THE ROLE OF OPIOID GROWTH FACTOR IN ENHANCING THE SUCCESS OF TRABECULECTOMY

A Dissertation in

Anatomy

by

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Abstract

Over 2.2 million people have glaucoma in the U.S. Trabeculectomy surgery is commonly used to prevent complications of glaucoma, and the success of the trabeculectomy depends on the reaction of Tenon's Capsule Fibroblasts (TCFs) to the surgical trauma. The studies depicted in this thesis were aimed at establishing the role of OGF and OGFr in the modulation of cell proliferation of rabbit TCFs (RTCFs) in vitro as well as in vivo.

RTCFs were isolated and established in culture. The presence of opioid growth factor (OGF) and its receptor (OGFr) was determined by immunoreactivity. The kinetics of OGFr were established in receptor binding assays. The ability of OGF to inhibit RTCF proliferation was assessed with dose response growth curves. Receptor-mediated effects of OGF were ascertained by treatment with OGF and naloxone (NAL). The toxicity of OGF action was examined in reversibility experiments. Interference of OGF-OGFr interaction by opioid antagonists or OGF antibody was conducted. The mechanism of action of the OGF/OGFr axis on the survival of RTCFs was evaluated with siRNA, BrdU, and TUNEL techniques.

Treating RTCFs with OGF over a 10 million-fold range of dosages revealed that OGF works to inhibit proliferation of RTCFs in a dose dependent manner. Through receptor binding assays, OGF was seen to work in a one-site model of binding. When treating RTCFs with the long-acting opioid antagonist, naltrexone (NTX), RTCFs proliferated at a much higher rate than control or OGF treated cells. Cell counts of RTCFs treated with mitomycin-C (MMC) were
significantly lower than control, OGF, and NTX treatment groups. MMC and MMC/OGF treated RTCFs did not differ in regard to cell number. The unique ability of OGF to alter cell proliferation was not evident when RTCFs were treated with an array of traditional opioids. OGF works in a receptor-mediated manner. When treated with the short-acting opioid antagonist naloxone, RTCF cell numbers were not significantly elevated compared to controls or RTCFs treated with OGF and naloxone simultaneously.

Specificity of OGF was determined through treatment of RTCFs with an antibody to OGF. Cell counts of antibody treated RTCFs were significantly higher than control counterparts, thus indicating that endogenous OGF plays an integral role in the regulation of cell proliferation.

Reversibility experiments showed that the inhibition of cell proliferation characteristic to OGF is not permanent. This is an important factor in demonstrating the non-toxic inhibitory effects of OGF compared to other anti-proliferative therapies. OGFr siRNA experiments, demonstrated that OGFr is necessary for OGF to inhibit RTCF proliferation. OGF also decreases DNA synthesis in RTCFs compared to NTX, MMC, and control groups. MMC did not alter DNA synthesis. OGF and NTX did not induce apoptosis as evident through caspase 3 and TUNEL assays indicting that both compounds displayed non-toxic characteristics. MMC treated RTCFs had significantly higher caspase 3 activity and TUNEL labeling when compared to control, OGF, and NTX treatment groups. TUNEL labeling revealed that MMC/OGF treated RTCFs possessed
significantly less TUNEL labeling compared to MMC alone. This decrease in late apoptotic activity suggests that OGF has some protective effects.

In the *in vivo* studies, a modified Scheie procedure was performed on New Zealand White (NZW) rabbits. Animals were treated intraoperatively with PBS (control), Hydron + OGF, MMC, or Hydron alone. Intraocular pressure was measured daily by a tonometer. Daily measurements of bleb height, area, and vascularity were assessed via a slit lamp microscope. H&E, trichrome, Giemsa, and Sirius Red staining were used to assess cellularity, blood vessels, inflammatory cells, and collagen fiber orientation. Cell proliferation and apoptosis assays were also performed.

Filtering blebs of the Hydron + OGF group survived more than twice as longer as controls, while also maintaining a reduced IOP for a significantly longer period of time. Filtering blebs of Hydron + OGF, and MMC animals had comparable survival times.

Bleb height and area of the MMC and Hydron + OGF treated animals were comparable throughout the study. IOPs of surviving blebs did not differ between any treatment group, while vascularity of control, Hydron, and Hydron + OGF group was significantly increased compared to MMC.

Histopathology evaluation revealed that Hydron and Hydron + OGF specimens had developed exuberant granulation tissue inside of the surgical bleb at their respective late time points, while the MMC specimen did not. Also, the Hydron + OGF specimen had less small and large collagen fibers within the bleb area compared to the Hydron specimen. DNA synthesis was markedly
increased in the Hydron specimen in the areas of the scleral fistula and Tenon’s capsule compared to control, MMC, and Hydron + OGF groups, whereas the Hydron + OGF specimen had significantly less DNA synthesis than all other treatment groups in the scleral fistula and Tenon’s capsule areas. MMC specimens had an increased amount of TUNEL labeling in the scleral fistula and conjunctiva areas compared to control, Hydron + OGF, and Hydron groups. This finding illustrates that treatment with MMC results in more toxic side effects than OGF, which has been documented to be safe and non-toxic.

In summary these data indicate that OGF is effective at inhibiting RTCFs in a safe and non-toxic manner. However, an optimal vehicle for OGF administration is needed to improve delivery of OGF, and thus optimize the inhibitory effects of OGF in vivo.
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<td>angle closure glaucoma</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>β</td>
<td>beta</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BSS</td>
<td>balanced salt solution</td>
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<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<td>CAIs</td>
<td>carbonic anhydrase inhibitors</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cm$^2$</td>
<td>centimeter squared</td>
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<td>COAG</td>
<td>chronic open angle glaucoma</td>
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<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
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<tr>
<td>ºC</td>
<td>degrees centigrade</td>
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<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<td>[D-Ala$^2$, D-Leu$^5$] enkephalin</td>
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<td>Dulbecco’s Modified Eagle Medium</td>
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<td>deoxyribonucleic acid</td>
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<td>[D-Pen$^{2,5}$] enkephalin</td>
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<tr>
<td>EGTA</td>
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<td>fetal calf serum</td>
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<td>glaucoma drainage devices</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>g/mm²</td>
<td>grams per millimeter squared</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>³H</td>
<td>tridium</td>
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<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>IACUC</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>intraocular pressure</td>
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<td>IRs</td>
<td>implantable reservoirs</td>
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<td>kg</td>
<td>kilogram</td>
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<td>Met</td>
<td>Methionine</td>
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<td>mg</td>
<td>milligram</td>
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<td>mm</td>
<td>millimeter</td>
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<td>millimolar</td>
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<td>MMC</td>
<td>mitomycin C</td>
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<td>mm/Hg</td>
<td>millimeters of mercury</td>
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<td>M</td>
<td>molar</td>
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<td>μ</td>
<td>mu</td>
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<td>μg</td>
<td>microgram</td>
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</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
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<tr>
<td>μm²</td>
<td>micrometers squared</td>
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<tr>
<td>μl</td>
<td>microliter</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<td>NIVM</td>
<td>non-invasive measurement</td>
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<td>nM</td>
<td>nanomolar</td>
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<td>NAL</td>
<td>naloxone</td>
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<tr>
<td>NTX</td>
<td>naltrexone</td>
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<td>OBBS</td>
<td>ocular beta blockers</td>
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<td>OGF</td>
<td>opioid growth factor</td>
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<td>OGFr</td>
<td>opioid growth factor receptor</td>
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<tr>
<td>%</td>
<td>percent</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PGs</td>
<td>prostaglandins</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>PMSF</td>
<td>phenylmethanesulphonylfluoride</td>
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<td>peroxidase</td>
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<td>rabbit Tenon’s capsule fibroblasts</td>
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<td>S.E.M.</td>
<td>standard error of the mean</td>
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<td>SPB</td>
<td>Sorensen’s phosphate buffer</td>
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<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
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CHAPTER I

INTRODUCTION
Glaucoma

Glaucoma refers to a group of diseases that damage the optic nerve, and lead to visual field loss that may progress to blindness [1]. Nevertheless, for the sake of simplicity, “glaucoma” will be used in the singular throughout this report. These devastating diseases are the leading cause of irreversible blindness (5.1 million persons) throughout the world, as well as the second leading cause of bilateral blindness [1, 2]. 66.8 million people suffer from glaucoma worldwide, and it is estimated that by the year 2020 that number will double to more than 130 million people [1]. 2.2 million Americans of age 40 and older suffer from the disease, and it is estimated that 2 million more have the disease and are undiagnosed [1, 2]. Of the numerous types of glaucoma, chronic open-angle glaucoma (COAG) is the most prevalent in black and white populations (85-90% of all glaucomas) [1]. The specific event that initiates COAG has not been determined, however it is likely that a genetic predisposition to COAG starts a series of pre- and post-trabecular mechanisms that lead to a characteristic decrease in aqueous outflow resulting in an increase in intraocular pressure and subsequent optic nerve damage [1]. COAG is most common in black populations (4.7%) compared to whites (1.3%) with the majority of Asian and Hispanic populations being comparable to whites [1]. It is interesting to note that in some Asian populations (Mongolians), angle-closure glaucoma (ACG) is the most prevalent of the glaucomas (64%). Singapore, China, and India also have a large prevalence of ACG compared to the remainder of the world’s population [1, 3]. Although ethnicity is a
predominant factor for developing glaucoma, age has a more commanding influence on prevalence. In white populations a more than 10-fold increase in prevalence of COAG is seen from age groups 40-49 (0.6%) to 80+ (7.3%). This same trend is seen in black populations with a 3-4 times higher prevalence than whites [4]. In regards to the incidence of glaucoma, blacks are over 5 times more likely to have the disease at age 40 than whites, as well as having an increased incidence in the 4th and 5th decades of life.

**Economic Cost of Glaucoma**

The massive economic cost of treating the glaucoma is hard to estimate. However, in the U.S. alone, the total cost of glaucoma treatment is estimated at 3.9 billion dollars [5]. 2.47 billion dollars are spent on medications, while the remainder (1.43 billion dollars) is spent on other glaucoma treatments (e.g., filtering surgery) [5]. It can be extrapolated that the average glaucoma patient’s annual cost of treatment is approximately 2,000 dollars. These economic costs do not take into account the economic impact due to blindness on glaucoma patients. The federal cost to assist blindness is roughly 11,000 dollars per person [6]. Therefore, federal costs of blindness due to glaucoma patients can be approximated to 1.8 billion dollars in the U.S. This brings a total estimated cost of glaucoma in the U.S. to 5.7 billion dollars, although this does not include productive loss of family members caring for a blind relative.

**Current Treatments**

In the numerous types of glaucoma, the two overriding findings are optic neuropathy and increased intraocular pressure (IOP) [1]. In the majority of
glaucomas, the increase in IOP is the most prevalent causative risk factor for the development of optic nerve atrophy, although almost one-third of those with glaucoma in the U.S. have IOPs within the statistically normal range (low tension glaucoma or normal pressure glaucoma). While the optic neuropathy of glaucoma is not completely understood, decreasing the IOP either by medication or surgery still remains the most effective treatment for glaucoma [1]. In categorizing the types of glaucomas for most glaucoma patients, the final common pathophysiologic pathway leads to an alteration of aqueous humor dynamics, which results in an elevated IOP [1]. Currently, early detection and correct IOP management are the only practical means to prevent the detrimental effects of glaucoma. Blindness from glaucoma is preventable; however, blindness is irreversible [1].

Current, medical treatments to lower intraocular pressure in glaucoma patients include the following classes of drugs: prostaglandins, β-blockers, selective adrenergic agonists, carbonic anhydrase inhibitors, miotic agents, and epinephrine compounds [7]. It has been shown that these anti-glaucoma medications can effectively decrease IOP; however, long-term alterations in tissue constituents such as, increased numbers of conjunctival inflammatory cells and Tenon’s capsule fibroblasts have been documented in patients receiving these topical medications [8-10]. After the medications cease being effective, these histological changes have been postulated to lead to an increased rate of glaucoma filtration surgery failure due to fibrosis. All of these
medications should be used only with consultation with an obstetrician during pregnancy.

**Prostaglandins**

Prostaglandins (PGs) are currently the most commonly used and most effective ocular hypotensive agent [11]. The most effective PGs for reducing IOP are: latanoprost, travoprost, bimatoprost, and unoprostone. PGs increase outflow of aqueous humor through the uveoscleral outflow pathway, therefore they do not have an effect on decreasing aqueous production [11]. PGs may increase aqueous outflow by relaxation of the ciliary muscle [12-14]. Also, PGs may cause dilated spaces between ciliary muscle bundles, which are thought to stimulate collagenases and other matrix metalloproteinases [14, 15].

Since 2002, Latanoprost has been approved as a second line glaucoma therapy for open-angle glaucoma and ocular hypertension [11]. In clinical studies PGs have decreased IOP in patients with normal tension glaucoma, exfoliation syndrome, pigment dispersion syndrome, and chronic angle-closure glaucoma [16-18]. Advantages of PGs are the lack of systemic side effects, and the potency and efficacy with once daily dosing. PGs are usually administered once daily in the evening, which may block the early morning diurnal spike in IOP that is normally seen [19]. PGs also are well tolerated by patients, and are as effective at lowering nocturnal IOP as they are during the day unlike other anti-glaucoma medications [19]. PGs can potentially decrease IOP below episcleral venous pressure unlike medication that only decreases
outflow facility. This unique attribute poses an advantage in normal tension glaucoma, which may require very low IOP for adequate control [11].

Although PGs have very few contraindications, the therapy does have some localized side effects. PGs should not be the first medication of choice for patients with cystoid macula edema or those predisposed to it such as aphatics [11]. The most common side effects of PGs are: eye irritation, conjunctival hyperemia, eyelash changes (e.g., increased length, number, and thickness), burning, pain, superficial punctate keratitis, blurred vision, cataracts, and headaches [11]. Although there are few systemic side effects, the biological active acid of latanoprost does reach systemic circulation. However, the active acid is metabolized by the liver through fatty acid-beta oxidation, and it is eliminated by the kidneys. In contrast to beta-blockers such as timolol, PGs have no effect on blood pressure, heart rate, or pulmonary function [20].

**Beta-Blockers**

In 1964, propranolol was the first β-adrenergic antagonist to have widespread clinical applications. Propranolol was used to treat systemic hypertension, angina, and cardiac arrhythmias [11]. Phillips observed a decrease in IOP following systemic administration of propranolol [21]. However, local side effects (e.g., ocular stinging, irritation, and corneal anesthesia) limited propranolol as an IOP hypotensive agent. A β-1-selective antagonist, practolol, has similar hypotensive effects as propranolol, but without the negative side effects [11]. However, adverse effects related to
immunologically mediated oculomucocutaneous syndrome limited the exposure of practolol to patients [11].

Starting in the 1970’s timolol was the favored choice of initial therapy to decrease IOP. Available therapies prior to ocular β-blockers (OBBs) were nonselective adrenergic agonists, parasympathomimetic agonists, and oral carbonic anhydrase inhibitors; however, all of them have significant limitations [11]. Although PGs have replaced ocular β-blockers as a first line therapy, OBBs are still a good alternative.

OBBs are effective because they decrease aqueous formation by as much as 50% without altering aqueous outflow [22]. Timolol and other β-blockers inhibit cAMP production in the ciliary body. It is interesting to note that some studies show conflicting data related to the increase of cAMP to decrease in IOP, while another study revealed no direct relationship [23, 24]. These conflicting results show that IOP decrease may not be completely due to competitive inhibition of ocular β-receptors in the ciliary body [11].

OBBs should not be used by patients with: chronic obstructive pulmonary disease, sinus bradycardia, overt compensated cardiac failure, cardiogenic shock, or second or third degree AV block (without a pacemaker) [11]. β2 receptor blockade causes a decrease in sympathetic activity, which leads to constriction of bronchiole smooth muscle. This blockade commonly causes aggravation of preexisting asthma and other obstructive airway diseases [25]. β2 receptor blockade also depresses myocardial contractibility and excitability leading to unwanted cardiac decompensation in patients that rely on
sympathetic drive because of abnormal cardiac function [25]. Unwanted side effects of OBBs can vary by anatomical location and dosage of the particular therapy. Common local side effects of OBBs are: corneal anesthesia, burning and stinging (due to pH, active molecule, preservative, and vehicle). OBBs are also noted to decrease tear production, and goblet cell density [26, 27].

