ENHANCEMENT OF SCLERAL MACROMOLECULAR PERMEABILITY WITH PROSTAGLANDINS*

BY *Robert N. Weinreb, MD*

ABSTRACT

Purpose: It is proposed that the sclera is a metabolically active and pharmacologically responsive tissue. These studies were undertaken to determine whether prostaglandin exposure can enhance scleral permeability to high-molecular-weight substances.

Methods: Topical prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was administered to monkeys to determine if this altered the amount of scleral matrix metalloproteinases (MMPs). Experiments also were performed to determine whether the prostaglandin F (FP) receptor and gene transcripts are expressed in normal human sclera. Permeability of organ-cultured human sclera following prostaglandin exposure then was studied and the amount of MMP released into the medium measured. Finally, the permeability of human sclera to basic fibroblast growth factor (FGF-2) was determined following prostaglandin exposure.

Results: Topical prostaglandin administration that reduced scleral collagen also increased scleral MMP-1, MMP-2, and MMP-3 by $63 \pm 35\%$, $267 \pm 210\%$, and $729 \pm 500\%$, respectively. FP receptor protein was localized in scleral fibroblasts, and FP receptor gene transcript was identified in sclera. Exposure to prostaglandin $F_{2\alpha}$, 17-phenyltrinor, $PGF_{2\alpha}$, or latanoprost acid increased scleral permeability by up to 124%, 183%, or 213%, respectively. In these cultures, MMP-1, MMP-2, and MMP-3 were increased by up to 37%, 267%, and 96%, respectively. Finally, transscleral absorption of FGF-2 was increased by up to 126% with scleral exposure to latanoprost.

Conclusions: These studies demonstrate that the sclera is metabolically active and pharmacologically responsive to prostaglandins. Further, they demonstrate the feasibility of cotreatment with prostaglandin to enhance transscleral delivery of peptides, such as growth factors and high-molecular-weight substances, to the posterior segment of the eye.

INTRODUCTION

Peptides, including growth factors and other high-molecular-weight substances, are potential therapeutic agents for delivery to the posterior segment in glaucoma, age-related macular degeneration, and other ocular disorders.[1-4] The potential benefit of such treatments is suggested by enhanced survival and differentiation of retinal neurons in cultures[5-7] and improved neuronal viability in experimental models[8-11] to which certain growth factors or neurotrophins have been added. However, targeted delivery of even low-molecular-weight drugs to the optic nerve, retina, and choroid has been problematic. Delivery of high-molecular-weight drugs is even more challenging. Methods for simple and safe drug delivery to the posterior segment clearly are needed if such neuroprotection strategies are to be effective.

It is proposed here that enhancement of scleral macromolecular permeability with prostaglandins may be such a method. The hypothesis investigated in this thesis is that the sclera is metabolically active and that prostaglandin cotreatment activates enzymes that enhance scleral macromolecular permeability.

CURRENT METHODS OF DRUG DELIVERY AND THEIR APPLICABILITY TO THE POSTERIOR SEGMENT

The applicability of current methods of drug delivery to the posterior segment is limited by poor drug absorption, particularly of high-molecular-weight substances.

Topical Application

Topical application is the primary route of drug delivery to the anterior segment of the eye. This route of administration is noninvasive, simple for the patient to use, and relatively free of systemic side effects, particularly when applied with punctal occlusion or gentle lid closure to minimize systemic drug absorption. However, topical application requires rigorous patient compliance over an extended time to effectively treat chronic disease, and it is largely

ineffective for drug delivery to the posterior segment.

