510.

McKeon RJ, Schreiber RC, Rudge JS, Silver J. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J Neurosci*, **11**:3398-3411.

McNatt L, Stropki K, McCartney M, Clark A. (Unpublished observations).

Modur V, Zimmerman GA, Prescott SM, McIntyre TM. (1996) Endothelial cell inflammatory responses to tumor factor α. Ceramide-dependent and – independent mitogen-activated protein kinase cascades. *J Biol Chem*, **271**:13094-13102.

Murphy G, Cockett MI, Stephens PE, Smith BJ, Doherty AJ. (1987). Stromelysin is an activator of procollagenase A. A study with natural and recombinant enzymes. *Biochem J*, **248**:265-268.

Mycek MJ, Gertner SB, Perper MM. Cholinergic agonists (chap. 4). In "Lippincott's Illustrated Reviews: Pharmacology." Eds. Harvey RA and Champe PC. Pp 35-44, 1992. JB Lippincott Company, New York.

Nagase H, Enghild JJ, Suzuki K, Salvesen G. (1990) Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl) mercuric acetate. *Biochemistry*, **29**:5783-5789.

Neufeld AH. (1998) New conceptual approaches for pharmacological neuroprotection in glaucomatous neuronal degeneration. *J Glauc*, **7**:434-438.

Okada Y and Nakanishi I. (1989) Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 ('gelatinase') by human neutrophil elastase and cathepsin-G. *FEBS Lett*, **244**:473-476.

Oldenburg HS, Pruitt JH, Lazarus DD, Rogy MA, Chizzonite R, Lowry SF, Moldawer LL. (1995) Interleukin 1 binding to its type I, but not type II receptor modulates the in vivo acute phase response. *Cytokine*, **7**:510-516.

Olson J. Peripheral nervous system (chap. 2). In "Clinical Pharmacology Made Ridiculously Simple." Pp 1-33, 1994. MedMaster, Inc., Miami.
O'Neill LAJ. (1995) Interleukin-1 signal transduction. *Int J Clin Lab Res*, **25**:169-177.

Opdenakker G, Fibbe WE, Van Damme J. (1998) The molecular basis of leukocytosis. *Immunol Today*, **19**:182-189.

Osborne NN, Chidlow G, Nash MS, Wood JPM. (1999a) The potential of neuroprotection in glaucoma treatment. *Curr Opin Ophthalmol*, **10**:82-92.

Osborne NN, Ugarte M, Chao M, Chidlow G, Bae JH, Wood JP, Nash MS. (1999b) Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv Ophthalmol*, **43(suppl 1)**:S102-S128.

Pang I-H, Shade DL, Clark AF, Steely HT, DeSantis L. (1994) Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. *Curr Eye Res*, **13**:51-63.

Pang I-H, Shade DL, Matsumoto S, Steely HT, DeSantis L. (1996) Presence of functional type B natriuretic peptide receptor in human ocular cells. *Invest Ophthalmol Vis Sci*, **37**:1724-1731.

Pang I-H, McCartney MD, Steely HT, Clark AF. (2000) Human ocular perfusion organ culture: A versatile ex vivo model for glaucoma research. *J Glauc* (accepted).

Parker JM, Adam RM, Peters CA, Guthrie PD, Sun Z, Klagsbrun M, Freeman MR. (1999) AP-1 mediates stretch-induced express of HB-EGF in bladder smooth muscle cells. *Am J Physiol*, **277**:C294-C301.

Parshley DE, Bradley JMB, Fisk A, Hadaegh A, Samples JR, Van Buskirk EM, Acott TS. (1996) Laser trabeculoplasty induces stromelysin expression by trabecular juxtacanalicular cells. *Invest Ophthalmol Vis Sci*, **37**:795-804.

Peplow PV. (1997) Actions of cytokines in relation to arachidonic acid metabolism and eicosanoid production. *Prostaglandins, Leukotriennes and Essential Fatty Acids*, **54**:303-317.

Polansky JR, Wood IS, Maglio MT, Alvarado JA. (1984) Trabecular meshwork cell culture in glaucoma research: evaluation of biological activity and structural properties of human trabecular cells in vitro. *Ophthalmology,* **91**:580-595.

Quigley HA. (1995) Ganglion cell death in glaucoma: pathology recapitulates ontogeny. *Aust N Z J Ophthalmol*, **23**:85-91.

Quigley HA. (1996) Number of people with glaucoma worldwide. *Br J Ophthalmol*, **80**:389-393.

Quinones S, Saus J, Otani Y, Harris ED, Kurkinen M. (1989) Transcriptional regulation of human stromelysin. *J Biol Chem*, **264**:8339-8344.

Quinones S, Buttice G, Kurkinen M. (1994) Promoter elements in the transcriptional activation of the human stromelysin-1 gene by the inflammatory cytokine, interleukin 1. *Biochem J*, **302**:471-477.

Rada JA and Brenza HL. (1995) Increased latent gelatinase activity in the sclera of visually deprived chicks. *Invest Ophthalmol Vis Sci*, **36**:1555-1565.