OBBs are absorbed systemically via the nasolacrimal system, and the nasal mucosa [28]. It is important to note that plasma levels of topically applied timolol can approach the trough levels of systemic administration. These topically applied levels of timolol are much less than the peak plasma levels of the systemic dose [29]. Although topically applied levels are markedly lower than trough levels during systemic administration, thoughtful patient selection would curb many possible systemic side effects. Even with careful consideration of the patients needs, some side effects to the central nervous system do occur, such as: anxiety, depression, fatigue, lethargy, confusion, sleep disturbance, memory loss, dizziness, and decreased libido [30].

Effects of OBBs on the cardiovascular system can present as decreased heart rate, which can lead to bradycardia. Early in timolol's life as an OBB 41% of 32 deaths attributed to topical timolol were cardiovascular in nature. Later, evidence surfaced that the deaths were circumstantial and not causative [31]. After timolol became a widely used OBB pulmonary complications in patients receiving the therapy started to become evident [32]. In its first eight years of use, timolol was responsible for 12 deaths because of its adverse respiratory effects [33]. In a study performed with patients who were using both
bronchodilators and timolol, it was found that 47% of patients needed additional bronchodilators compared to patients who were using bronchodilators alone [34]. OBBs also affect lipid metabolism. Patients using timolol have a 12% increase in triglyceride levels as well as a 9% increase in HDL levels [35].

Adrenergic Agents

Adrenergic agents work by decreasing the aqueous production of the ciliary epithelium by creating a net inhibition of adenylate cyclase. The final messengers of this mechanism are uncertain, however the net inhibition of adenylate cyclase, and the decrease of intracellular cAMP are necessary steps in decreasing aqueous production [11]. This mechanism explains why both β-receptor antagonists, which block endogenous stimulation of adenylate cyclase, and α₂ agonists, which actively inhibit adenylate cyclase, both work to decrease IOP [11].

Epinephrine is a non-selective adrenergic agonist. It was the first topical adrenergic agent used to decrease IOP in patients with open angle glaucoma. Epinephrine causes vasoconstriction to vasculature of the conjunctiva, which in turn causes blanching and slight mydriasis. The decrease in IOP is time dependent. At first, an elevation in IOP is seen, followed by a 12 to 24 hour decrease [36]. Systemic side effects of epinephrine include: tachycardia, extrasystoles, hypertension, palpitation, and anxiety; however, in regards to the eye, epinephrine causes excessive tearing and stinging, and long-term use can lead to allergic blepharitis and conjunctivitis [37, 38]. Epinephrine treatment also is contraindicated for patients with narrow chamber angles, because the induced
mydriasis can precipitate pupillary block, thus creating an angle closure attack [11].

Epinephrine lately has been replaced in the treatment of glaucoma by selective alpha agonists such as, clonidine, apraclonidine, and brimonidine. These drugs have a very safe systemic safety profile after topical administration in adults but are contraindicated in very young children.

Clonidine was originally tested as a nasal decongestant and shaving astringent. On the other hand, it was the first alpha-agonist that was used either systemically or topically for glaucoma treatment [11]. Because of the lipophilic nature of clonidine it can easily penetrate corneal epithelium, endothelium, and the blood brain barrier [39]. It was originally noted to decrease IOP following intravenous administration [40]. Initial studies have shown that one topical administration of clonidine was safe and effective [41]. In later studies it was found that clonidine caused dangerous swings in systemic blood pressure as well as hypotension, syncope, and sedation [42].

Aпрaclonidine is a hydrophilic derivative of clonidine. It has the same effects as clonidine while lacking the negative side effects [43]. The hydrophilic nature of apraclonidine makes it less absorbable, which decreases systemic absorption and blood brain barrier penetration [11]. Apraclonidine decreases IOP by decreasing aqueous production while improving trabecular outflow and decreasing episcleral venous pressure [11]. Apraclonidine has some unwanted side effects such as, dry nasal mucosa, oral mucosa, and decreased oxygen tension in the conjunctiva [11]. Apraclonidine has no cardiovascular side
effects. In one study, after argon laser trabeculoplasty, apraclonidine 1% was seen to decrease the incidence of IOP elevation from 59% to 21% [44]. Apraclonidine also has been used as an adjunct therapy to short and long-term timolol [11]. Apraclonidine has also been used in patients with angle-closure glaucoma attacks [11].

Brimonidine is a highly selective $\alpha_2$ agonist, and it has a lipophilicity between that of clonidine and apraclonidine [11]. Brimonidine has been shown to decrease IOP by reducing aqueous outflow by 20%, while increasing uveoscleral outflow [45]. Brimonidine has similar side effects as apraclonidine, and is a good alternative therapy for patients who have limited success with other glaucoma medications. Some speculate that brimonidine may have neuroprotective properties. One study showed improvement in visual field tests of glaucoma patients currently taking brimonidine [46], while another study showed significant preservation of retinal nerve fibers by scanning laser polarimetry [47].

**Carbonic Anhydrase Inhibitors**

Carbonic anhydrase inhibitors (CAIs) have been an option for patients that were resistant to other glaucoma medications. CAIs are versatile in a wide array of ages and comorbidities, and can be administered either topically or systemically [11]. Many of the aforementioned side effects of other antiglaucoma medications such as, shortness of breath and fatigue are rarely present in CAI treatment. The first CAI used to treat glaucoma was acetazolamide, however a modification to acetazolamide, methazolamide,
offered many advantages such as, better absorption and longer duration of action [11]. CAIs occupy the active portion of carbonic anhydrase making it inactive, thus limiting the accumulation of carbonate in the posterior chamber. The decrease in carbonate is coupled with a decrease in sodium and the associated fluid movement that is linked to the bicarbonate ion [11]. Methazolamide is well tolerated, and usually administered twice a day with the dose ranging from 25-50 mg. Acetazolamide can be given in sustained-release capsules (500 mg) twice daily. This treatment regimen has improved compliance, and has an advantage in decreasing IOP [48]. Side effects of CAIs are many, they are: numbness, paresthesia, malaise, anorexia, nausea, flatulence, diarrhea, depression, decreased libido, poor tolerance of carbonated beverages, myopia, hirsutism, increased serum urate, and rarely seen are thrombocytopenia, and idiosyncratic aplastic anemia [49]. Urolithiasis is common in patients taking CAIs. A decrease in excretion of renal citrate coupled by higher urine levels of calcium forms urate stones [11]. Patients taking acetazolamide have a 15 fold higher incidence of kidney stones than before treatment [11]. These medications can potentiate other diuretics, and lead to rapid loss of potassium or dehydration, particularly in elderly individuals.

Dorzolamide and brinzolamide are both topical CAIs that were introduced in the mid 1990s. Topical CAIs have markedly lower side effects compared to their systemic counterparts, which have greatly reduced the use of systemic CAIs to treat glaucoma [11]. Topical CAIs work in the same way as
systemic CAIs, and it has been shown that topical CAIs are better as adjunct therapies rather than first line agents [50].

*Miotic Agents*

Although miotic therapies have been displaced by newer therapies, they still serve a purpose in angle-closure glaucoma and elderly open-angle glaucoma patients [11]. These therapies mimic acetylcholine at postganglionic parasympathetic junctions as well as other autonomic, somatic and central synapses. Acetylcholine is synthesized by choline acetyltransferase, and it produces its effects by binding to cholinergic receptor at the effector site [51]. Acetylcholine has minimal effects on aqueous formation and episcleral venous pressure [51], but effects are seen on aqueous outflow. Ciliary muscle contraction has two effects on outflow: 1) since there is no barrier between the trabecular lamellae and the ciliary muscle bundles, in the absence of cholinergic stimulus, aqueous can flow from the lamellae to the bundles to the suprachoroidal space through the sclera then finally into the orbit [51]. This unconventional (uveoscleral route) can account for one-third of aqueous outflow. 2) Cholinergic stimulation increases conventional outflow. The ciliary muscle contraction results in unfolding of the trabecular meshwork and the widening of Schlemm’s canal. These two events assist in aqueous outflow from the anterior chamber through the meshwork into Schlemm's canal to venous channels then finally to venous circulation [51]. The conventional drainage route corresponds to the majority of the drainage of aqueous; therefore it
overcompensates for the uveoscleral route, which results in a net lowering of IOP.

Pilocarpine, a non-selective muscarinic receptor agonist, was used in a study that found that removal of the iris from a monkey does not alter the outflow facility response of pilocarpine [52]. These results state that neither miosis nor the presence of the iris is necessary for the aqueous outflow response. Another study indicates that the effect that pilocarpine has on aqueous outflow is nullified if the tendons of the ciliary muscle are severed [53]. This study indicates that the muscle-meshwork attachment is necessary for proper aqueous outflow, and that there are no direct effects of cholinergic stimulation on the cells of the trabecular meshwork or Schlemm’s canal that facilitate outflow.

Cholinergic agonists should not be used by patients with severe asthma, bronchial obstruction, acute cardiac failure, active peptic ulcers, hyperthyroidism, gastrointestinal spasms, urinary tract obstruction, Parkinson Disease, recent myocardial infarction, or poorly controlled blood pressure [11]. Pilocarpine is usually used as a long-term treatment for glaucoma at 0.5-4% by volume and administered up to 4 times daily. However, in acute-angle closure glaucoma with pupillary block 1-2% pilocarpine is used 2-3 times over the span of thirty minutes to try to open the angle by constricting the pupil thus pulling the iris away from the angle structures, and not through its effect of the trabecular meshwork [11].
Prolonged use of cholinergic agents may increase inflammatory cells in the conjunctiva, which makes trabeculectomy more likely to fail [54]. Cholinergic agents also can increase the risk of post-operative inflammation and hyphema [55]. Other ocular side effects include: decreased night vision, corneal toxicity, conjunctiva and intraocular vascular congestion as well as lacrimal canaliculi stenosis, iris cysts, angle closure, and cataracts [11]. Systemic toxicity is rare, however an overdose could produce sweating, salivation, nausea, tremors, slowing of the pulse, and decrease in blood pressure [11].

**Glaucoma Filtering Surgery**

*Trabeculectomy*

The traditional trabeculectomy was first performed by Cairns in 1968 and Watson in 1970 [56, 57]. It is currently the procedure of choice when surgical intervention is needed to decrease IOP. The trabeculectomy functions by the creation of a corneal-scleral fistula. This fistula permits passive diffusion of aqueous humor into the subconjunctival space. As a result, the aqueous bypasses the trabecular meshwork, Schlemm’s canal, and episcleral venous drainage routes. The surgical fistula is covered by a partial thickness scleral flap, which decreases complications (e.g., hypotony, flat anterior chamber, and endophthalmitis) associated with previous full-thickness procedures without compromising control of IOP [58]. In most cases, the aqueous that flows into the subconjunctival space elevates the conjunctiva over the surgical site creating a filtering bleb [58]. The aqueous can then diffuse through the
conjunctiva into the tear film, the conjunctival lymph system, or the conjunctival vessels [59, 60].

Scheie’s Procedure

Scheie’s procedure was the most commonly used filtration procedure until the advent of the guarded trabeculectomy, and it was still used in reoperation procedures until antifibrotic therapies started to become commonplace [61]. Scheie’s procedure is a full thickness procedure that starts with the subconjunctival space being elevated by local anesthetic [62]. After a limbal bases conjunctival flap is made, a thermal cautery is applied to the sclera 1 mm posterior to the limbus. The anterior chamber is entered through the cauterized sclera, and the cautery is applied to the fistula edges to expand the scleral fistula. A peripheral iridectomy is then performed, and the conjunctiva is then sutured water tight [62]. The most common side effects of the Scheie’s procedure are hypotony, flat anterior chamber, and cataract [61]. Although Scheie’s procedure is now considered an obsolete filtering procedure, it still has many beneficial uses in animal models for studying potential antifibrotic therapies.

Glaucoma Drainage Devices

Glaucoma Drainage Devices (GDDs) are usually placed into two categories, nonrestrictive (nonvalved) or restrictive (valved) devices [58]. Nonrestrictive shunts (e.g., Molteno, Baerveldt, and Schocket implants) allow free flow of aqueous from the anterior chamber to the area of the implant. Because of the nonrestrictive nature of these implants there is a high risk of
hypotony and flat anterior chamber prior to their encapsulation by Tenon’s capsule [58]. Restrictive shunts (e.g., Krupin and Ahmed implants) have valves that are designed to hinder the flow of aqueous when IOP drops below a predetermined level [58]. However, some researchers have concluded that the valves of these shunts are functional when tested in air, but lack the intended action when immersed in liquid [63]. Selection of which implant to use is based on a number of factors such as, size and shape of the shunt, previous ocular procedures, severity of glaucoma, and the surgeon’s preference [58]. Although GDDs may be beneficial for certain patients there also are many side effects such as tube shunt occlusion, disturbance in ocular motility, plate and tube migration, and corneal decompensation, which can manifest as corneal endothelial cell loss [58].

**Antifibrotics**

Current intra- or post-surgery adjuvant therapies include treatment with mitomycin-C (MMC) or 5-fluorouracil (5-FU). 5-FU acts by interfering with the synthesis of thymine nucleotides, thus inhibiting DNA synthesis resulting in cell death [64, 65]. 5-FU initially undergoes anabolic transformation into ribosyl and deoxyribosyl nucleotide metabolites. In particular, one metabolite, 5-fluoro-2’deoxyuridine 5’-phosphate (F-dUMP), binds to thymidylate synthetase and its cofactor N5,10-methylene. Tetrahydrofolate forms a tenary complex thereby interfering with synthesis of thymine nucleotides, which results in inhibition of DNA synthesis leading to cell death [64, 65]. *In vitro* and *in vivo* studies have demonstrated that a 5 minute exposure of 5-FU leads to growth arrest [66]. It
has also been documented that 5-FU interferes with qualitative fibroblast functions such as collagen lattice contraction [67]. 5-FU is a useful adjuvant therapy for trabeculectomy because it only inhibits proliferating cells, and it is easy to administer [68].

One of the largest clinical studies done on 5-FU was the Fluorouracil Filtering Surgery Study [69]. After surgery, 5-FU was administered to patients via subconjunctival injection (5 mg) 180° opposite of the surgical site. For the first 7 days 5-FU was given twice daily, and once daily from days 8 to 14. Failure was defined as a return of IOP to control levels or an IOP greater than 21 mm Hg. At the 5 year follow up 51% of trabeculectomies receiving 5-FU had failed versus 74% of control surgical sites. At the 6 month follow up 90% of the blebs of 5-FU group were functional compared to only 65% of the control group. This study also revealed some interesting pre-operative risk factors. The blebs of patients who had a previous ocular surgery within 30 days of the trabeculectomy had failed by post-operative month 12. The time interval between previous surgery and the trabeculectomy was inversely proportional. Patients with primary open-angle glaucoma had the best trabeculectomy results while patients with secondary angle-closure glaucoma faired the worst. Other factors that negatively influenced the time to failure were: poor preoperative visual acuity, high preoperative IOP, Hispanic ethnicity, and the location of the trabeculectomy. 5-FU has been documented to decrease the early onset of fibrosis in filtration surgery, however late fibrosis and remodeling (>2 years post-surgery) is consistent with untreated controls [7, 69].
The most common side effect of 5-FU is corneal toxicity. Epitheliopathy can be present in greater than two-thirds of the patients treated with 5-FU. Epitheliopathy can lead to severe secondary complications such as, corneal ulceration, corneal melting, and perforation [7]. Concurrent use of corticosteroids after surgery can potentiate these side effects [7]. It is also noted that titrating the dose of 5-FU can minimize side effects while not sacrificing surgical success [68]. Eye infection (endophthalmitis) has been documented in 5.7% of patients given 5-FU, and often presents as a late onset endophthalmitis [70]. It also is interesting to note that trabeculectomies performed at the inferior limbus have a 3-fold higher risk of developing late endophthalmitis compared to the superior limbus [70]. 5-FU enhances trabeculectomy success, however high doses result in some level of toxicity. In addition, multiple subconjunctival injections of 5-FU are inconvenient and uncomfortable for patients, which means that a less toxic and safer adjunct therapy is needed.