Many drugs are capable of penetrating intact corneal epithelium to achieve significant levels in the cornea, anterior chamber, iris, and ciliary body. Topically applied drugs also may enter the eye by crossing the conjunctiva and diffusing through the sclera, but do so only to a minor extent. Although the transcorneal penetration of drugs is essential to achieve therapeutic drug levels in anterior segment tissues, drugs applied to the cul-de-sac typically do not achieve pharmacologically active concentrations in posterior segment tissues following topical administration. An important factor that limits topical application as a means of drug delivery to the posterior segment is the loss of drug from the precorneal area. Induced lacrimation because of an instilled volume into the conjunctival sac, blinking, physiologic tear turnover, drug-protein binding, and enzymatic degradation of drug in tear fluid allow only a small amount of an applied dose to pass into the aqueous humor and surrounding tissues in the anterior chamber. Edelhauser and Maren found that lower corneal permeability in humans than rabbits may result, in part, from our fourfold greater blinking rate and twofold greater tear turnover.[12]

The corneal epithelium is another contributing factor that limits topical application, as it is a barrier to drug absorption, particularly for high-molecular-weight substances such as growth factors. As a result of these hurdles, the absorption of drugs applied topically to the eye is quite poor compared with the systemic route of administration. The extent of absorption, as measured by taking the ratio of the total amount of drug that has entered the eye divided by the instilled dose, ranges from 1% to 7% for ophthalmic drugs.[13,14] In contrast, the extent of absorption of systemic drugs is usually greater than 75%.

Within the anterior chamber, drug dilution and removal also reduce the amount of drug available for diffusion posteriorly to the optic nerve, retina, and choroid. Aqueous secretion within the anterior segment dilutes the aqueous humor drug concentration, and normal aqueous drainage removes drug that has penetrated the corneal tissues. Drug also may diffuse into blood vessels within the anterior segment and then be removed from ocular tissues.

A drug that is absorbed into the anterior chamber also must redistribute from the aqueous to the vitreous humor. However, a drug topically applied to the eye in general does not enter the vitreous in significant concentrations. Two main factors explain the relative lack of penetration in the vitreous cavity. One factor is that there is only a minute space between the ciliary processes and the lens, and drugs must diffuse against an aqueous humor flow gradient. Another factor is the relatively slow diffusion of drugs in the vitreous. Molecular charge and lipophilicity have little effect on the diffusion of drugs of ocular

interest.[13-15] Drug diffusion depends on molecular movement through an aqueous environment. Although it is not restricted by the presence of collagen in the vitreous, drug diffusion is too slow to allow significant drug accumulation within the vitreous. Finally, cell junction barriers, as discussed subsequently, can limit the diffusion of drugs within the vitreous into the optic nerve, retina, and choroid.[15]

Systemic Administration

Despite the excellent absorption of systemic drugs, systemic administration of biologically active agents is ineffective at achieving therapeutic levels in the posterior segment of the eye. In the intact eye, systemic routes, such as oral or parenteral, may not produce a high enough concentration of drug because of resistance to entry from blood-ocular barriers, metabolism of drug to an inactive species, or significant uptake into another organ or tissue. Drug absorption into the eye is increased during ocular inflammation, which is associated with a disruption of the blood-aqueous barrier, the blood-retinal barrier, or both. Even in this case, however, systemic drug administration at doses sufficient to be absorbed into the eye may cause unacceptable systemic side effects, since the drug actions are unlikely to be localized to the eye. As an example, neurotrophins, which are high-molecular-weight drug candidates for optic nerve and retinal neuroprotection, have many properties aside from their roles in neuronal survival and axonal growth associated with retrograde transport. Neurotrophins also are anterogradely transported and released from presynaptic to postsynaptic targets.[16] Further, they modulate membrane excitability, induce neuronal hypertrophy, and affect cell differentiation.[2] If cells throughout the body were exposed to exogenously administered neurotrophins, the systemic side effects would likely be ubiquitous and deleterious.