Ridley SH, Sarsfield SJ, Lee JC, Bigg HF, Cawston TE, Taylor DJ, DeWitt, DL, Saklatvala J. (1997) Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase. *J Immunol*, **158**:3165-3173.

Rohen JW and Witmer R. (1972) Electron microscopic studies on the trabecular meshwork in glaucoma simplex. *Graefe's Arch Klin Exp Ophthalmol*, **183**:251-266.

Rohen JW. (1983) Why is intraocular pressure elevated in chronic simple glaucoma? *Ophthalmology*, **90**:758-765.

Ruiz C, Montes MJ, Abadia-Molina AC, Olivares EG. (1997) Phagocytosis by fresh and cultured human decidual stromal cells: opposite effects of interleukin-1 alpha and progesterone. *J Reprod Immunol*, **33**:15-26.

Samples JR, Alexander JP, Acott TS. (1993) Regulation of the levels of human trabecular matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. *Invest Ophthalmol Vis Sci*, **34**:3386-3395.

Schumer RA and Podos SM. (1994) The nerve of glaucoma! *Arch Ophthalmol*, **112**:37-44.

Shade DL, Clark AF, Pang I-H. (1996) Effects of muscarinic agents on cultured human trabecular meshwork cells. *Exp Eye Res*, **62**:201-210.

Shen TY and Winter CA. (1977) Chemical and biological studies on indomethacin, sulindac, and their analogs. *Adv Drug Res*, **12**:90-245.

Shields MB. Primary open-angle glaucoma (Chap. 9). In "Textbook of Glaucoma." Ed.: Brown C-L. 3rd ed, pp 172-197, 1992(a). Williams and Wilkins, Baltimore.

Shields MB . Visual function in glaucoma (Chap. 6). In "Textbook of Glaucoma." Ed.: Brown C-L, 3rd ed, pp 126-159, 1992(b). Williams and Wilkins, Baltimore.

Shields MB. Anatomic principles of glaucoma surgery (Chap. 31). In "Textbook of Glaucoma." Ed.: Brown C-L. 3rd ed, pp 522-526, 1992(c). Williams and Wilkins, Baltimore.

Shirato S, Bloom E, Polansky J, Alvarado J, Stillwell L. (1988) Phagocytic properties of confluent cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*, **29**(suppl.):125.

Shirato S, Murphy CG, Bloom E, Franse-Carman L, Maglio MT, Polansky JR, Alvarado JA. (1989) Kinetics of phagocytosis in trabecular meshwork cells. *Invest Ophthalmol Vis Sci*, **30**:2499-2511.

Sims JE, March CJ, Cosman D, Widmar MB, MacDonald HR, McMahon CJ, Grubin CE, Wignall JM, Jackson L, Call SM, Friend D, Alpert AR, Gillis S, Urdal DL, Dower SK. (1988) cDNA expression cloning of the IL1 receptor, a member of the immunoglobulin superfamily. *Science*, **241**:585-589.

Sims JE, Gayle MA, Slack JL, Alderson MR, Bird TA, Giri JG, Colotta F, Re F, Mantovani A, Shanebeck K, Gradstein KH, Dower SK. (1993) Interleukin 1 signaling occurs exclusively via the type I receptor. *Proc Natl Acad Sci*, **90**:6155-6159.

Smith ME, van der Maesen K, Somera FP, Sobel RA. (1998) Effects of phorbol myristate acetate (PMA) on functions of macrophages and microglia in vitro. *Biochem Res*, **23**:427-434.

Snyder RW, Stamer WD, Kramer TR, Seftor RE. (1993) Corticosteroid treatment and trabecular meshwork proteases in cell and organ culture supernatants. *Exp Eye Res*, **57**:461-468.

Steely HT, Browder SL, Julian MB, Miggans ST, Wilson KL, Clark AF. (1992) The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*, **33**:2242-2250.

Steinhausen K, Stumpff F, Strauss O, Thieme H, Wiederholt M. (2000) Influence of muscarinic agonists and tyrosine kinase inhibitors on L-type Ca(2+) channels in human and bovine trabecular meshwork cells. *Exp Eye Res*, **70**:285-293.

Steinkamp GW, Hattenbach LO, Heider HW, Scharrer I. (1993) Plasminogen activator and PAI. Detection in aqueous humor of the human eye. *Ophthalmologie*, **90**:73-75.

Stromberg U. (1962) Ocular hypertension. *Acta Ophthalmol*, **Suppl**: 69.

Tamura M, Iwamoto Y. (1989) The effect of platelet-derived growth factor on phagocytosis of cultured human trabecular cells. *Exp Eye Res,* **48**:761-770.

Tao W, Prasanna G, Dimitrijevich S, Yorio T. (1998) Endothelin receptor A is expressed and mediates the [Ca2+]i mobilization of cells in human ciliary smooth muscle, ciliary nonpigmented epithelium, and trabecular meshwork. *Curr Eye Res*, **17**:31-38.