Prior to the initial 5-FU pilot studies, Chen et al [71] found that intraoperative treatment of MMC during trabeculectomy significantly increases the success rate of the surgery. However, regular use of MMC did not start until the 1990s. MMC is an antibiotic isolated from soil fungus (*Streptomyces caespitosus*), and is classified as an alkylating agent that cross-links DNA (mediated by cytochrome P-450 reductase) [65]. MMC can act on any phase of the cell cycle making it a non-specific alkylating agent, thus hindering DNA synthesis, mitosis, and protein synthesis [7]. MMC is effective at inhibiting the
proliferative phase of the wound healing pathway by killing fibroblasts and endothelial cells [7].

MMC has been well established as an effective antifibrotic agent, and is administered in a one-time application during filtration surgery at dosages of 100 fold less than 5-FU. MMC treated blebs appear thinner and more avascular than blebs treated with 5-FU. When comparing the effects of 5-FU and MMC on Tenon's capsule fibroblasts the results are striking. Fibroblasts cultured from MMC treated rabbits still had evidence of growth inhibition one month after treatment, while 5-FU treated fibroblasts recovered after 7 days [72, 73]. It is of interest to note that most studies performed on MMC are retrospective, and that many factors in each study (e.g., drug concentration, size, number, and type of sponge used, duration of application, and surgical approach) were variable. Also, most studies were of very short duration (several months to 1 year of follow up) [74, 75]. Two studies with the longest time of follow-up, Cheung [76] and Perkins [77], reported 3 year success rates of 88.7 and 47%, respectively. Regardless of the variability of the studies, MMC was found to significantly increase the survival of filtering blebs.

Despite the success of MMC, it has been discovered that the side effects are far more severe than that of 5-FU. Focal bleb leaks are 3 times more frequent in MMC treated blebs compared to 5-FU treated blebs [78]. MMC treated blebs also are more likely to develop endophthalmitis [79], while the incidence of endophthalmitis is similar in 5-FU and MMC treated blebs [80].
There also is an increase in bleb infection associated with full-thickness procedures, and intraoperative MMC administration [81].

5-FU and MMC help to control scarring, but also have serious side effects, such as keratitis, bleb leakage, chronic hypotony with maculopathy, and endophthalmitis [82, 83]. A reliable, non-toxic, physiologic therapy for reducing TCFs proliferation after glaucoma filtration surgery would increase the success rate of the procedure, thereby preventing further irreversible vision loss from glaucoma, while reducing the incidence of important short- and long-term surgical complications.

More recent studies have focused on the use of growth factor inhibitors to modulate wound healing after trabeculectomy. Growth factor suppression and immune modulation without direct cytotoxicity has been utilized in treatments of breast cancer, melanoma, renal cell carcinoma, and endocrine tumors [7]. Khaw et al, have performed in vitro studies on EGF, basic-FGF, TGF-β, and IGF-1, and it has been demonstrated that these growth factors can stimulate fibroblast proliferation, migration, and collagen production [84]. Sala-Prato et al, demonstrated that PDGF and bFGF can stimulate fibroblast proliferation, migration, and collagen production, and that Tenon's capsule fibroblast can be effectively inhibited by rapamycin [85]. There also is mounting evidence that the TGF-β isoforms play an important role in ocular wound healing. TGF-β1, 2, 3 can exhibit regulatory actions on cell differentiation, and cell growth. Furthermore, TGF-β1, 2 can induce epithelial cell proliferation, stimulation, and mesenchymal cell growth [7]. TGF-β2 is present in the vitreous
of normal individuals, and it has been discovered that levels are significantly elevated in patients with glaucoma [86, 87]. Interestingly, the aqueous humor from glaucoma patients can increase fibroblast proliferation by 60% compared to aqueous from normal patients [88].

After the proliferative properties of TGF-β2 had been discovered, Khaw et al, started to study an antibody to TGF-β2, CAT 152, to evaluate its effectiveness at combating fibroblast proliferation, migration, and collagen production. *In vitro* studies established that fibroblast proliferation, migration, and collagen production decreased when fibroblasts were treated with CAT-152 [84]. *In vivo* studies of CAT-152 performed in rabbits showed that bleb vascularity was no different in the CAT-152 group compared to control animals. CAT-152 treated animals did not develop inflammation after trabeculectomy [89, 90]. Histopathology studies concluded that control eyes had significantly greater numbers of fibroblasts and densely packed collagen compared to CAT-152 treated eyes, which exhibited a loose collagen construction, and a visible subconjunctival bleb. When CAT-152 specimens were compared to MMC treated specimens there was no evidence of conjunctival destruction in the CAT-152 animals as was present in MMC treated animals. Also, MMC treated animals had significantly fewer fibroblasts compared to CAT-152 treated animals. Although preliminary studies have gone well, there are still more pre-clinical studies to be performed.
Drug Delivery Systems

Liposomes

Liposomes are microscopic vesicular structures consisting of lipid bilayers separated by water or an aqueous buffer, and are used to transport drug therapies [11]. Liposomes usually consist of a single lipid bilayer or multiple bilayer components. The location of therapeutic drugs within the liposome depends on the drugs’ properties, and they are released by diffusion. Hydrophilic drugs are located in the internal aqueous compartment, while lipophilic drugs are contained within the phospholipid bilayers [91]. The phospholipid bilayers usually are formed by phosphatidylcholine, phosphotidylethanolamine, phosphatidylserine, phosphatidic acid, sphingomyelins, cardiolipin, plasmalogens, and cerebrosides [11]. Liposomes can be prepared a number of ways, including: sonication of dispersions of phospholipids, reverse-phase evaporation, solvent injection detergent removal or calcium-induced fusion [92].

Liposomes have many advantages in ocular drug delivery. Therapeutics administered via liposomes have the ability to evade cell membrane permeability barriers by cell membrane-liposome interactions. Liposomes also can control the rate of drug release, protect the drug from metabolic enzymes, decrease drug toxicity, enhance therapeutic effects, and possibly, increase ocular drug absorption [11].

The rate of efflux of the drug depends on the drug’s properties, and the properties of the liposomal membrane. The phospholipid bilayer used to create
lipsomes changes from a gel crystalline state to a liquid crystalline state at a
given transition temperature [11]. The bilayer becomes more permeable at the
liquid crystalline state thus releasing more therapeutic drug. The incorporation
of cholesterol also can have an affect on drug release increasing the
permeability of the bilayer when more cholesterol is incorporated [93]. Also, a
decrease in acyl chain length and degree of unsaturation can increase
permeability. Charged phospholipids may affect efflux of the confined drug as
well [93].

Five different mechanisms are proposed for liposome-cell interaction,
which are: intermembrane transfer, contact release, adsorption, fusion, and
endocytosis. Adsorption and endocytosis are the most prominent of the five
methods [11]. Increasing the liposomes residence time in the targeted area is
key to allowing the therapeutic drug to work effectively. Viscosity enhancing
polymers (e.g., hydroxypropyl methylcellulose or provinyl alcohol) have been
used to suspend liposomes [93]. Liposomes that are suspended in these
substances have been shown to maintain retention for significantly longer than
traditional liposomes thus allowing the therapeutic drug to stay in contact with
targeted area for a longer duration [93].

The subconjunctival injected drugs in the liposomal form provided higher
drug concentrations in the sclera and cornea up to 24 hours after injection. One
study indicated that liposome-bound cyclosporine injected subconjunctivally
achieved a concentration 40% higher than injected free cyclosporine [94].
Unlike topical drug delivery, which is limited by short shelf life, limited drug-
loading capacity, and difficulty in stabilizing the drug preparation, liposomal delivery prolongs the therapeutic effects of drugs when administered subconjunctivally or intravitreally [93].

*Implanted Reservoirs*

Implantable reservoirs (IRs) are characterized by a central core of drug, which is implanted in either permeable or impermeable polymers. IRs are designed to provide a sustained release of the drug when implanted either subconjunctivally or intravitreally [11]. IRs allow a small amount of drug (5-6 mg) to be compressed in a 2.5 mm tablet die. The pellets are then coated in polyvinyl alcohol (permeable polymer) and ethylene vinyl acetate (impermeable polymer). The reservoir is then treated to change the crystalline structure of polyvinyl alcohol [11].

The release rate of IRs is controlled by size and permeability of the device. After the IR is implanted, water dissolves the outer part of the pellet making it a saturated drug solution [11]. The advantage of IRs is the ability to deliver the active compound directly into the eye to achieve a therapeutic effect [11]. Also, constant delivery of a drug nullifies the need for multiple subconjunctival or intravitreal injections [11]. 5-FU has been used in a sustained release device, which was designed to maintain low levels of therapeutic drug when implanted subconjunctivally. The pellets contained 12 mg of 5-FU coated with permeable and impermeable polymers. When the IR was implanted in the subconjunctival space of a rabbit it released 1 mg of 5-FU daily for 10 days [11]. The 5-FU IR was administered to high-risk glaucoma
patients in Phase I clinical trials. Three out of four patients had maintained successful filtering blebs, while one patient’s bleb failed within 2 months of the surgery [95].

The success of implanting sustained release drugs relies on how the eye reacts to the IR. Risk of endophthalmitis, retinal detachment, vitreous hemorrhage, inflammation, device dislocation, cataract formation, and astigmatism have to be studied [11].

**Hydron**

Slow release polymers have been studied in detail for decades, and some can cause inflammation in the cornea and are not suitable for ocular drug delivery [96]. Langer et al [97], have revealed that hydroxyethylmethacrylate (Hydron) and ethylene-vinyl acetate copolymer do not induce inflammation in the rabbit cornea. Studies whereby sterile polymer pellets (1.5 x 1.5 x 0.5 mm) were implanted in rabbit corneas, and evaluation of histological sections took place 3 and 4 weeks after implantation of the pellets showed no foreign cells in the region surrounding the polymer or in other areas of the cornea [97]. On the other hand, polyacrylamide and polyvinylpyrrolidone polymers produced significantly inflammation in the rabbit cornea. Regarding the polymer release, polyvinylalcohol, Hydron, and ethylene-vinyl acetate copolymers were examined for their ability to release soybean trypsin inhibitor. The study showed that an increase in polymer concentration decreased the rate of release from the polymer. A threshold was reached at a 10% polyvinylalcohol concentration, further increase of the polymer did not yield a decrease in the
release of protein [97]. Interestingly, the release of the protein was dependent on the water-polymer ratio. The Hydron polymer is an attractive vehicle for the time-released administration of ocular therapeutics.

**Opioid Growth Factor (OGF) and Opioid Growth Factor Receptor (OGFr)**

An endogenous pentapeptide, [Met\textsuperscript{5}]-enkephalin, termed opioid growth factor (OGF) because of its regulatory role on cell growth, has been found in a variety of groups of the phylum Chordata, which include Mammalia, Aves, Reptilia, Amphibia, and Osteichthyes [98]. Endogenous opioid systems have been shown to alter development of the mammalian retina. Furthermore, mRNA of the prohormone for OGF (preproenkephalin A, PPE) is present in the developing and mature rat retina [99]. OGF has also been found in the developing rat retina [100]. Previous studies have shown, OGF and its receptor (OGFr) are also present in the corneal epithelium of rat, rabbit, and humans [101-103]. Preliminary studies have suggested that OGF and OGFr are present and functional in human and rabbit TCFs.

The OGFr is unique from other classical opioid receptors (\(\mu, \kappa, \delta, \zeta, \) and \(\epsilon\) receptors) in function, spatial and temporal expression, ligand specificity and subcellular location [104]. Also, OGFr does not share any structural homology with these receptors [104]. Previous receptor binding studies have documented the ligand specificity, saturability, and stereospecificity of OGFr, as well as the presence of an opioid agonist (OGF) [104]. OGF reacts with OGFr on the outer nuclear envelope of the cell, and transport of the OGF-OGFr complex from the cytoplasm into the nucleus sets off a cell signaling cascade [104, 105]. The
OGF-OGFr axis serves as a tonically repressive pathway that regulates cell proliferation through cyclin-dependent inhibitory kinases, causing a delay in the cell cycle at the G₀/G₁ phase [106, 107]. The potent opioid antagonist naltrexone (NTX) accelerates growth, interrupting the OGF-OGFr axis, indicating that the OGF-OGFr system is critical to maintenance of homeostatic equilibrium. OGF acts as an inhibitory growth factor in homeostasis, various cancers, and in the physiologic repair processes such as corneal re-epithelialization following experimental corneal abrasions [101-104, 108, 109].

Along with the known inhibitory effects of OGF on cell proliferation, OGF may also help govern tissue organization [110]. Rabbit corneal explants (endothelium removed) exhibited a smaller outgrowth region in response to OGF, whereas the outgrowth region was increased by the presence of NTX. The effects of OGF were blocked by concomitant treatment with naloxone (NAL), a weak opioid antagonist that is specific for OGFr [110]. OGF treated explants also exhibited a decrease in organization of the outgrowth as determined by morphometric analysis for cell concentration, as well as immunocytochemical analysis for cell type and content.

In conclusion, although IOP may be controlled by surgery and adjunct therapy with MMC and 5-FU, the toxicity of these compounds reduces their overall advantages. The potential use of the OGF/OGFr axis, a tonically active, regulator of cell proliferation, to retard TCF replication warrants experimentation. The hypothesis of this thesis is that the OGF/OGFr axis is present and functions to reduce the proliferation of TCFs in a safe and non-toxic
manner thereby increasing filtering bleb survival. This is the first time that an endogenous or biological substance (OGF) will be used exogenously to down regulate proliferation of TCFs. This treatment has the possibility of eliminating the toxic and detrimental side effects of other antifibrotic therapies used in trabeculectomies. OGF treatment could prolonging the function of trabeculectomies, and benefit the 2.2 million people in the U.S. that have glaucoma, as well as over 66 million people diagnosed with glaucoma throughout the world. This new treatment also could have economic implications by possibly decreasing the cost of repeated surgical procedures to repair failing blebs, and costs due to late onset fibrosis of the trabeculectomy surgical site, which can result in failure of the procedure.
CHAPTER II

THE ROLE OF OPIOID GROWTH FACTOR AND TENON’S CAPSULE FIBROBLASTS:

IN VITRO STUDIES
Abstract

**Purpose:** Over 2.2 million people have glaucoma in the U.S. Trabeculectomy surgery is commonly used to prevent complications of glaucoma, and the success of the trabeculectomy depends on the reaction of Tenon's capsule fibroblasts (TCFs) to the surgical trauma. This study examines whether the opioid growth factor (OGF) and its receptor (OGFr) are present in TCFs and whether addition of exogenous OGF suppresses proliferation of (TCFs) in a safe and non-toxic manner. **Methods:** Rabbit TCFs (RTCFs) were isolated and established in culture. The presence of OGF and its receptor (OGFr) was determined by immunoreactivity. The kinetics of OGFr were established in receptor binding assays. The ability of OGF to inhibit RTCF proliferation was assessed with dose response growth curves. Receptor-mediated effects of OGF were ascertained by treatment with OGF and naloxone (NAL). The toxicity of OGF action was examined in reversibility experiments. Interference of OGF-OGFr interaction by opioid antagonists or OGF antibody was conducted. The mechanism of action of the OGF/OGFr axis on the survival of RTCFs was evaluated with siRNA, BrdU, and TUNEL techniques. **Results:** OGF and OGFr were detected in RTCF cells in vitro using immunohistochemistry. RTCFs treated with $10^{-6}$ M OGF for 72 hours showed a 28%, 30%, and 30% decrease in cell proliferation compared to TCFs treated with sterile water, NAL, and OGF/NAL, respectively. RTCFs treated for 72 hr with naltrexone or antibody to OGF had significant increases in cell number demonstrating OGF specificity. RTCFs transfected with OGFr siRNA showed a 32% increase in cell
number compared to non-transfected RTCFs signifying that OGFr is required for inhibition of the proliferation of RTCFs. OGF treated cells had a 40% decrease in BrdU labeling compared to controls. BrdU labeling in MMC treated cells did not differ from controls. Apoptosis or necrosis was not evident after 72 hr of OGF treatment. Apoptotic indexes of MMC treated cells were approximately 2-fold higher than MMC/OGF treated cells demonstrating possible protective effects of OGF. **Conclusion:** OGF and OGFr are present and function to decrease RTCF proliferation in a safe and non-toxic manner.