Intravitreal Injection

Intravitreal injection, most often via the pars plana, offers a direct route to the posterior segment and often can provide adequate tissue drug levels. For some infectious or inflammatory diseases of the posterior segment, intravitreal injections are an effective and essential component of treatment. Local delivery of drugs to the eye via intravitreal injection offers several advantages over other routes of administration. First, it avoids many of the side effects associated with systemic therapy. This is particularly of benefit in the case of medications that may be too toxic for systemic administration but are well tolerated by the eye. Second, it bypasses the blood-ocular barrier, allowing higher intraocular drug levels than might otherwise be achieved. This may be particularly advantageous for high-molecular-weight substances.

Even if a drug can be delivered intravitreally,

however, the barrier presented by the internal limiting membrane is an important factor that may preclude intravitreal delivery of many peptides, including growth factors and other high-molecular-weight substances, to the retina. In rabbits with experimental subretinal detachments, Takeuchi and associates[17] observed that fluorescein isothiocyanate albumin (67 kDa) injected intravitreally can diffuse, but only slowly, across the sensory retina into the subretinal space. Kamei and associates[18] injected tissue plasminogen activator (70 kDa) labeled with fluorescein isothiocyanate and rhodamine B isothiocyanate–labeled dextran (20 kDa) into the midvitreous cavity of normal rabbits and those with experimental subretinal hemorrhage. The smaller-molecular-weight dextran diffused slowly throughout the neural retina in each of the eyes. Intravitreal tissue plasminogen activator did not diffuse through the intact neural retina in any of them. Distribution of an intravitreal drug also may limit drug delivery to the retina. A drug with a rapid rate of clearance from the vitreous may require large boluses and frequent injections to ensure therapeutic levels over an extended period.

Intravitreal injections have the inherent potential side effects of retinal detachment, endophthalmitis, cataract formation, and vitreous hemorrhage. The benefits of treatment must supersede these risks. In chronic diseases, long-term intravitreal treatment also might need frequent injections. Repeated injections have incremental risks, and they generally would not be well tolerated by the patient. Therefore, sustained-release intravitreal drug delivery, which would require fewer injections, may be particularly advantageous for treatment of chronic eye diseases.[19]

The use of sustained-release drug delivery systems that are placed within the vitreous is being investigated for a number of eye diseases, including cytomegalovirus retinitis,[20-23] uveitis,[24-26] proliferative vitreoretinopathy,[27-30] and choroidal neovascularization. In addition to the advantages of intravitreal injection, sustained drug delivery offers the promise of relatively constant drug levels in the vitreous. On the other hand, drugs that may be safe to the eye when used for a short time may prove to be toxic with sustained intraocular levels. Further, once placed intraocularly, they would need to be surgically removed if there were untoward side effects. The devices, too, have risks and complications associated with their placement that may preclude their routine use in eyes with glaucoma or age-related macular degeneration. These limitations have delayed their introduction into clinical use, particularly for treatment of chronic diseases.

Periocular Injection
Directly introducing a drug into the tissues surrounding the posterior segment by anterior or posterior sub-Tenon's, subconjunctival, or retrobulbar injection is another method of delivering drugs to the posterior segment of the eye. For many reasons, this is an attractive route for delivering drugs to the optic nerve, retina, or choroid. This route has a major advantage of bypassing the epithelial barriers of the cornea and conjunctiva, which limit drug absorption with topical application.

Despite the frequent clinical use of periocular injection for a plethora of ocular disorders, the mode of drug transfer from the periocular location into the ocular tissues is not clearly understood. The drug may leak from the conjunctival injection site and penetrate the cornea or may enter the eye in part via systemic absorption. Also, the drug may enter the eye through intrascleral vascular channels or through perivascular and perineural spaces surrounding penetrating blood vessels and nerves. Perhaps most significantly, the drug also may enter the eye after penetrating directly through the sclera.[31,32]