Teski R. (1996) Sphingomyelin breakdown and cell fate. *Trends Biochem Sci*, **21**:468-471.

Tripathi RC and Tripathi BJ. (1982) Human trabecular endothelium, corneal endothelium, keratocytes, and scleral fibroblasts in primary cell culture. A comparative study of growth characteristics, morphology, and phagocytic activity by light and scanning electron microscopy. *Exp Eye Res,* **35**:611-624.

Tyagi SC, Lewis K, Pikes D, Marcello A, Mujumdar VS, Smiley LM, Moore CE. (1998) Stretch-induced membrane type matrix metalloproteinase and tissue plasminogen activator in cardiac fibroblast cells. *J Cell Physiol*, **176**:374-382.

Van Buskirk EM, Pond V, Rosenquist RC, Acott TS. (1984) Argon laser trabeculoplasty. Studies of mechanism of action. *Ophthalmology*, **91**:1005-1010.

Van Buskirk EM. (1989) Pathophysiology of laser trabeculoplasty. *Surv Ophthalmol*, **33**:264-272.

van der Zee E, Everts V, Hoeben K, Beertsen W. (1995) Cytokines modulate phagocytosis and intracellular digestion of collagen fibrils by fibroblasts in rabbit

periosteal explants. Inverse effects on procollagenase production and collagen phagocytosis. *J Cell Sci*, **108**:3307-3315.

Vaughan-Thomas A, Gilbert SJ, Duance VC. (2000) Elevated levels of proteolytic enzymes in the aging human vitreous. *Invest Ophthalmol Vis Sci*, **41**:3299-3304.

Vossler MR, Yao H, York RD, Pan M-G, Rim CS, Stork PJS. (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell*, **79**:73-82.

Weinreb RN, Kashiwagi K, Kashiwagi F, Tsukahara S, Lindsey JD. (1997) Prostaglandins increase matrix metalloproteinase release from human ciliary smooth muscle cells. *Invest Ophthalmol Vis Sci*, **38**:2772:2780.

Welgus HG. (1991) Stromelysin: structure and function. *Prog Inflamm Res Therapy (Agents Actions Suppl)*, **35**:61-67.

Welsh N. (1996) Interleukin-1β-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH_2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. *J Biol Chem*, **271**:8307-8312.

Wheeler LA, Lai R, Woldemussie E. (1999) From the lab to the clinic: activation of an alpha-2 agonist pathway is neuroprotective of retinal and optic nerve injury. *Eur J Ophthalmol*, **9(suppl 1)**:S17-S21.

Wilson K, McCartney MD, Miggans ST, Clark AF. (1993) Dexamethasone-induced ultrastructural changes in cultured human trabecular meshwork cells. *Curr Eye Res*, **12**:783-793.

Wise JB, Witter SL. (1979) Argon laser therapy for open angle glaucoma: a pilot study. *Arch Ophthalmol*, **97**:319-322.

Wise JB. (1981) Long-term control of adult open-angle glaucoma by argon laser treatment. *Ophthalmology*, **88**:197-202.

Withers DJ. (1997) Signaling pathways involved in the mitogenic effects of cAMP. *Clin Sci Colch*, **92**:445-451.

Woessner JF. The matrix metalloproteinase family. In: Parks WC, Mecham RP, eds. *Matrix Metalloproteinases*. San Diego: Academic Press; 1998:1-14.

WoldeMussie E, Ruiz G, Feldmann B. (1990) Muscarinic receptor subtype involved in signaling mechanisms in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*, **31(suppl)**:338.

WoldeMussie E and Ruiz G. (1992) Effect of histamine on signal transduction in cultured human trabecular meshwork cells. *Curr Eye Res*, **11**:987-995.

Yan X, Tezel G, Wax MB, Edward DP. (2000) Matrix metalloproteinases and tumor necrosis factor α in glaucomatous optic nerve head. *Arch Ophthalmol*, **118**:666-673.

Yang J-H, Briggs WH, Libby P, Lee RT. (1998) Small mechanical strains selectively suppress matrix metalloproteinase-1 expression by human vascular smooth muscle cells. *J Biol Chem*, **273**:6550-6555.

Yue BYJT. (1996) The extracellular matrix and its modulation in the trabecular meshwork. *Surv Ophthalmol*, **40**:379-390.

Zhong H, SuYang H, Erdjument-Bromage H, Tempst P, Ghosh S. (1997) The transcriptional activity of NFκB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell*, **89**:413-424.

Zhou L, Fukuchi T, Kawa JE, Higginbotham EJ, Yue BY. (1995) Loss of cell-matrix cohesiveness after phagocytosis by trabecular meshwork cells. *Invest Ophthalmol Vis Sci*, **36**:787-795.

Zhou L, Li Y, Yue BY. (1999) Alteration of cytoskeletal structure, integrin distribution, and migratory activity by phagocytic challenge in cells from an ocular tissue – the trabecular meshwork. *In Vitro Cell Dev Biol Anim*, **35**:144-149.