**Introduction**

Glaucoma refers to a group of diseases that can damage the optic nerve, and lead to decreased vision, and often, blindness [1]. It is estimated that 67 million people have glaucoma worldwide. These devastating diseases are the leading cause of irreversible blindness (5.1 million persons) throughout the world, as well as the second leading cause of bilateral blindness [1, 2]. 66.8 million people suffer from glaucoma worldwide, and it is estimated that by the year 2020 that number will double to more than 130 million people [1].

One of the most effective ways to treat glaucoma is through glaucoma filtration surgery. However, a serious complication of this surgery is the over proliferation of Tenon’s capsule fibroblasts, which can cause surgical failure. Current therapies (e.g., 5-fluorouracil and mitomycin-C) reduce the fibrosis associated with surgical failure; however unwanted complications such as bleb leakage and infection can occur.
A reliable, non-toxic, and physiologic treatment with minimal complications is desired. Methionine enkephalin (Met<sup>5</sup>-enkephalin), termed opioid growth factor (OGF) is a tonically active, endogenous peptide that has been documented to inhibit cell proliferation. The hypothesis of the proposed research is that the OGF-OGFr axis is present in isolated rabbit Tenon’s capsule fibroblasts (RTCFs), and that an endogenous opioid functions to inhibit the proliferation of these cells. This study will establish in vitro the feasibility of in vivo trabeculectomy surgery by examination of efficacy, toxicity, and mechanism of action.

**Materials and Methods**

**Rabbit Tenon’s capsule fibroblasts.** RTCFs were dissected from mature New Zealand white rabbits (~ 4 kg) that had been euthanized with sodium pentobarbital (100 mg/kg; i.p.). These rabbits were obtained under IACUC protocol 2006-020-2 (Dr. Zagon, P.I.), and were unused or control animals purchased for other studies in the laboratory.

After Tenon’s capsule dissection, primary explant technique was utilized to isolate RTCFs [111]. After initial dissection, the tissue was immediately placed in a sterile petri dish (9 cm) and rinsed in balanced salt solution (BSS). The tissue was transferred to another 9 cm petri dish while unwanted tissue was dissected off (e.g., fat and necrotic tissue). The remaining tissue was cut into 1 mm cubes using a cross scalpel technique. The 1 mm cubes were transferred with a 10 ml wide tipped pipette into a 50 ml sterile centrifuge tube containing BSS. The pieces were washed three times by allowing the tissue to
settle in the centrifuge tube. The supernatant was poured off and fresh BSS added. The pieces were transferred to a 25-cm$^2$ flask and spread evenly. 1 ml of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum, 1.2% sodium bicarbonate, and antibiotics (5,000 units/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin) were added to the flask and maintained in a 37°C incubator with a 5% CO$_2$/95% air mixture. Once the tissue had adhered to the flask an additional 1 ml of DMEM with 20% FCS was added to the flask daily until a total volume of 5 ml was obtained. Media was changed weekly until substantial outgrowths were observed. Once the outgrowths had reached approximately 50% confluence the tissue was removed and the RTCFs were subcultured. Falcon 75-cm$^2$ flasks and DMEM with 10% FBS was used for subculture. Each experiment was performed twice.

**Purity of RTCF Population.** The purity of the harvested RTCFs was assessed via cell counting (quantitatively) and phalloidin staining (qualitatively). RTCFs were seeded on 22 mm coverslips (100,000 cells per well) allowed to adhere for 24 hours then stained with hematoxylin and eosin or phalloidin. Human umbilical vein endothelial cells (HUVEC) were used as positive controls for phalloidin staining. The phalloidin staining was performed as follows, cells were fixed and permeabilized in 95% ethanol for 5 minutes and acetone for 5 minutes at -20°C and stored at -20°C. Fibroblasts were stained within 3 days of fixation. Cover slips were rinsed with phosphate buffered saline (PBS), and then stained with Alexfluor-488 phalloidin (1:40, Molecular Probes, Eugene, OR) diluted in
PBS with 1% bovine serum albumin (BSA) for 30 minutes. Coverslips were then washed in PBS for 30 minutes and mounted. Cells that did not appear to be fibroblasts were not counted as such. Six random grids were counted on each coverslip, and six coverslips were used. The number of fibroblasts counted per total number of cells was used to create a percentage of fibroblasts in the cell population.

**In vitro analysis of OGF and OGFr immunocytochemistry.** RTCFs were stained with antibodies specific for OGF and OGFr using well established protocols [25, 32]. In brief, log phase RTCFs were seeded (50,000 cells per well) onto 22 mm diameter cover slips, which were placed in 6-well Falcon culture plates. Cells were fixed and permeabilized in 95% ethanol for 5 minutes and acetone for 5 minutes at -20°C and stored at -20°C. Fibroblasts were stained within 3 days of fixation. Cover slips were rinsed with Sorensen’s phosphate buffer (SPB), and blocked with SPB and 3% normal goat serum and 0.1% Triton –X-100 at 22°C for 15 minutes. Ammonium sulfate purified anti-[Met5]-enkephalin IgG was diluted (1:100) in SPB with 1% normal goat serum and 0.1% Triton-X-100 was added for 2 hours at 4°C. Cover slips were washed and incubated with goat anti-rabbit IgG (1:1000) conjugated to rhodamine. Some cells were incubated with secondary antibodies preabsorbed with either an excess of OGF or excess OGFr-fusion protein, and served as controls.
In vitro analysis of OGFr by receptor binding assays. To quantify the kinetics of OGFr in RTCFs, receptor binding assays were performed. Preparation of protein fractions were completed by harvesting log phase RTCFs grown in 75 cm² flasks by scraping (trypsin inactivates the protein), and the cells were pelleted by centrifugation. The pellet was washed and homogenized in a 1:10 (vol/vol) solution of cold 50 mM Tris-HCl with 0.1 mg/ml bacitracin, 1 μg/ml leupeptin, 6nM thiorphan, 1mM EGTA, and 3.5 mM PMSF (pH 7.4) at 4°C (Tris/all). Homogenates were layered over a 1.4 M sucrose cushion, and centrifuged at 4000 rpm for 30 minutes. This procedure obtained a clean, relatively pure population of nuclei.

Nuclear pellets were diluted with Tris/all to the appropriate protein concentration and incubated at room temperature (22°C) for 20 minutes to remove endogenous opioid peptides. Aliquots of protein were incubated with 50 μl of [³H]-[Met⁵]-enkephalin (PE-NEN, Boston, MA) in Tris/all buffer. Saturation assays were carried out for 60 minutes at 22°C. Incubations were terminated by rapid filtrations through Whatman GF/B filters under vacuum pressure with a Brandel Cell Harvester. Filters were rinsed 3 times with 5-ml volumes of ice cold 50 mM Tris/all, dried at 60°C for 1 hour, and counted by liquid scintillation spectrometry (Beckman LS-2800). Non-specific binding was determined in the presence of 100 nM unlabeled [Met⁵]-enkephalin. Duplication tubes of homogenates were assayed for each concentration. Protein concentrations were determined by the BioRad method with γ globulin as a standard. Specific and saturable binding was analyzed by GraphPad
prism software; the software also generated Bmax and Kd values.

**Cell proliferation assays.** To assess growth in the presence of OGF, NTX, mitomycin-C (MMC), MMC/OGF fibroblasts were seeded (5 x 10^4 per well) into 6-well or 24-well plates and incubated for 24, 48, 72, and 96 hours in media and compounds. Media and compounds (OGF, NTX, and sterile water) were replaced daily. MMC (4 μg/ml) was administered once for 5 minutes. Over a 36 day study of the effects of MMC on human TCFs, it can be extrapolated that this dose was the average ID_{50} dose of MMC [66]. After 5 minutes the media and drug were pipetted off, and the cells were rinsed with sterile PBS, then new media was replenished. Sterile water served as a control for all experiments. Cells were harvested from the monolayer with 0.25% Trypsin/0.53 mM EDTA, centrifuged, and re-suspended in trypan blue stain. Viable cells were counted using a hemocytometer at 20X magnification. Dead cells were noted as well.

**Dose Response.** To determine if OGF was dose dependent, RTCFs were treated with OGF (10^{-10} to 10^{-4} M) for 72 hours. Sterile water served as control, and drug and media were changed daily.

**Competing opioids.** To determine the effects of competing opioid peptides, other competing opioid peptides [DAla^{2}, MePhe^{4}, Glycol^{8}] enkephalin (DAMGO), morphine, [D-Pen^{2,5}] enkephalin (DPDPE), [D-Ala^{2}, D-Leu^{5}] enkephalin (DADLE), dynorphin A, endomorphin-1, U-59693, and β-endorphin (all in 10^{-6} M) were added to 96-well cultures for 72 hours; sterile water served as control. Drug or vehicle, and media were changed daily. 1000 cells/well
were seeded into 96 well Falcon tissue culture plates. After 24 hours in culture drug treatments were added to the wells. The final volume of media/drug in each well was 200 $\mu$l. At 72 hours of drug exposure, 100 $\mu$l of media was removed from each well and 20 $\mu$l/well of Promega Cell Titer 96 Aqueous One Solution Cell Proliferation Assay was added and incubated for 4 hours. Cell proliferation was measured by recording absorbance at 290 nm and a 750 nm filter on a BioRad Plate Reader. The plate consisted of 10 wells/treatment and the mean of those absorbencies were plotted.

**Opioid receptor mediation.** To determine if the effects of OGF were receptor mediated, RTCF cultures in 24-well plates were exposed to OGF and the short-acting opioid antagonist naloxone at concentrations of $10^{-6}$ M [33]; cultures treated only with OGF or naloxone were included. Drugs and media were changed daily; and cells were treated for 72 hours then counted.

**OGF antibody.** RTCFs were treated with either sterile water, an antibody to OGF, or pre-immune serum for 72 hours. Compounds and media were changed daily. RTCFs were harvested and counted as described earlier.

**Reversibility of OGF growth inhibition.** The ability to reverse OGF’s inhibitory action on growth was assessed as a measure of the non-toxic nature of OGF. Cells were seeded into 6-well plates and treated with OGF or an equal volume of sterile water for 72 hours at which time; half of the OGF treated wells were replenished with media not containing OGF. The other half of the OGF treated cells continued to receive OGF. All cells were counted 48 hours later (e.g., 120 hours in culture).
Unless otherwise stated for all cell proliferation assays, three wells per treatment were established for each time point; two aliquots per well were counted providing 6 cell counts for each condition. Cell viability was determined by trypan blue staining.

**Mechanisms.**

To determine the specificity of OGF and to establish the pathway of OGF activity, OGF was knocked down with siRNA technology. Mechanisms of action by which the OGF/OGFr axis functioned were investigated by assessment of DNA synthesis and cell death.

**siRNA transfections.** siRNA transfections were performed to establish the specificity for OGF and other competing opioids for OGFr. OGFr siRNAs were obtained as ready-annealed, purified duplex probes (Invitrogen, Carlsbad, CA). For transfections, 5 x10^4 cells were seeded into 24 well plates with antibiotic free media. Transfections took place 24 hours after seeding. Non-transfected cells and cells transfected with negative control siRNA (Ambion Inc., Austin, TX) served as controls. For each well, 0.4 µl of siRNA stock solution (50 µM) was added to 50 µl of serum and antibiotic free media. 1 µl of oligofectamine was diluted in 50 µl of serum and antibiotic free media. These two mixtures were incubated for 5 minutes at room temperature (20°C). The oligofectamine and siRNA volumes were mixed together and incubated for 20 minutes at room temperature. 100 µl of this mixture was added to each well. Cells were incubated for 4 hours at 37°C prior to the addition of OGF, NTX, competing opioids, or sterile water. 24 hours after transfection, media was
replaced with 1 ml of complete media and drug or sterile water. Cells were counted at 72 hour after transfection, and RNA for northern blot analysis was collected at 72 hours after transfection. Two independent experiments were conducted.

Total RNA was extracted using the Paris Kit (Ambion), separated on an agarose gel, and transferred to a nylon membrane (Immobilon, Bio-Rad Laboratories, Hercules, CA). Membranes were probed with 32P-dCTP-OGFr cDNA. To control for equal loading, blots were stripped and re-probed with radiolabeled GAPDH and optical density of each band was determined and analyzed by QuickOne (Bio-Rad Laboratories). Each value was normalized to GAPDH from the same blot. Means and SE were determined from at least 2 independent experiments.

DNA synthesis. RTCFs were treated for 72 hours as described in cell proliferation assays. DNA synthesis was evaluated as the number of cells incorporating BrdU. RTCFs grown on 22mm cover slips and subjected to BrdU were processed with BrdU-POD antibodies and labeled cells counted. At least 1000 fibroblasts were evaluated.

Cell Death. RTCFs were treated for 72 hours as described in cell proliferation assays then evaluated via caspase-3 ICC or TUNEL.

After treating RTCFs for 24 hours, caspase 3 activity was assessed by immunocytochemistry using an anti-caspase 3 antibody (1:100, Invitrogen, Carlsbad, CA) via immunofluorescence. RTCFs were fixed in 4% paraformaldehyde in PBS for 30 minutes, and washed in PBS, 0.1% Tween 20
for 20 minutes. RTCFs were blocked with PBS 0.1% Triton X-100, 1% BSA for at least 2 hours at room temperature, incubated with primary antibody overnight at 4°C, washed with PBS 0.3% Triton X-100, 0.1% BSA, 2% NGS three times for 1 hour, and incubated with secondary antibody conjugated to Alexa-Fluor 488 (Molecular Probes, Eugene, OR) 1:5000 for 2 hours at room temperature [112].

After treating RTCFs for 72 hours, nuclear fragmentation was assessed using a DermaTACS In Situ Apoptosis Detection Kit (Trevigen; Gaithersburg, MD). At least 1000 cells were evaluated both experiments.

Statistics. Data on cell counts, number of dead cells, and presence of BrdU positive cells were analyzed by one-way analysis of variance with Newman-Keuls posts tests. Non-linear regression curved fit analysis was performed to established the doubling time of RTCFs. A p-value of <0.05 was considered reliably significant.
Results

Purity of RTCFs

Based on microscopic observations of H&E and fluorescent phalloidin stained RTCFs, initial RTCF populations were 99.2% pure. There is a distinct difference in the distribution of fluorescent phalloidin in RTCFs compared to HUVEC cells indicating that isolated cultures of RTCFs were relatively devoid of other cell types (Figure 2.1). The initial 96-hour growth curve produced a doubling time of 28.6±3.8 hours.

Presence of OGF and OGFr

Staining RTCFs with antibodies specific for either OGF or OGFr demonstrated the presence of both OGF and OGFr in RTCFs (Figure 2.2). Both OGF and OGFr were seen in the cytoplasm, and directly outside of the nuclear membrane. The localization of OGF and OGFr outside of the nuclear envelope is supported by previous research on the distribution of subcellular OGF and OGFr in epithelial cells [105, 113].