McCartney and associates[33] documented the direct penetration through underlying sclera of subconjunctival tritium-labeled hydrocortisone (molecular weight [MW], 362 Da), a low-molecular-weight drug, through the ocular tissues in the rabbit eye. In their study, the percentage of the total dose that penetrated into the eye appeared to be small (~1% to 2%). Studies in normal squirrel monkeys by Barza and associates[34] demonstrated that gentamicin (MW, 450 Da to 477 Da), a mixture of 3 similar low-molecular-weight compounds, also can penetrate directly through underlying sclera when administered by subconjunctival or retrobulbar injection. They also found the highest drug concentrations in the superior and inferior segments of the rabbit sclera, but no detectable drug in the nasal and temporal areas.[35] On the basis of these data, they hypothesized that the drug solution tends to remain localized and spreads over the superior segment nearest the injection site for a period long enough to be absorbed by the sclera. It then moves to the inferior segment, possibly as a result of gravity. Depending on the position of the subject, it is absorbed through the superior and inferior scleral surfaces rather than nasal and temporal areas. They observed significant levels in the retina and choroid, but not the vitreous.

Lim and associates[36] detected tissue plasminogen activator (MW, 70 kDa) in rabbit vitreous after subconjunctival injection, but the concentrations were very low. Subsequently, Lincoff and associates[37] observed that recombinant human interferon α-2a (MW, 20 kDa) diffused into the rabbit choroid, but only in small amounts, after retrobulbar injection. The total choroidal concentration was only 3% of the amount injected in the retrobulbar space, and the serum concentration was less than 1% of the choroidal concentration. Weijtens and associates[38,39]

studied dexamethasone concentrations in subretinal fluid of patients with a rhegmatogenous retinal detachment undergoing a scleral buckling procedure with drainage. They found that a subconjunctival injection, preceded by topical cocaine to disrupt the corneal and conjunctival epithelial barrier, resulted in a higher maximal vitreous concentration of dexamethasone (MW, 393 Da) than a sub-Tenon's or retrobulbar injection. However, even the highest vitreous concentrations in their study were lower than those needed for a therapeutic effect based on in vitro testing of human retinal pigment epithelial cell proliferation.[39]

Although periocular injection can deliver some low-molecular-weight substances in clinically relevant concentrations to the posterior segment, the rate of delivery is not as effective with high-molecular-weight substances. Improvement in the extent of absorption can come from better retention at the site of absorption through the use of gels,[40-42] nanospheres,[43] liposomes,[44-49] or other biodegradable carriers. Improvement in the extent of absorption also can be achieved with improved penetrability. Methods to improve the penetrability of the sclera have been sparsely investigated.

SCLERA

Basics

The sclera is a densely collagenous, hypocellular and elastic tissue that is composed of a proteoglycan matrix and closely packed collagen fibrils.[50-53] It has been thought to be relatively inactive metabolically, having no intrinsic capillary bed and few fibroblasts. Duke-Elder[54] described the sclera as "inert and purely supportive in function." Watson and Hazleman[55] stated that it is "metabolically relatively inert."

The outer surface of the sclera is covered by the loosely organized episclera and its inner surface by the lamina fusca and suprachoroidal space. The sclera is perforated by the emissarial canals for arteries, veins, and nerves. Unlike corneal collagen, scleral collagen bundles do not lie in orderly, regular lamellae but are interlaced in an irregular fashion, which accounts for the lack of transparency. Collagen forms 75% of the dry weight of the sclera;[56] the remainder is made up of noncollagenous proteins and mucopolysacharides.[57,58] Approximately 70% of the weight of intact sclera is water.

Spitznas[59] studied the ultrastructure of human scleral collagen posterior to the ora serrata and reported that the diameter of the fibrils in the outer layers is significantly larger than that of the inner layers. There is a ratio of 1:2 between the diameter of collagen fibrils in the innermost and outermost layers of human posterior sclera, respectively.[59,60] Shields and associates[61] observed that there is