In vitro analysis of OGFr by receptor binding assays

Standard pharmacological assays were utilized to determine if there was specific and saturable binding of radiolabeled methionine enkephalin (OGF) in the nuclear fraction of RTCFs. Log phase cultures were harvested and the nuclear fraction isolated. Binding data suggested a one-site model of binding,
Figure 2.1. RTCFs and HUVEC cells stained with fluorescent phalloidin. Fluorescence indicates the presence and distribution of F-actin. RTCFs isolated and established in culture showed a distinct distribution of F-actin indicating the presence of fibroblasts.
Figure 2.2  Photomicrographs of immunocytochemical staining of RTCFs for OGF and OGFr. Cells were seeded on 22 mm coverglasses and grown for 48 hrs before staining with anti-OGF or anti-OGFr IgG and rhodamine conjugated secondary antibody. Cells were photographed with an Olympus Spot RT camera. DIC= direct interference contrast. Insert= 2 antibody only Bar= 15 mm.
with binding capacity ($B_{\text{max}}$) of 6.5 ± 0.8 fmol/mg protein and a binding affinity ($K_d$) of 4.6 ± 1.4 nM (Figure 2.3).

Dose Response

OGF in dosages ranging from $10^{-4}$ to $10^{-10}$ M was added to 24-well cultures for 72 hours; sterile water served as control. Cell counts of RTCFs treated with $10^{-7}$, $10^{-6}$ and $10^{-5}$ M OGF were 28%, 33%, and 37% respectively lower than controls ($p<0.001$, Figure 2.4). RTCFs treated with $10^{-4}$ M OGF showed a decrease in proliferation of 47% relative to controls ($p<0.001$), as well as a 26% ($p<0.01$) and 22% ($p<0.05$) decrease in cell proliferation compared to RTCFs treated with $10^{-7}$ or $10^{-6}$ M OGF, respectively. Cell counts of RTCFs treated with $10^{-10}$, $10^{-9}$ and $10^{-8}$ M OGF did not differ from controls. These data demonstrate that OGF works in a dose dependent manner to decrease the proliferation of RTCFs.

Cell Proliferation 96 hour Growth Curve

The inhibitory effects of OGF were further studied in conjunction with the long acting opioid antagonist naltrexone (NTX), MMC, and a MMC/OGF combination. At 24 hours, cell counts of NTX treated cells were 37% greater than controls while cell counts of MMC and MMC/OGF treated groups were 70% less than controls (Figure 2.5). Cell counts of OGF treated RTCFs were 16 to 33% lower than controls starting at 48 hours and continuing through 96 hours ($p<0.001$).
Figure 2.3. Representative saturation isotherm of specific binding of \[^{3}H\]-[Met\(^5\)] enkephalin (■). Mean ± S.E.M. binding affinity (K\(_d\)) was 4.6 ± 1.4 nM and binding capacity (B\(_{max}\)) was 6.5 ± 0.8 fmol/mg protein.
Figure 2.4. RTCFs treated for 72 hr with OGF (10^{-10} to 10^{-4} M) or sterile water. 40,000 cells per well were seeded into 24-well plates. Statistically significant from controls and OGF (10^{-10} M to 10^{-8} M) at p<0.001 (**). Values represent means ± S.E.M.
Figure 2.5. RTCFs were treated daily with either OGF (10^{-6} M), NTX (10^{-6} M), or sterile water. MMC (4\mu g/ml) was administered one time for 5 minutes. Media was changed daily. 50,000 cells per well were seeded in 6-well plates. Statistically significant from controls at p<0.001 (***) and controls and OGF at p<0.001 (+++), and controls, NTX and OGF at p<0.001 (^^^). Values represent means ± SEM.
Competing Opioids

To determine the effects of competing opioids on the proliferation of RTCFs, eight classic opioids were chosen; DAMGO, morphine, DPDPE, DADLE, dynorphin A1-8, endomorphin-1, endomorphin-2, and β-endorphin. After 72 hours of treatment, no significant increase or decrease in cell proliferation was seen in any of the competing opioids (Figure 2.6). OGF treated RTCFs had a 33% decrease in proliferation ($p<0.001$), while NTX treated RTCFs had a 29% increase in cell proliferation ($p<0.05$) compared to controls at 72 hours. Thus, OGF is unique from other classic opioids in its ability to decrease cell proliferation.

Opioid Receptor Mediation

To determine if the effects of OGF are receptor mediated, RTCF cultures in 24-well plates were exposed to OGF and the short-acting opioid antagonist naloxone (NAL) at concentrations of $10^{-6}$ M [33]; cultures treated only with OGF or NAL were included. RTCFs treated with $10^{-6}$ M OGF for 72 hours showed a 28%, 30%, and 30% decrease in cell proliferation compared to RTCFs treated with sterile water, NAL, and OGF/NAL, respectively ($p<0.001$, Figure 2.7.). Cell counts of RTCFs treated with sterile water or OGF/NAL did not differ from each other. These data demonstrate that OGF acts in a receptor mediated fashion.
Figure 2.6. RTCFs were treated for 72 hr with competing opioids (10^{-6} M), OGF (10^{-6} M), NTX (10^{-6} M), or sterile water. Statistically significant from controls at p<0.001 (***) and p<0.05 (*). Values represent means ± S.E.M.
Figure 2.7. RTCFs treated for 72 hr with OGF (10^{-6} M), NAL (10^{-6} M), OGF/NAL, or sterile water. 40,000 cells were seeded per well into 24-well plates. Statistically significant from controls, NAL, and OGF/NAL at \(p<0.001\) (**). Values represent means ± S.E.M.
Specificity of OGF

To demonstrate if endogenous OGF present in RTCF cultures regulates cell proliferation, an antibody to OGF, or pre-immune serum was added for 72 hours. Cell counts of RTCFs treated with OGF antibody were 21% greater than control and pre-immune serum treated cells (Figure 2.8), suggesting that OGF neutralization by antibody can increase cell proliferation. Cell counts of control and pre-immune serum groups were comparable.

Reversibility of OGF growth inhibition

Reversibility experiments were performed in order to evaluate whether the inhibitory effects of OGF were permanent and toxic. After 48 hours of treatment with OGF, cell counts were 31% less than controls (p<0.001) (Figure 2.9). At 72 hours, OGF treatment for half of the RTCFs ceased (OGF-Control). OGF-Control treated RTCFs started to return to a normal proliferative state, while RTCFs that were continuously treated with OGF through 120 hours were significantly less in number compared to controls and the OGF-Control group. Non-linear regression curve fit analysis revealed that the doubling times for control, OGF-control, and OGF-OGF groups were 36 ± 5.9, 39 ± 5.8, and 44 ± 8.3 hours respectively.

siRNA

In order to determine the specificity of OGFr as the receptor for OGF, RTCF cultures were transfected with OGFr siRNA and subsequently treated with OGF. RTCFs were transfected for 24 hours with OGFr siRNA, a scrambled siRNA control, or not transfected. The efficacy of transfection was
Figure 2.8. RTCFs were treated with an antibody to OGF (1:50), serum, or sterile water for 72 hr. 50,000 cells per well were seeded into 24-well plates. Statistically significant from controls and pre immune serum at \( p < 0.01 \) (**). Values represent means ± S.E.M.
Figure 2.9. RTCFs were treated with OGF (10^{-6} M) or sterile water for 72 hr at which time, half the OGF treated cells were then treated with sterile water for another 48 hours, while the other half continued receiving OGF. 50,000 cells per well were seeded in 6-well plates. Statistically significant from controls at p<0.001 (***) and p<0.01 (**); from OGF-Control group at p<0.01 (++). Values represent means ± S.E.M.
determined by Northern blot quantitation, and revealed that OGFr siRNA transfected RTCFs had a 60% reduction in OGFr RNA compared to wild type RTCFs and RTCFs transfected with scrambled siRNA (Figure 2.10A). Cell counts of RTCFs transfected with OGFr siRNA were significantly higher than those of scrambled siRNA or non-transfected groups independent of treatment (Figure 2.10B). Non-transfected and scrambled siRNA RTCFs treated with OGF showed a 25% and 18% decrease in cell number compared to non-transfected controls (p<0.001). These data demonstrate that OGFr is necessary for inhibition of cell proliferation.

**Cell Proliferation BrdU Assay**

To evaluate whether OGF decreases cell proliferation by altering DNA synthesis, the amount of incorporated BrdU was assessed. After 72 hours of treatment, the percentage of BrdU positive cells in the OGF group was 44% lower than controls (Figure 2.11). NTX treated RTCFs had a labeling index 1.3 times higher than controls (p<0.01). Labeling indexes of MMC/OGF treated cells were comparable to OGF treated cells. MMC did not significantly alter DNA synthesis.

**Apoptosis**

*Caspase 3 ICC.* Staining RTCFs for caspase 3 had a two-fold purpose, which was to demonstrate that MMC did in fact induce apoptosis at an early stage after treatment, and that OGF did not push cells into the apoptotic path to cell death. This is important in evaluating the non-toxic and possible protective properties of OGF. RTCFs treated with either MMC or MMC/OGF showed a
Figure 2.10. OGFr is required for OGF's inhibitory action on growth. A) Northern blot analysis and quantitative densitometry demonstrating the specificity and level of OGFr knockdown in RTCFs. Log phase cells were transfected for 24 h with either scrambled siRNA or OGFr siRNA. Forty-eight h after transfection, cells were harvested and RNA isolated. Data (percent of OGFr/GAPDH ratio) represent mean ± SEM for 2 blots from independent experiments. Significantly different from non-transfected and scrambled siRNA cultures at p<0.05 (*). B) Growth of RTCF cultures transfected with OGFr siRNA or scrambled siRNA for 24 h and treated with either OGF (10^{-6} M), NTX (10^{-6} M), or an equivalent volume of sterile water for 72 h; compounds and media were changed daily. Values represent mean ± SEM cell counts for at least 2 aliquots/well and least 2 wells/treatment. 50,000 cells were seeded per well into 24-well plates. Statistically significant from controls or OGF treated controls at p<0.001 (**), and p<0.01 (*).
Figure 2.11. RTCFs treated with OGF (10⁻⁶ M), NTX (10⁻⁶ M), MMC (4μg/ml), MMC/OGF or sterile water for 72 hr. Cells were then incubated with BrdU (30 mM) for 5 hours before staining. Over 1,000 cells were counted. Statistically significant from Control at p<0.01 (**), p<0.05 (*), OGF at p<0.05 (+), NTX at p<0.001 (^^^), and p<0.01 (^). Values represent means ± S.E.M.
1.39 to 1.46 fold increase in caspase 3 staining compared to RTCFs treated with OGF, NTX, or sterile water (Figure 2.12). Caspase 3 activity was comparable in MMC and MMC/OGF treatment groups.

*TUNEL Assay.* Cell death was assessed by TUNEL staining. MMC treated RTCFs showed a 26-fold increase in TUNEL labeling compared to controls ($p<0.001$, Figure 2.13). In contrast, MMC/OGF treated cells had significantly higher apoptotic index than controls, however MMC/OGF RTCFs had 46% less labeling than MMC treated cells. NTX and OGF did not induce apoptosis.
Figure 2.12. Caspase 3 activity measured in RTCFs that were treated with either OGF (10⁻⁶ M), NTX (10⁻⁶ M), MMC (4μg/ml), MMC/OGF, or sterile water for 24 hours. A) Photomicrographs of RTCFs stained for the apoptotic marker Caspase-3 (green), cell nuclei were stained blue with DAPI. B) Mean grey scale measurements of the caspase 3 present in RTCFs. Significantly different from control at p<0.001(***) from OGF at p<0.001(+++), and from NTX at p<0.001(^^^). Values represent means ± SEM.
Figure 2.13. Number of apoptotic cells measured by the TUNEL technique after RTCFs were treated with OGF (10^{-6} M), NTX (10^{-6} M), MMC (4\mu g/ml), MMC/OGF, or sterile water for 72 hr. Statistically significant from Control at $p < 0.001$ (***) and $p < 0.01$ (**), OGF at $p < 0.001$ (+++), and $p < 0.05$ (+), NTX at $p < 0.001$ (^^^), and $p < 0.01$ (^^), and MMC/OGF at $p < 0.01$ (##). Values represent means ± S.E.M.
**Discussion**

In this study, the OGF/OGFr axis was found to be present, and to actively inhibit proliferation of RTCFs. Receptor binding data indicate that OGF functions in a one-site model of binding. Treating RTCFs with naltrexone significantly increased cell number compared to control or OGF treated cells. MMC significantly lowered cell number compared to control, OGF, and NTX treatment groups. RTCFs treated simultaneously with MMC and OGF did not yield an additive effect. The action of OGF on RTCF proliferation was receptor mediated. Synthetic and natural opioids that are selective for classical opioid receptors (µ, κ, and δ) did not alter cell proliferation. OGF also has a reversible action in regards to modulating cell proliferation supporting the evidence that OGF does not induce apoptosis or necrosis. MMC, unlike OGF, significantly increases the number of apoptotic cells in culture as observed through early and late markers for apoptosis. Therefore, for the first time, OGF has been shown to modulate cell proliferation of RTCFs, which could potentially translate into a new therapy to combat fibrosis in glaucoma filtration surgery.

OGF and OGFr have been found in a variety of groups of the phylum Chordata, which include Mammalia, Aves, Reptilia, Amphibia, and Osteichthyes [98]. The inhibitory effects of OGF have been previously documented in a variety of cancer and normal cell lines [102-104, 114-117]. This study has demonstrated the same specific, reversible, and inhibitory effects of OGF, but now in RTCFs. Furthermore, along with its known inhibitory effects on cell proliferation, OGF may also help govern tissue organization.
Decreasing tissue organization may have a beneficial role in preventing fibrosis of glaucoma filtration surgery.

OGF actively modulates cell proliferation without causing cell death, which is unlike current antifibrotic therapies. The inhibitory effect of OGF on cell proliferation in RTCFs is consistent with previous studies [102, 103, 108-110, 117]. Moreover, siRNA experiments have shown that when OGFr is knocked down, addition of exogenous OGF does not alter cell proliferation. Moreover, following knockdown of OGFr, RTTCFs do not respond to NTX, demonstrating that other opioid receptors are not involved in cell proliferation. It can therefore be stated that OGFr is necessary for OGF to function in RTCFs. Conversely, neutralizing endogenous OGF with an antibody to OGF produces an increase in cell number, which confirms that endogenous OGF has a role in maintaining normal cell replication. The decrease in cell number of RTCFs treated with OGF correlates with the decreased number of BrdU positive cells seen in culture. These data combined with the lack of cell death associated with OGF treated RTCFs, demonstrates that OGF works by inhibiting cell proliferation through DNA synthesis and not apoptosis. Having demonstrated the effectiveness of OGF in inhibiting proliferation of RTCFs in culture, the next step in evaluating OGF as a novel antifibrotic therapy is to test its inhibitory effects in an animal model of glaucoma filtration surgery.

In the realm of glaucoma filtering surgery, many adjunct therapies have been studied; however, most have harmful side effects (e.g., infection, loss of vision) that can nullify any beneficial attributes of that treatment. It is necessary
to examine other therapies that are as effective as traditional adjunctive therapies (e.g., 5-FU and MMC) without killing healthy cells located in and around the surgical area. OGF is an endogenous, non-toxic, and safe biotherapy that does not invoke apoptosis, but instead, works through cyclin-dependent inhibitory kinase pathways to inhibit proliferation of normal cells [106, 107, 118]. Having demonstrated the effectiveness of OGF in cell culture, it is necessary to test it in an in vivo model of glaucoma filtering surgery to fully assess the efficacious properties of OGF.
CHAPTER III
THE ROLE OF OPIOID GROWTH FACTOR IN ENHANCING TRABECULECTOMY: IN VIVO STUDIES
Abstract

Purpose: Trabeculectomy commonly is used to prevent complications of glaucoma, and the success of this procedure depends on the reaction of Tenon's capsule fibroblasts (TCFs) to the surgical trauma. Current adjuvant therapies used to reduce cell proliferation and fibrosis such as 5-fluorouracil and mitomycin-C (MMC) have serious side effects such as toxicity and bleb leakage that may be complicated by endophthalmitis. This study examined the efficacy of OGF as a potential non-toxic, antifibrotic adjuvant treatment to trabeculectomy. Methods: A modified Scheie procedure was performed on New Zealand White (NZW) rabbits. Animals were treated intraoperatively with PBS (control), Hydron + OGF, MMC, or Hydron alone. Postoperatively, intraocular pressure was measured daily by a tonometer. Daily measurements of bleb height, area, and vascularity were assessed via a slit lamp microscope. H&E and Sirius Red were used to assess cellularity and collagen fiber orientation. Cell proliferation and apoptosis assays also were performed.

Results: A Kaplan-Meier survival curve showed a mean bleb survival of 19.5 days for Hydron + OGF treated animals compared to 21 days for the MMC group. Both Hydron + OGF and MMC treated animals had blebs that functioned significantly longer than controls (9 days) (p=0.0005, log rank). Bleb area and height of MMC treated animals were larger and higher than controls starting at post-operative days 4 and 8 respectively (p<0.01). Most importantly, MMC and OGF treated groups were comparable to each other in bleb area and height. Cell proliferation was significantly increased in the Hydron group.
compared to all other groups, while apoptosis was evident in MMC treated animals. Hydron + OGF animals displayed a decrease in cell proliferation compared to all other groups, and minimal apoptotic labeling was evident.

**Conclusion:** Post-operative evaluation revealed that OGF is comparable to MMC in prolonging bleb function in NZW rabbits. These data suggest that OGF could serve as a new non-toxic treatment to reduce the resultant fibrosis following filtration surgery for glaucoma.

**Introduction**

Glaucoma is the leading cause of irreversible blindness in the world, as well as the second leading cause of bilateral blindness [1, 2]. 2.2 million Americans of age 40 and older suffer from the disease, and it is estimated that 2 million more have the disease and are undiagnosed [1, 2]. Although ethnicity is a predominant factor for developing glaucoma, age has a more commanding influence on prevalence. In white populations, a more than 10-fold increase in prevalence of chronic open angle glaucoma is seen from age groups 40-49 (0.6%) to 80+ (7.3%). This same trend is seen in black populations with a 3-4 times higher prevalence than whites [4]. In regards to the incidence of glaucoma, blacks are over 5 times more likely to have the disease at age 40 than whites, as well as having an increase incidence in the 4th and 5th decades of life.

Current non-invasive treatments to lower intraocular pressure in glaucoma patients range from a variety of medications such as: prostaglandins, β-blockers, selective adrenergic agonists, carbonic anhydrase inhibitors, miotic
agents, and epinephrine compounds [7]. It has been shown that these anti-glaucoma medications can effectively decrease intraocular pressure (IOP); however, in many cases, these medications cease being effective and a trabeculectomy is needed. A serious complication of this surgery is the over proliferation of Tenon’s capsule fibroblasts (TCFs), which can cause surgical failure. Current therapies (e.g., 5-fluorouracil and mitomycin-C) reduce the fibrosis associated with surgical failure; however unwanted complications such as bleb leakage and infection can occur. What is needed is an adjuvant therapy with minimal side effects that successfully decreases TCF proliferation.

Methionine enkephalin, termed opioid growth factor (OGF) is a tonically active, endogenous peptide that has been documented to inhibit cell proliferation. The OGF-OGFr axis is present in rabbit Tenon’s capsule fibroblasts (RTCFs), and my in vitro studies demonstrate its ability to inhibit the proliferation of these cells in a non-toxic manner. Using the New Zealand White rabbit as the animal model; OGF was explored as an adjuvant treatment to prevent failure of trabeculectomy surgery.

**Materials and Methods**

**Animals.** All experiments were performed on 20-week-old adult New-Zealand white rabbits weighing approximately 4 kg (Covance). All animals were randomized to treatment groups and observers were masked as to treatment. All protocols were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the Department of Comparative Medicine of The Pennsylvania State University.
College of Medicine.

**Surgical Procedure.** A modified Scheie’s procedure was performed on the left eye of 53 adult male New Zealand White rabbits. An intramuscular injection of ketamine (70 mg/kg), xylazine (7 mg/kg), and acepromazine (10 mg/kg), as well as topically applied Proparacaine Hydrochloride Ophthalmic Solution 0.5% (Baush & Lomb Inc.; Tampa, FL) was given to sedate the animals. Conjunctiva and Tenon’s capsule layer were incised 5 mm posterior to the limbus, and the incision was enlarged parallel to the limbus. Tenon’s layer was blunt-dissected anteriorly to the area of the limbus. A Bovie low temperature fine tip thermal cautery (Aaron Medical, St. Petersburg, FL) was applied to the sclera to make a 3 mm wide line 1 mm posterior to the limbus. An incision was made through the cauterized area to enter the anterior chamber. The cautery was again applied to the lips of the incision to cause retraction of the wound edges. A peripheral iridectomy was performed after cauterization. The incision of the conjunctiva was then closed with a continuous 10-0 vicryl suture (Ethicon, Somerville, NJ).

**Treatment Regimen.** Rabbits were randomly assigned to treatment groups receiving OGF, MMC, or PBS by various applications. OGF (5 mg) was administered with two enkephalinase inhibitors (Bestatin and Thiorphan, $10^{-4}$ M), which have been documented to impede degradation of enkephalins in mucous membranes [119]. OGF and enkephalinase inhibitors were impregnated into a slow release polymer hydroxyethylmethacrylate (Hydron; Interferon Sciences, New Brunswick, NJ) containing sucralfate. Construction of
Hydron pellets was described in Kenyon et al [120]. MMC (0.4mg/ml) was administered intraoperatively via a K-sponge (4x1mm dry area) (Katena Products, Denville, NJ). The saturated sponge contained 0.1 ml of the MMC solution, and it was applied underneath the conjunctival flap for 5 minutes. After administration of MMC the surgical area was irrigated with 10 ml of sterile BSS. Hydron alone was administered as a control. PBS was administered in a subconjunctival injection (0.1 ml) once daily for the first 5 days after surgery. Animals were given topical anesthetic (proparacaine hydrochloride 0.5% eye drops, 1 drop per eye) before subconjunctival injections.

Clinical assessment of bleb height, area, and leakage, as well as inflammation, vascularity, depth of the anterior chamber, and intraocular pressure was evaluated daily until bleb failure. Rabbits were euthanized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). Both eyes were proptosed and enucleated and histologically evaluated. The non-surgical eye served as comparison. One rabbit from each treatment group was euthanized on the 5th day after surgery to assess cell proliferation and apoptosis.

**Baseline Evaluation.** Before the filtration surgery was performed, baseline recordings of intraocular pressure and evaluation of the conjunctiva was performed. IOP was recorded in both eyes with a handheld tonometer (Tonopen XL Tonometer; Medtronic, Jacksonville, FL), after topical proparacaine hydrochloride ophthalmic solution 0.5% was administered. An observer masked to each treatment group made all measurements. Bleb
survival was used as the primary efficacy endpoint. A flat, vascularized, scarred appearance and an IOP equal to or higher than that of the unoperated eye characterized a failed bleb.

**Postoperative Clinical Evaluation.** Measurements of the bleb’s appearance, size, and vascularity were graded and recorded with a hand-held slit-lamp (Zeiss HSO 10 Hand Held Slit Lamp; Dublin, CA). Bleb width, length, and height were measured by using Castroviejo Calipers (Katena Eye Instruments, Denville, NJ) to define the margins of the bleb. Bleb height was graded semi quantitatively (0, flat; 1, shallow/formed <1mm; 2, elevated <2 mm; 3, high >2 mm). Bleb vascularity was graded on a scale of 0-3 based on blood vessel encroachment on the filtering bleb (0, avascular; 1, normal vascularity; 2, hyperemic; 3, very hyperemic).

**Preliminary Studies.**

Preliminary studies were conducted with a variety of treatment groups in order to evaluate the efficacy of OGF as a possible antifibrotic therapy for trabeculectomy surgery. Treatment groups were as follows: a) OGF (0.4 mg/ml) administered via subconjunctival injection (0.1 ml) once daily for the first 5 days after surgery, b) OGF (2.5 mg/ml + Bestatin and Thiorphan \(10^{-4} \text{M}\)) administered intraoperatively (same method as MMC with exception of irrigation) as well as a once daily subconjunctival (0.1 ml) injection until surgical failure, c) low dose naltrexone (LDN, 0.01 mg/kg) administered daily via intraperitoneal (i.p.) injections at the same time each day until failure, d) high dose naltrexone (HDN, 30 mg/kg) administered in the same manner as LDN, e)
OGF (5 mg + Bestatin and Thiorphan (10⁻⁴ M)) impregnated in Hydron (Hydron + OGF) and administered intraoperatively, f) OGF (20 mg + Bestatin and Thiorphan (10⁻⁴ M)) impregnated in Hydron (Hydron + OGF 20 mg) and administered intraoperatively, g) Control (0.1 ml of PBS administered via subconjunctival injection for the first 5 post-operative days), h) Hydron implanted intraoperatively without OGF or enkephalinase inhibitors.

**Histological Evaluation.**

During the enucleation of both eyes the upper lid was removed along with the whole eye to preserve the bleb and superior conjunctiva. The eyes were fixed in 10% neutral buffered formalin for 24 hours, processed, embedded in paraffin, and 8-μm sections were cut. The sections were evaluated with a variety of stains including: a) H&E for cellularity and inflammation, b) Giemsa for lymphocytes, monocytes, and leukocytes, c) trichrome for blood vessels, and d) a standard protocol for Sirius Red staining was used to evaluate collagen fiber orientation as a result of the trabeculectomy surgery. Sirius Red is a strong anionic dye that stains collagen fibers via its sulphonic acid group [121]. The elongated dye molecules attach to the collagen fibers in a parallel fashion. As a result, collagen can be qualitatively evaluated while viewing the specimens under polarized light. Sections were stained with Sirius Red by the technique of Constantine and Mowry [121]. Sections were postfixed in Bouin's solution for 15 minutes, and then incubated for 30 minutes in saturated picric acid solution containing picrosirius red at 1 mg/ml. The sections were viewed with an Olympus microscope (Olympus BX51) equipped for light polarization.
**DNA Synthesis.** DNA synthesis was assessed by bromodeoxyuridine (BrdU) labeling. Five days after surgery, BrdU, 100 mg/kg body weight was injected intraperitoneally 3 and 6 hours before euthanasia by intraperitoneal injection of sodium pentobarbital (Euthansol, Ft. Worth, TX). Specimens were stained with anti-BrdU antibodies (Invitrogen, Eugene, OR) and counterstained with hematoxylin.

**Apoptosis.** Apoptosis was assessed using a kit (DermaTACS in situ Apoptosis Detection Kit: Trevigen, Gaithersburg, Maryland).

BrdU and TUNEL techniques were quantified through labeling indexes (labeled cells/total cells x 100); At least 3 sections per treatment group of Tenon’s capsule layer, borders of the scleral fistula, and conjunctiva were counted (6 grids/section). Non-surgical eyes were stained and used as a reference for appearance of normal conjunctival tissue.

**Statistical Analysis.** Statistical analysis was performed to determine the differences between the treatment groups. Analysis of number of cells (at least 6 counts/condition/time point), IOP, bleb width, length, height, and vascularity, as well as anterior chamber inflammation, and histological evaluation was performed by one-way analysis of variance with Newman-Keuls posts tests. A p-value of <0.05 was considered reliably significant. Bleb survival estimates were calculated by Kaplan-Meier method of survival.
Results

Bleb Survival and IOP Maintenance

Post-operative examination of the surgical area revealed that all blebs were functional for at least 2 days after the Scheie’s procedure was performed. Log rank tests indicate that both Hydron + OGF 5 mg and Hydron + OGF 20 mg treated blebs were functional for significantly longer than controls (p=0.0004, log rank). As healing progressed, controls (both PBS and Hydron) as well as all preliminary treatment groups (except MMC, Hydron + OGF 5 mg, and Hydron + OGF 20 mg groups) failed at or before 11 days after surgery (Figure 3.1); however, rabbits treated with Hydron + OGF had functional blebs with a median survival of 19.5 days versus Hydron + OGF 20 mg (median survival 14 days, p=0.01, log rank). Bleb survival of Hydron + OGF 5 mg was comparable to MMC treated animals, which had a median survival of 21 days.

Post-operative IOP measurements all were below fellow eye IOP levels for at least two days after surgery. Hydron + OGF treated animals maintained IOP levels below that of the fellow eye for significantly longer periods than controls (p=0.0071, log rank). MMC treated animals did not maintain IOP levels for significantly longer than controls (Figure 3.2A). Hydron + OGF treated animals maintained lower IOP levels for significantly longer than LDN, HDN, and OGF 2.5 mg treated animals (Figure 3.2B). Hydron + OGF treated animals did not maintain IOP levels for significantly longer than Hydron + OGF 20 mg and OGF 0.4 mg treated animals.
Figure 3.1. A) Hydron + OGF and MMC significantly prolonged bleb survival compared to control (PBS) and Hydron as shown in the Kaplan-Meier survival curve (p=0.0004, log rank). B) Hydron + OGF significantly prolonged bleb survival compared to treatment groups with exception of MMC, which was comparable (p=0.9499, log rank).
Figure 3.2. A) Eyes treated with Hydron + OGF maintained IOP levels below the fellow eye for significantly longer than Controls and Hydron. MMC treated eyes did not maintain IOP levels for significantly longer than Controls or Hydron. IOP maintenance of MMC and Hydron + OGF groups were comparable. B) Eyes treated with Hydron + OGF maintained IOP levels below the opposite eye for significantly longer than OGF 2.5 mg, LDN, and HDN groups. Failure to maintain IOP occurred in all cases by day 21. IOP failure was defined as a return to or an increase from opposite eye IOP.
Using slit lamp examination, it was evident that by day 7 filtration blebs of the control groups were failing (e.g., flat appearance, numerous new blood vessels) (Figure 3.3), while the MMC and Hydron + OGF treated animals still showed signs of functional filtration blebs (e.g., elevated bleb, avascularity). Further studies were limited to MMC, Hydron + OGF, Hydron, and control groups because all other routes of administering OGF did not significantly exceed Hydron or control groups in time to failure or IOP maintenance. Experiments were repeated with these 4 groups, and rabbits were euthanized at the time of bleb failure or on day 5.

Post-Operative Evaluation

_Bleb Height_

Heights of all filtering blebs were comparable for the first three post-operative days (Figure 3.4). Starting at day 8 and continuing until failure, PBS control animals had bleb heights that were significantly flatter than their MMC and Hydron + OGF counterparts (p<0.01). Bleb heights of Hydron animals were flatter than MMC and Hydron + OGF groups beginning on day 11. MMC and Hydron + OGF treated animals had comparable bleb heights throughout the study (~22 days).

_Bleb Area_

Bleb areas of control animals were significantly smaller than those of MMC treated animals starting at post-operative day 4 and continuing until surgical failure of all control animals (Figure 3.5). Bleb areas of MMC and Hydron + OGF treated animals were comparable throughout the study.
Figure 3.3. Photographs of the surgical site on post-operative days 1, 7, 11, 14, and 22. As healing progressed bleb appearance became vascularized, and scarred. Hydron + OGF and MMC treated blebs survived significantly longer than controls. Bar = 1 cm
Figure 3.4. Bleb height was graded semi quantitatively (0, flat; 1, shallow/formed <1 mm; 2, elevated <2 mm; 3, high >2 mm). Animals receiving PBS (control) were significantly different from MMC at p<0.05(\*), p<0.01(\**), p<0.001(\***), from Hydron + OGF at p<0.01(\++), p<0.001(\+++), and from Hydron at p<0.05(\*). Control n=9, Hydron n=3, Hydron + OGF n=4, MMC before day 14 n=6 after day 14 n=2. Values represent mean ± SEM.
Figure 3.5. Bleb areas of rabbits treated with PBS (control), Hydron, Hydron + OGF, or MMC. Measurements were taken daily until the time of surgical failure. Untreated animals (controls) were significantly different from MMC at p<0.05(*), p<0.01(**), p<0.001(***), from Hydron + OGF at p<0.05(+), and from Hydron at p<0.05(*). Control n=9, Hydron n=3, Hydron + OGF n=4, MMC before day 14 n=6 after day 14 n=2. Values represent mean ± SEM.
**Bleb Vascularity**

Bleb vascularity was assessed in a semi-quantitative manner. At post-operative day 8 the blebs of MMC treated animals had significantly less blood vessel development than control, Hydron, and Hydron + OGF animals (Figure 3.6). Throughout the remainder of the study the vascularity of MMC treated blebs was significantly less than Hydron + OGF (p<0.01).