significantly less difference in average collagen fibril diameter between the inner and outer portions of anterior compared with more posterior human sclera. According to them, Purnell and McPherson[61] suggested that the larger fibril diameter, which they extrapolated to looser fibril arrangement, might provide less resistance to aqueous flow through the intervening ground substance. Bundles of thinner fibers, possibly precollagen, are found near scleral fibroblasts. The length of the bundles is not known. The bundles have a slightly fusiform shape with tapering ends and dichotomous branches. The turnover rate of scleral collagen is also unknown. The flat and elongated cells, the scleral fibroblasts, are few in number and are separated by collagen. The long axis of the cell and nucleus is parallel to the surface. Long, thin cytoplasmic extensions from the cells are attenuated to a diameter one-third to one-half the size of the collagen bundles. Experimental studies in an avian model suggest that the sclera is derived from 2 sources, the ectodermal neural crest and mesoderm.[62]

The mean total scleral surface area is approximately 17.0 ± 1.5 cm^2.[63] Mean scleral thickness $\pm$ SD is 0.53 ± 0.14 mm at the corneoscleral limbus, significantly decreasing to 0.39 ± 0.17 mm near the equator and increasing to 0.9 to 1.0 mm near the optic nerve.[64] The sclera thins with age. The large surface area and thinness of the sclera are desirable features of a route for targeted drug delivery.

In a normal eye, blood vessels only traverse the sclera and do not supply it directly. Therefore, the stroma of the sclera derives its nutrition from a distance and not through intimate contact with the capillary bed. This implies that the sclera must be permeable to fluids and metabolites, as reported first by Bill.[65] The external movement of substances through the sclera results from a pressure difference between the suprachoroidal space, where the pressure is 1 to 2 mm Hg lower than intraocular pressure, and the episcleral tissue, where the pressure is near 0 mm Hg. Transscleral movement should theoretically be slowed by reducing intraocular pressure. Whether this flow forms part of the normal flow of fluid from the suprachoroidal space is uncertain.[66]

Transscleral Fluid Movement

Drug penetration across the sclera is a route of entry into the eye for some ocular drugs, particularly those with low molecular weight. However, the details of transscleral fluid movement are poorly understood.

In addition to being the outer surface of the globe, the sclera is the distal component in the uveoscleral outflow pathway. Histologic analysis of sclera following injection of various tracers into the anterior chamber indicated the presence of transscleral fluid flux through the scleral

stroma, as well as possibly through narrow spaces around penetrating nerves and blood vessels.[65,67-69] However, there is little information regarding the character of this fluid movement. What factors influence scleral permeability? Does the sclera allow only unidirectional flow from within the eye to the outside? Or might the flow be bidirectional, and also from outside to within? Is it always passive, as might occur with porous diffusion through a fiber matrix? Or might it also be regulated by endogenous signals?

Assessment of drug diffusion through the sclera by in vitro permeability studies is a useful approach to estimate drug movement for in vivo conditions. Maurice and Polgar[70] examined diffusion across bovine sclera with a broad range of different molecular weight solutes and ions, including methylene blue (MW, 320 Da) and serum albumin (MW, 69 kDa). They showed first that the diffusion of the ions and solutes was inversely related to molecular weight. Shields and associates[71] performed an in vitro study with postmortem human eyes to assess the permeability of outer anterior sclera following trabeculectomy. They recognized the permeability of sclera to ferritin or india ink, and they speculated that the route of flow was through vessels in the flap or through the "collagen ground substance between individual fibrils." Ahmed and Patton[72] recognized that under certain conditions, even some topically applied drugs–timolol maleate (MW, 433 Da) and inulin (MW, 5 kDa)–can enter the eye via the transscleral route and bypass the anterior chamber. Edelhauser and Maren[12] reported that scleral permeability was greater than corneal permeability except for highly lipid soluble compounds, for which scleral and corneal permeability were the same.