**Intraocular Pressure**

All operated eyes had a decrease in IOP ranging from 33% to 52% 24 hours after surgery compared to baseline measurements (Figure 3.7). IOPs of all operated eyes regardless of treatment were not significantly different from each other. IOPs of Hydron + OGF and MMC treated eyes were comparable throughout the study.

**Histopathology**

Evaluation of H&E and Sirius Red stained sections showed that the scleral fistulas of animals treated with either MMC or Hydron + OGF remained patent at post-operative day 5 (Figure 3.8). Scleral fistulas of control and Hydron animals were occluded or partially occluded by immature collagen (birefringent green). Filtering blebs of Hydron + OGF and MMC animals were elevated, and had sparse connective tissue throughout. Control and Hydron filtering blebs had evidence of more robust connective tissue, and in the case of Hydron animals, the beginnings of granulation tissue formation.

Tissue also was harvested at a late time point (MMC – 40 days, Hydron + OGF – 33 days, Hydron – 11 days). Filtering blebs of MMC treated animals
Figure 3.6. Bleb vascularity of rabbits treated with PBS (control), Hydron, Hydron + OGF, or MMC. Vascularity was scored on a semi-quantitative scale from 0 (avascular) to 3 (very hyperemic). MMC treated animals were significantly different from Control at p<0.05(*), p<0.01(**), p<0.001(++*), from Hydron + OGF at p<0.05(+), p<0.01(++) and from Hydron at p<0.05(∗), p<0.01(∗∗). Control n=9, Hydron n=3, Hydron + OGF n=4, MMC before day 14 n=6 after day 14 n=2. Values represent mean ± SEM.
Figure 3.7. Ratio of operated eye IOP to unoperated eye IOP. Failure to maintain a below normal IOP was defined as a value of 1.00 or greater. Values did not differ significantly between treatment groups. Control n=9, Hydron n=3, Hydron + OGF n=4, MMC before day 14 n=6 after day 14 n=2. Values represent mean ± SEM.
Figure 3.8. Photomicrographs of the surgical site on post-operative day 5. The left column represents H&E stained specimens. The right column represents specimens stained with Sirius Red and viewed under polarized light. Control = A,B. MMC = C,D. Hydron + OGF = E,F. Hydron = G,H.
were flat, and the Tenon’s layer appeared normal (Figure 3.9). The filtering blebs of Hydron + OGF treated animals exhibited exuberant granulation tissue, and numerous blood vessels infiltrated the Tenon’s layer (seen in trichrome stained specimens). Giemsa staining revealed the presence of lymphocytes and monocytes within the area of granulation tissue. Interestingly, immature collagen fibers (birefringent green) were sparsely evident compared to the appearance of the MMC specimen. The Hydron specimen exhibited the same exuberant granulation tissue and blood vessel appearance as the Hydron + OGF specimen. However, smaller immature collagen fibers were more abundant in the Hydron specimen compared to the Hydron + OGF and MMC specimens.

**Cell Proliferation**

The number of BrdU labeled cells located at the border of the sclera fistula conjunctiva, and Tenon’s layer were comparable in control and MMC treated animals (Figure 3.10A, B, C). DNA synthesis of the scleral fistula and Tenon’s layer of Hydron + OGF treated animals was reduced by 100% and 78%, respectively, from MMC treated animals. Interestingly, DNA synthesis of Hydron animals was elevated between 2.5 to 3.5 fold when compared to control animals (p<0.001). BrdU labeled cells in the conjunctiva were comparable for all treatment groups.

**Apoptosis**

Cell death was evaluated by the TUNEL technique on tissues harvested on post-operative day 5. For one rabbit in each group
Figure 3.9. Photomicrographs of the surgical site at a late time point. MMC = 40 days. Hydron + OGF = 33 days, and Hydron = 11 days. The left column represents H&E stained specimens. The right column represents specimens stained with Sirius Red and viewed under polarized light. MMC = A, B. Hydron + OGF = C, D. Hydron = E, F.
Figure 3.10. Labeling indexes in cells located in the area of A) Borders of the scleral fistula, B) conjunctiva, C) Tenon’s layer, as measured by BrdU incorporation 5 days after surgery. Significantly different from Control at $p<0.001^{(***)}$, and $p<0.01^{(**)}$, from Hydron + OGF at $p<0.001^{(+++)}$, from Hydron at $p<0.001^{(###)}$, and from MMC at $p<0.001^{(^^^)}$ and $p<0.01^{(^^)}$. Data was recorded from 6 grids (50 x 290 μm) /section, 2 sections per region. Values represent means ± SEM.
(control, Hydron, Hydron + OGF, or MMC) three separate areas of the anterior chamber were examined for TUNEL positive cells: borders of the scleral fistula, conjunctiva, and Tenon’s layer. At post-operative day 5, MMC treated animals showed a 93% increase in apoptotic cells in the area of the scleral fistula compared to controls (Figure 3.11). Apoptotic cells were not evident at the borders of the scleral fistula in the Hydron or Hydron + OGF treated animals. MMC treated animals showed a 100% increase in apoptotic cells in the conjunctiva of the filtration bleb when compared to controls, Hydron, and Hydron + OGF. In all treatment groups no more than 1% of cells in Tenon’s layer were apoptotic.
Figure 3.11. The number of apoptotic cells as measured by the TUNEL technique in scleral fistula, conjunctiva, and Tenon's layer. Apoptotic Indexes showed no significant differences between treatment groups. Values represent means ± SEM.
Discussion

In this study, a modified Scheie’s procedure was performed in rabbits to assess the possible antifibrotic effects of OGF. Blebs of animals treated with Hydron + OGF survived for more than double the time of control animals, and were comparable in survival to MMC animals. Rabbits with trabeculectomy treated with Hydron + OGF maintained IOPs for significantly longer than controls, while MMC treated animals did not. Bleb height and area of the MMC and Hydron + OGF treated animals were comparable throughout the study, while being significantly higher and larger than blebs of control animals.

Histopathology evaluation revealed that Hydron and Hydron + OGF specimens had developed exuberant granulation tissue inside of the surgical bleb at their respective late time points, while the MMC specimen did not. However, DNA synthesis was markedly increased in the Hydron specimen in the areas of the scleral fistula and Tenon’s capsule compared to control, MMC, and Hydron + OGF groups. In contrast, the Hydron + OGF specimen had significantly less DNA synthesis than all other treatment groups in the scleral fistula and Tenon’s capsule areas. MMC specimens had an increased amount of apoptotic cells in the scleral fistula and conjunctiva areas compared to control, Hydron + OGF, and Hydron groups. This illustrates that MMC could possibly be a more toxic and detrimental therapy than OGF, which has been documented to be safe and non-toxic. Once more, these results are an extension of what was discovered during in vitro studies.

To accurately study OGF, a reproducible and consistent animal model
was chosen. Although there are numerous variations of glaucoma filtration surgeries used as experimental models, we chose to utilize the modified Scheie’s procedure because of its ease of use and consistent reproducibility. Our data regarding time to failure of control and MMC blebs were consistent with historical data [122-124], indicating that the procedure was performed properly and with little variation. Historical data regarding apoptosis and cell proliferation on MMC and control groups was consistent with the present data in this study [89, 125]. These data support the conclusion that MMC induces apoptotic cell death and does not alter cell proliferation through regulating DNA synthesis.

*In vitro* studies have documented that OGF and MMC reduce the number of RTCFs in cell culture. MMC reduces cell number by actively killing cells, while OGF has not been acknowledged to induce apoptosis. It has also been discovered that OGF inhibits proliferation of RTCFs in culture through DNA synthesis, while MMC does not. Now, for the first time *in vivo*, the same antiproliferative effects of OGF and MMC have been revealed. Complementing, these findings with the postoperative clinical results it can be stated that OGF is comparable to MMC as an antifibrotic agent. However, OGF does not induce cell death, therefore, possibly minimizing the detrimental side effects seen with MMC treatment.

OGF could have a far-reaching impact as a new and novel anti-fibrotic therapy to combat scarring of trabeculectomy surgery. Recent estimates show the patients pay on average over 6 thousand dollars for trabeculectomy surgery
and medications over a five year time span, with postoperative complications having the greatest impact [126]. As seen in this study, OGF has the potential to minimize the surgical complications associated with current antifibrotics (e.g., 5-FU and MMC), because of its ability to inhibit cell proliferation through decreasing DNA synthesis and not via cell death. Although OGF has positive effects on extending the life of filtering blebs in rabbits, an optimal vehicle for administering the biotherapy needs to be elucidated. Liposomal vehicles may be a viable option for OGF administration because they can control the rate of drug release, protect the drug from metabolic enzymes, decrease drug toxicity, enhance therapeutic effects, and possibly increase ocular drug absorption [11]. However, a new formulation of Hydron also may help to optimize OGF treatment. It may be discovered that once a proper sustained release model of treatment is developed, OGF could stay in the surgical area for a longer time period. If the presence of OGF can be extended past the remodeling phase of wound healing, then it is possible that minimal scarring and fibrosis will occur. This event would allow the surgical area to stay intact for a much longer, if not indefinite period of time, which would maintain normal IOP levels, and in turn, prevent further optic neuropathy.
CHAPTER IV
DISCUSSION
Glaucoma is a group of diseases that damage the optic nerve, and causes irreversible blindness [1]. Glaucoma occurs when IOP is too high for the health of the optic nerve, resulting in optic neuropathy [1]. 66.8 million people have glaucoma worldwide, and this number is expected to double by 2020 [1]. This group of diseases is the leading cause of irreversible blindness in the world [1]. The most prevalent risk factor for glaucoma is elevated IOP, though the specific pathophysiology of the optic neuropathy of glaucoma is not fully understood, decreasing IOP by either surgery or medication remains the most effective treatment [1, 127, 128].

Current medications such as prostaglandins and beta-blockers can effectively decrease IOP, but after a period of time the treatments cease working once again elevating IOP. Additionally, the anti-glaucoma medications can have long term complications leading to an increased likelihood that subsequent surgical intervention may fail [8-10].

Traditional trabeculectomy is the current procedure of choice when surgical intervention is needed to reduce IOP. At first, trabeculectomy is successful at reducing IOP; however, scarring of the surgical area may eventually occlude the filtering bleb resulting in surgical failure [129, 130]. Antifibrotic treatments such as 5-FU and MMC have been used effectively to decrease scarring of filtration blebs [69, 73, 74]; however, these treatments cause unwanted cell death and may be associated with severe complications [7, 72, 125, 131, 132]. Therefore, a novel and non-toxic adjunct therapy is needed.
OGF was proposed as a new and novel antifibrotic therapy because the OGF/OGFr axis has been documented to modulate cell proliferation not only in a variety of cancer cell lines, but also in normal cells as well [102-104, 114-117]. Historical data demonstrates that the OGF/OGFr axis inhibits cell proliferation through DNA synthesis [103, 108-110, 117], and it does not induce apoptosis [133]. Previous studies also demonstrate that OGF and OGFr are present in the corneal epithelium of rats, rabbits, and humans [101-103]. In normal cells (human umbilical vein endothelial cells and human epidermal keratinocytes) the OGF/OGFr axis utilizes the cyclin-dependent kinase inhibitors p16 and p21 causing a delay in the G₀/G₁ phase of the cell cycle [106, 107]. Through siRNA experiments it was discovered that both p16 and p21 are required to inhibit proliferation of normal cells [118]. Furthermore, previous immunohistochemistry and immunoelectron microscopic studies have found that OGF and OGFr are colocalized in the cytoplasm and the nucleus [105, 113].

Analysis of the nucleotide structure of OGFr reveals 3 nuclear localization sequences (NLS). NLSs usually interact with karyopherin β in order to transport their contents into the nucleus [134]. Immunoelectron microscopy, suggests that karyopherin β may function as a nucleocytoplasmic transport receptor [105, 113]. Of the 3 NLS sites, the two monopartite sequences (NLS_{383-386}, NLS_{456-460}) are essential to carrying the OGF/OGFr through the nuclear pore complex [135]. This study also demonstrated the spatial and temporal factors of OGFr trafficking between the cytoplasmic and nuclear areas. OGFr can be observed solely in the nucleus after 24 hours. This finding
tell us that the OGF/OGFr pathway is unidirectional from the cytoplasm to the nucleus, and recycling of OGF and OGFr does not occur [135]. These data support the conclusion that a sustained and constant release of OGF would be beneficial in combating the fibrosis that occurs after glaucoma filtration surgery.

In order to evaluate the effects of the OGF/OGFr axis on TCF proliferation, a relatively pure population of fibroblasts needed to be established for my research. The majority of in vitro studies utilizing TCFs were obtained from human donors [66, 67, 82, 84, 88], and the studies which used RTCFs were extremely brief in their methods of isolating a pure cell population [125, 136]. As a result, it was necessary to develop an accurate method to demonstrate the purity of the RTCF population in vitro. After portions of Tenon’s capsule were dissected, and successfully subcultured [111] it was necessary to characterize the cells by counting RTCFs versus non-RTCFs, staining the cells with FITC conjugated phalloidin, and performing an initial temporal growth curve. Utilizing FITC-conjugated phalloidin, F-actin microfilaments of RTCFs could be observed [137], and compared to endothelial cell controls. Typical fibroblasts should present with F-actin fibers throughout the cytoplasm, while endothelial cells should have F-actin in the periphery. A microscopic observation revealed that the cell population obtained from Tenon’s capsule harvest was over 99% fibroblasts. The doubling time of the harvested RTCFs was 28.6 hours, indicating that these results are similar to unpublished data of human TCFs.
Since a relatively pure population of cells had been established, the next step to characterizing OGF and OGFr was to evaluate if both were present and functional in RTCFs. In previous studies, the presence of OGF and OGFr have been documented in both non-normal (cancer) and normal cells [101, 103, 117, 138-140]. Using polyclonal antibodies specific for either OGF or OGFr it was revealed that OGF and OGFr were present in RTCFs. This conclusion is further supported by a study documenting the presence of OGF and OGFr in many groups of the phylum Chordata [98]. Receptor binding assays have been traditionally utilized to characterize a one site model of binding of OGF to OGFr in cells and tissue [140-144]. Binding analysis of RTCFs revealed a one-site model of binding with a $K_d$ of $4.6 \pm 1.4$ nM and a $B_{max}$ of $6.5 \pm 0.8$ fmol/mg. These data revealed that a new and novel system (OGF/OGFr axis) is present and functional in RTCFs.

To establish the effectiveness of OGF in decreasing the proliferation of RTCFs, cells were treated with OGF in a wide range of dosages from $10^{-10}$ to $10^{-4}$ M. Dosages higher than $10^{-7}$ M decreased RTCF proliferation ranging from 28 to 47% of controls, whereas lower dosages of OGF ($10^{-8}$ to $10^{-10}$ M) were comparable to controls. Further experiments were performed with $10^{-6}$ M OGF because this dosage of OGF has been documented to be efficacious and non-toxic in numerous cancer cell lines and normal cells [102, 103, 115, 145, 146].

The next question posed was whether the inhibitory actions of OGF were mediated by an opioid receptor. This was evaluated through treating RTCFs with the short-acting opioid antagonist naloxone. When RTCFs were treated
with a dosage of NAL \((10^{-6} \text{ M})\) that does not affect cell proliferation, cell numbers were comparable to controls. RTCFs treated with OGF showed a 28% decrease in proliferation from controls. Interestingly, when NAL and OGF were administered simultaneously the inhibitory effects of OGF were nullified indicating that OGF functions in a receptor mediated fashion as supported by previous literature [115, 117, 145, 146]. A unique characteristic of OGF is that its action is reversible. Evidence of this reversibility is documented in numerous cancer cell lines [115, 145, 146]. After being treated with OGF for 72 hours, some RTCFs stopped receiving the drug while others continued treatment for another 48 hours. RTCF cultures that ceased OGF treatment had 22% more cells than those RTCFs that had continuous OGF administration. These data suggest that in RTCFs, OGF functions in a dose-dependent, reversible, and receptor mediated manner.