Olsen and associates[64] examined the diffusion across human sclera by using a range of different molecular weight solutes as high as dextran (MW, 70 kDa). They confirmed in human sclera that the diffusion of the ions and solutes was inversely related to molecular weight. Moreover, they observed that the absorption of molecular weight substances greater than 5 kDa was poor. As they studied only tissue without macroscopically visible perforating channels or light and electron microscopic perforating vascular channels, their studies were most consistent with direct penetration through the sclera as an important vector for transscleral drug transfer into the eye. More recently, Ambati and associates[73] measured the permeability of rabbit sclera to a series of fluorescein-labeled hydrophilic compounds with a wide range of molecular weights as high as 150 kDa. Scleral permeability decreased with increasing molecular weight, a finding consistent with previous human and bovine data. They also suggested that molecular radius might be a better predictor of scleral permeability than molecular weight. These studies all are consistent with the sclera being permeable, and the permeability being inversely related to the molecular weight of the permeating substance.

Influencing Drug Penetration and Scleral Permeability
The rate and extent of absorption are determined by both the physicochemical behavior of a substance and the permeability of the sclera. Absorption can be enhanced for a particular substance by increasing drug penetration or by increasing scleral permeability. Few methods have been tested to enhance either of these, particularly for molecular weight substances higher than 5 kDa.

Drug penetration into the eye may be increased with iontophoresis, the process of moving a charged molecule by an electric current across the cornea or sclera. Transcorneal iontophoresis has been shown to result in significantly higher drug levels than those found after multiple drop treatments.[74] However, it is questionable whether significant drug concentrations within the optic nerve, retina, or choroid can be achieved with it. Experimental studies by Lam and associates[75] have shown that toxic tissue-damaging effects might accompany drug delivery by transscleral iontophoresis. They observed local retinal and choroidal lesions following transscleral iontophoresis of various drugs. Current density and duration of application affected the size and severity of the lesions. This technique also is not practical.

Few studies have investigated the factors that contribute to scleral permeability. Olsen and associates[64] did not find any significant correlation between scleral permeability to inulin and age. They also observed that cryotherapy did not significantly affect scleral permeability to 5-fluorouracil (MW, 130), inulin, or dextran (MW, 40 kDa).[64] Further, scleral permeability to sucrose (MW, 342), inulin, and dextran (10 kDa) was unaffected by transscleral diode laser retinopexy.

Scleral permeability does appear to be related to scleral thickness. Shields and associates[71] found that increased outflow was inversely related to the scleral flap thickness in a small number (N=6) of postmortem human eyes that underwent experimental trabeculectomy. Scleral permeability to the low-molecular-weight substances dexamethasone and methotrexate (MW, 455) also was increased significantly with one half surgical thinning of the sclera.[64] Dan and Yaron[76] found increased transscleral flow of saline through bovine sclera with application of clostridial collagenase. Interestingly, they also observed that collagenase applied directly to rabbit sclera after a fornix-based peritomy resulted in scleral thinning and lower intraocular pressure.[76] Even with the use of a microapplicator, this response could not be precisely controlled. In contrast to increased permeability with scleral thinning, abnormal and thickened sclera may be associated with reduced permeabilty. Trelstad and associates[77]

found that sclera from 2 nanophthalmic eyes was thicker than normal and contained unusually disordered collagen fibrils. Yue and associates[78] found that collagen fibers were twisted and more closely packed in nanophthalmic eyes, changes consistent with reduced scleral permeability. Interestingly, Gass[79] first suggested posterior sclerotomy, which increases transscleral flow, as an effective treatment before entering the anterior segment to prevent choroidal effusion in these eyes. As recommended by Brockhurst,[80] vortex vein decompression also may be effective.

Scleral permeability also may be influenced by intraocular pressure. Rudnick and associates[81] recently evaluated the permeability of human sclera to 3 low-molecular-weight compounds (carboxyfluorescein, dexamethasone, and water) and found a small effect of intraocular pressure. They suggested that pressure-related compression of collagen and narrowing of intracollagen pathways within the sclera slow diffusion of small molecules, yet may completely block transport of macromolecules.[81]

By enhancing scleral permeability, one might be able to more effectively deliver drugs to the posterior segment. Enhanced scleral permeability also might lower uveoscleral outflow resistance and lower intraocular pressure.