To examine the temporal effect of OGF on RTCFs, cells were treated with OGF \((10^{-6} \text{ M})\), NTX \((10^{-6} \text{ M})\), or sterile water for 96 hours. Actively blocking opioid receptors with NTX or treating RTCFs with OGF demonstrated that there is, in fact, an opioid system working to modulate RTCF proliferation. In addition, a wide variety of natural and synthetic opioids related to the traditional opioid receptors \((\mu, \delta, \sigma, \epsilon, \text{ and } \kappa)\) were assessed for their role in cell proliferation. Treating RTCFs with these traditional opioids revealed no effects on cell proliferation, unlike the inhibitory effects seen with OGF. These results showed an inhibitory actions of OGF in this specific tissue culture regimen are consistent with previous studies [115, 145, 146].
If OGF were tonically active in RTCFs, treating the cells with an antibody to OGF would potentially neutralize endogenous OGF thus increasing cell replication. When RTCFs were treated for 72 hours with an antibody to OGF, cell number of the antibody treated cultures increased 17% compared to controls. This result indicated that OGF is tonically active, and that by neutralizing endogenous OGF, normal cell replication can be disrupted. These results are consistent with previously published data [115, 145, 146].

Using siRNA technology specific for OGFr, it was determined that OGFr is necessary for OGF to function. Cell proliferation of RTCFs with silenced OGFr was not altered when OGF was present. Furthermore, RTCFs with silenced OGFr were not stimulated to proliferate when in the presence of NTX. This finding supports the conclusion that OGFr-NTX interaction up-regulates cell proliferation, and that the interaction of NTX with the classical opioid receptors is not involved [118].

OGF functions to inhibit cell proliferation in ocular surfaces through DNA synthesis [103, 108, 110, 117]. OGF treated RTCFs showed a 41% decrease in BrdU labeling compared to controls, while NTX treated cells showed a 32% increase in DNA synthesis. Current adjunct therapies for trabeculectomy (e.g., 5-FU and MMC) actively induce cell death [147-150]. It was necessary to assess if OGF had any cytotoxic effects on RTCFs via caspase 3 immunocytochemistry and TUNEL technique. After treating RTCFs for 24 hours, OGF did not increase caspase 3 activity in RTCFs; however, in contrast, MMC treated cells showed an almost 1.5 fold increase in the amount of
caspase 3 present compared to OGF treated RTCFs. Caspase 3 immunocytochemistry and TUNEL technique revealed that OGF treated RTCFs did not induce apoptosis, which is consistent with previous results [133, 151]. MMC treated cells increased TUNEL labeling by 28% compared to controls. Interestingly, when MMC and OGF were used together, TUNEL labeling was decreased by 40% compared to MMC treated RTCFs alone, indicating that OGF may have a protective effect. Evidence of this protective effect has been documented in combination chemotherapeutic and biotherapy studies in nude mice [152].

This study supported the hypothesis that the OGF/OGFr axis is present and functions in a nontoxic manner to inhibit proliferation of RTCFs in cell culture. The *in vitro* study revealed that in RTCFs, OGF functions in a dose dependent, tonically active, and receptor mediated manner. OGF is specific for OGFr, and acts through cyclin dependent inhibitory kinase pathways to modulate cell proliferation in normal cells [118]. Also, OGF did not induce apoptosis while inhibiting cell proliferation through DNA synthesis, which signifies its safety and non-toxic characteristics.

Previous clinical studies have indicated that, without antifibrotic treatment, 74% of trabeculectomy surgeries fail with in 5 years [69]. 5-year follow up studies of both 5-FU and MMC indicate that 51% and 40% of trabeculectomy surgeries that utilized these therapies failed, respectively [69, 153]. Despite the reduction of surgical failures with the adjunctive use of these medications, many side effects such as epitheliopathy and endophthalmitis are
prevalent in patients treated with these antiscarring agents [7, 79]. These data suggest that a more efficacious and non-toxic therapy is still needed for preventing fibrosis following trabeculectomy surgery.

In general, there are four animal models currently in use to evaluate potential antifibrotic therapies. This primate model (cynomolgus monkey) utilizes argon laser treatment of the trabecular meshwork in order to increase IOP creating a hypertensive state, followed by full thickness trabeculectomy [154-157]. However, the argon laser treatment produces variable hypertensive incidence along with irritation and inflammation, which is a drawback to the experimental model [158]. The increased inflammation may hinder the success of the experimental treatment even before the trabeculectomy is performed.

The second model is a modified partial thickness trabeculectomy which places a surgical cannula through the area of the scleral fistula [89, 159-162]. Although it is effective at reducing IOP, shunt migration and placement does pose some variability in the procedure.

The last two models are partial-thickness trabeculectomy and full-thickness trabeculectomy. Partial thickness trabeculectomy reduces many of the unwanted side effects seen in full-thickness procedures, such as a flat anterior chamber [163]; however, when translated to the rabbit, it is difficult to reproduce because of the differences in anatomy of the eye compared to humans (e.g., scleral thickness) [123, 124]. The full-thickness trabeculectomy was utilized in many previous glaucoma filtration surgery studies because of its reproducibility and effectiveness at forming functioning filtering blebs in the
rabbit [62, 122, 125, 164, 165]. Personally, the modified Scheie’s procedure was the best choice of experimental glaucoma filtering surgery in regards to its easy reproducibility and moderate learning curve. Although the rabbit model of glaucoma filtration surgery is reliable, it also has limitations. The major limitation of this animal model is that in order to achieve success, IOP has to be depressed from a normal state to an artificial hypotensive state. Surgical success usually is arbitrarily defined as attaining an IOP of below 21 mm Hg [166][167]. In the normal animal model the rabbit has an initial IOP of anywhere from 12 to 14 mm Hg. Another disadvantage of this animal model is the aggressive response to ocular injury compared to that of the primate and human [168]. Therefore, extending the life of a filtration bleb by weeks or even days is a very significant finding, and could be potentiated in humans.

The numerous variations within experimental glaucoma filtering surgery make exact comparisons to historical data difficult. A few of the variations include: conjunctival flap construction (e.g., limbal or fornix based), full or partial thickness sclerotomy, use of a tube shunt, dosage of antiscarring therapy, size of surgical sponge used for intraoperative drug administration, subconjunctival space dissection area, and suturing technique. Historically, MMC treated blebs of full thickness trabeculectomy survived for approximately 60 days [122, 164], which is 3 times the survival of the MMC blebs in this current study. These previous studies entailed excising a 1 x 3 mm scleral block before cauterization of the scleral fistula edges. This technique differs from the Scheie procedure,
which dictates a 3 mm wide scleral incision 1 mm behind the limbus before scleral cauterization.

Another variation was in the application of MMC. These studies saturated a 4 x 1 mm surgical sponge in either a 0.4 mg/ml or 0.5 mg/ml MMC, whereas in the current study 0.1 ml of a 0.4 mg/ml MMC solution was placed on the surgical sponge. This variation could be significant, because results from another MMC study state that size of the surgical sponge increases bleb area and overall survival [159]. The post-operative measurements of bleb height, and vascularity in the current study are similar to the data seen in previous publications [89, 159-161]. Bleb areas in the current study are significantly smaller than those in previous literature. However, this decrease was seen across all treatment groups, therefore variation in the surgical procedure (e.g., tube shunt versus Scheie procedure) could be the reason for the variation. It is of importance to note that in vivo studies of previous experimental treatments were, at best, comparable to either 5-FU or MMC treatment [89, 90, 125, 160, 161].

In the in vivo study, OGF (5 or 20 mg) and enkephalinase inhibitors were impregnated in Hydron, a slow release polymer that was most recently used as a vehicle for evaluating neovascularization in the cornea.[120]. In a sustained release vehicle, OGF is thought to be present within the surgical area for far longer than when administered intraoperatively or subconjunctivally. The blebs of Hydron + OGF treated animals lasted more than double the time of control blebs and 9.5 days longer than the best subconjunctival OGF treatment. Blebs
of Hydron + OGF treated animals lasted 4.5 days longer than Hydron + OGF 20 mg treated animals. It is interesting that ¼ the dose of OGF would result in more efficacy. One hypothesis is that the acetate salt present in the OGF powder caused an abnormal amount of irritation to the conjunctiva and sclera [169]. This irritation could easily trigger an inflammatory response, thus, decreasing the amount of time the filtration bleb was functional. An increased inflammatory response also could bring about further migration of fibroblasts from surrounding areas allowing the filtration procedure to fail prematurely.

The bleb survival of the Hydron + OGF group was comparable to MMC and significantly longer than controls. H&E and Sirius Red stained specimens indicated that, at post-operative day 5, filtering blebs of Hydron + OGF and MMC animals were elevated, and had sparse connective tissue throughout the subconjunctival space. Control and Hydron filtering blebs had evidence of more robust connective tissue, and in the case of Hydron animals, the beginnings of granulation tissue formation. One study that documented the wound healing events after trabeculectomy in rabbits stated that new collagen and granulation tissue was evident at postoperative day 3, and persisted until at least day 10 [168]. In regards to control and MMC specimens, these observations are comparable with those seen in previous publications [89, 90, 122-124, 159-161].

Upon histological evaluation, Massone’s trichrome staining confirmed that numerous blood vessels were seen within the bleb cavity of both Hydron and Hydron + OGF specimens. In previous studies, OGF impregnated in
methylcellulose significantly reduced the number and length of blood vessels present in chick chorioallantoic membrane indicating that OGF is antiangiogenic [170]. Upon further evaluation, Giemsa staining of the Hydron and Hydron + OGF specimens revealed that the tissue contained numerous monocytes, lymphocytes and leukocytes, while control and MMC specimens did not. 

Interesting enough, at postoperative day 5, the Hydron + OGF specimens had a significant decrease in cell proliferation compared to Hydron specimens. This finding could indicate that the vehicle, not OGF was the culprit stimulating granulation tissue, and that the formulation of Hydron was the causative factor. It also is possible that part of the plastic mesh used as a molding to create the Hydron pellets broke off with the Hydron and was placed in the subconjunctival space, causing the large inflammatory effect. The addition of sucralfate to the Hydron pellet could have caused the new blood vessel formation. Historical evidence states that sucralfate is angiogenic. When sucralfate was administered to rats, it significantly increased bFGF levels [171], therefore inducing angiogenesis.

Liposomal vehicles may be a viable option for OGF administration because they can control the rate of drug release, protect the drug from metabolic enzymes, decrease drug toxicity, enhance therapeutic effects, and possibly, increase ocular drug absorption [11]. The methylcellulose method implemented in previous studies [170, 172] also would be an effective vehicle for OGF treatment, in that when methylcellulose was administered as a control it did not increase blood vessel number or length. This vehicle would allow
OGF to be present in the surgical area for an increased amount of time reducing cell proliferation, while not producing unwanted inflammation.

DNA synthesis of the cells located at the borders of the scleral fistula and Tenon’s capsule layer of Hydron + OGF treated animals was reduced by 100% and 78%, respectively, compared to MMC treated animals. Interestingly, DNA synthesis in Hydron animals was elevated between 2.5 to 3.5 fold when compared to control animals. The effect of OGF on DNA synthesis in ocular tissue has been demonstrated in prior publications [102, 103, 110, 117]; however, this is the first time it has been evaluated in Tenon’s capsule layer and the sclera. Although the Hydron vehicle initiated a large inflammatory response, it is of value to note that the Hydron + OGF specimen still displayed a reduction in DNA synthesis compared to controls. These data display the inhibitory effects of OGF in a reliable animal model of glaucoma filtering surgery. There is limited evidence regarding quantitation of apoptosis in experimental animal models of trabeculectomy. However, one study indicated that MMC increased apoptosis in specimens at post-operative day 2 via qualitative analysis of the TUNEL technique [125]. The current study evaluated apoptosis using the TUNEL technique at post-operative day 5. The MMC specimen had evidence of significant apoptotic activity in the conjunctiva and borders of the scleral fistula compared to Hydron, Hydron + OGF, and controls. This finding substantiates the previous *in vitro* and historical data demonstrating that OGF is safe and non-toxic [133, 151].
These studies provide proof of principle that OGF is effective in reducing proliferation of RTCFs *in vitro*, while significantly extending the life of the filtering bleb *in vivo*. The future direction for these studies should focus on evaluation of a sustained release vehicle that optimizes the inhibitory effects of OGF without stimulating an inflammatory response. Once an efficacious dose of OGF can be consistently administered to the surgical area in a prolonged manner, the healing of the surgical area may be modulated in such a way that the scleral fistula could remain patent indefinitely. Tenon’s capsule and conjunctival layers void of excess fibrotic tissue would result in a functional bleb that could maintain a healthy IOP for an extended period of time. These results could have a far-reaching impact eliminating the use of traditional antifibrotic therapies as well as other adjunct therapies used to decrease IOP, and their associated complications. Reducing the need for follow up procedures and medications not only would increase patient health, it would markedly decrease economic cost.
APPENDIX
Appendix Figure 1. RTCFs were seeded onto 22 mm glass coverslips then treated with OGF (10^{-6} M), NTX (10^{-6} M), or sterile water for 72 hours. Cells were then fixed with 10% neutral buffered formalin, and stained with an antibody to alpha smooth muscle actin (myofibroblast differentiation, Sigma, St. Louis, MO), and counterstained with hematoxylin. A labeling index was computed as the number of labeled cells divided by the total number of cells with nuclei x 100. Values are means ± SEM.
Appendix Figure 2. p16 and p21 knockdown and OGF effects on cell number. RTCFs were transfected for 24 hr with scrambled siRNA, p16 siRNA, or p21 siRNA, and treated for 72 hr with OGF (10^{-6} M) or sterile water. Data represent mean ± SEM. Significantly different from non-transfected and scrambled siRNA transfected OGF treated RTCFs at p<0.001 (**).
Appendix Figure 3. A) Bleb area, B) Bleb Height, C) Bleb Vascularity, and D) IOP Ratio of MMC OGF 0.4 mg, and Control treated rabbits. Post-operative measurements were performed daily until failure. Significantly different from controls at p<0.05 (*), p<0.01 (**), p<0.001 (***) from OGF 0.4 mg at p<0.05(+), p<0.01(++), and p<0.001(+++). Values represent mean ± SEM.
Appendix Figure 4. A) Bleb area, B) bleb height, C) bleb vascularity, and D) IOP ratio of MMC, Hydron, Hydron + OGF 20 mg, and control treated rabbits. Post-operative measurements were performed daily until failure. Significantly different from controls at p<0.05 (*), p<0.01 (**), p<0.001 (***), from Hydron at p<0.05 (+), p<0.01 (++), p<0.001 (+++), from Hydron + OGF 20 mg at p<0.05 (*), p<0.01 (**), and p<0.001 (****). Values represent mean ± SEM.
Appendix Figure 5. A) Bleb area, B) bleb height, C) bleb vascularity, and D) IOP ratio of MMC, OGF 2.5 mg, and control treated rabbits. Post-operative measurements were performed daily until failure. Significantly different from controls at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and from OGF 2.5 mg at $p<0.05$ (+), $p<0.01$ (++), $p<0.001$ (+++). Values represent mean ± SEM.
Appendix Figure 6. A) Bleb area, B) bleb height, C) bleb vascularity, and D) IOP ratio of MMC, LDN, and control treated rabbits. Post-operative measurements were performed daily until failure. Significantly different from controls at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and from LDN at $p<0.05$ (+), $p<0.01$ (++)$p<0.001$ (***). Values represent mean ± SEM.
Appendix Figure 7. A) Bleb area, B) bleb height, C) bleb vascularity, and D) IOP ratio of MMC, HDN, and control treated rabbits. Post-operative measurements were performed daily until failure. Significantly different from controls at p<0.05 (*), p<0.01 (**), p<0.001 (***), and from HDN at p<0.05(+), p<0.01(++), p<0.001(+++). Values represent mean ± SEM.
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