Can Transscleral Fluid Movement be Enhanced by Prostaglandins?
The possibility that various prostaglandins (PGs) could modulate transscleral fluid movement and enhance scleral macromolecular permeability is suggested by several observations. First, topical treatment of monkey eyes with $PGF_{2\alpha}$-isopropyl ester (IE) for 5 days is known to enhance uveoscleral outflow and to reduce collagen type I and collagen type III immunoreactivity within sclera by 43% and 45%, respectively.[82] Second, scleral collagen is predominantly type I collagen and accounts for about one half of the total dry weight of sclera.[56] Finally, evidence that compaction of extracellular matrix affects transscleral permeability suggests that collagen density within sclera is an important determinant of permeability.[83] Hence, it is plausible that PG-mediated reduction of scleral collagens could significantly alter permeability. Increased local biosynthesis of matrix metalloproteinases (MMPs), a family of secreted neutral proteinases that can initiate specific degradation of key extracellular matrix components, may be regulating the reduction of scleral collagen following topical PG.[84,85]

PGs induced substantial remodeling of ciliary muscle extracellular matrix in situ that reflected MMP-mediated collagen reduction.[82] The sequence of cellular events underlying this response includes PG transduction at cell surface PG receptors, induction of MMP gene transcription, translation and secretion of proMMPs, activation of MMPs by proteolytic truncation, and MMP-mediated

initiation of collagen degradation in the ciliary muscle extracellular space.[86] There is immunohistochemical evidence that MMP-1, which can mediate normal turnover of fibrillar collagens, such as collagen type I and collagen type III, is present in normal human sclera.[87] Analysis with PG receptor agonists suggests that these PG responses are receptor-mediated.[87]

The biologic activity of a drug, whether it be therapeutic or toxic, is often proportional to the concentration of that drug at the receptor site. Moreover, the persistence of its effects is directly related to the residence time of the drug at the receptor. The PG receptor type that most specifically recognizes F family prostaglandins is the FP prostanoid receptor, a G-protein–coupled cell membrane receptor.[88,89] In situ hybridization and immunohistochemical studies have demonstrated FP receptor transcripts and protein in several anterior segment tissues of monkey eyes.[90] In the sclera, however, only FP receptor immunoreactivity has been observed,[90] and no evidence of FP receptor transcripts has been detected. It is possible that the sensitivity of the in situ hybridization technique used was insufficiently sensitive to detect small amounts of FP receptor mRNA. In view of the potential responsiveness of sclera to prostaglandins, direct assessment of FP receptor gene transcription and protein expression in human sclera clearly is needed.

If prostaglandin exposure does enhance scleral macromolecular permeability, the assertions that might be valid include the following:

- Topical prostaglandin administration reduces scleral collagen by increasing scleral metalloproteinase (MMP) activity.
- This effect is FP (prostaglandin F) receptor-mediated, and both gene transcription and protein expression are present in the sclera.
- Exposure of isolated sclera to specific FP receptor agonists increases scleral permeability in association with increased MMP expression.
- Transscleral absorption of a high-molecular-weight substance, basic fibroblast growth factor (FGF-2), increases with scleral exposure to prostaglandin.

METHODS

1. MEASUREMENT OF SCLERAL MATRIX METALLOPROTEINASES AFTER TOPICAL PROSTAGLANDIN

$PGF_{2\alpha}$-IE–Treated Monkey Eye Tissue
Young adult cynomolgus monkeys were evaluated by slit-lamp biomicroscopy on 2 occasions prior to initiation of treatment to confirm the absence of signs of ocular inflammation. In addition, integrity of the blood-aqueous barrier was confirmed by measuring the appearance and

disappearance of fluorescence in the anterior chamber (AC) following intravenous fluorescein administration. To qualify for further study, AC fluorescence levels and time course of appearance and decay in both eyes had to be similar and within the range of values obtained for control eyes in previous studies.[91] Monkeys meeting these conditions were presumed to have an intact blood-aqueous barrier.[91]

The following week each qualifying monkey received $2\mu g$ $PGF_{2\alpha}$-IE (in 5 μL) twice daily (morning and afternoon, approximately 7 hours apart) in 1 eye and 5 μL of vehicle in the other eye for 5 days, as previously described. On the fifth day of treatment, slit-lamp biomicroscopy was performed. Eight eyes of 4 monkeys were evaluated. AC cells or flare was not observed during treatment of these monkeys.

The animals were sacrificed on day 5.[82] The vascular bed was perfused with lactated Ringer's solution to remove circulating MMPs from the ocular tissues. The anterior segments were dissected and immediately fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) for 3 hours. Increased sensitivity of immunohistochemical staining of various antibodies has been demonstrated for many antigens after methacarn fixation.[92,93] Fixed anterior segments were transferred to cold 100% ethanol. The tissues were embedded in paraffin and sections were collected from the midsagittal region of each eye on Vectabond coated slides (Vector Laboratories, Burlingame, Calif). For histopathologic analysis, 3 or 4 sections from each eye were stained with hematoxylin and eosin. All procedures were conducted in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Tissue sections analyzed in the present study were cut from the same tissue blocks as sections analyzed in a previous study.[82]

Immunohistochemistry

Sclera was immunostained by a standardized protocol. Each step of the protocol was optimized as previously described.[94] The concentration of each solution containing antibodies or horseradish peroxidase-conjugated streptavidin was optimized to obtain submaximal (nonsaturating) staining intensity as determined by imaging densitometry (described in the next section). Finally, the incubation time with diaminobenzidine was optimized for each primary antibody to obtain the strongest signal (staining intensity) that still was increasing linearly with time. The elimination of saturating binding or development parameters from the protocol supports the position that the observed changes in staining intensity reflected differences in tissue content of target antigen.

Sections from the treated and control eyes were stained at the same time. Five sections, 10 μm thickness, from each eye were heated to 56°C for 20 minutes,

washed in 3 xylene changes to remove paraffin, and rehydrated through graded ethanols. The sections were treated with antigen retrieval solution (AR-10, Biogenex, San Ramon, Calif) at 95°C for 5 minutes. After cooling, the sections were exposed to 3% H_2O_2 for 10 minutes to suppress endogenous peroxidase activity. To remove intrinsic melanin, sections were treated successively with aqueous potassium permanganate (2.5 g/L) for 10 minutes and oxalic acid (5 g/L) for 3 minutes.[95-97] After rinsing, the sections were blocked for 30 minutes with 0.1% bovine serum albumin (Sigma Chemical Co, St Louis, Mo) and incubated for 2 hours with affinity-purified polyclonal sheep anti-porcine MMP-1 (dilution 1:25, AB772, Chemicon, Temecula, Calif), polyclonal rabbit anti-human MMP-2 (dilution 1:100, AB809, Chemicon), or rabbit anti-human MMP-3 (dilution 1:1,000, AB810, Chemicon). These concentrations had been optimized in pilot studies for quantitative analysis. Specificity of these antibodies has been previously confirmed.[87,98] After rinsing, the sections treated with antibody to MMP-1 were exposed to biotinylated donkey anti-sheep IgG for 30 minutes (Biotin-SP-Conjugated Affinipure, Jackson Immunoresearch Laboratories, Inc, West Grove, Pa, diluted 1:500). The sections exposed to antibodies to MMP-2 or MMP-3 were exposed to biotinylated goat anti-rabbit immunoglobulin (Biogenex) for 20 minutes. After rinsing, the sections were exposed to horseradish peroxidase-conjugated streptavidin for 20 minutes. Consecutively, each section was rinsed and incubated with 3,3-diaminobenzidine chromogen for 10 minutes (HRP-DAB Super Sensitive Immunodetection System, Biogenex). To facilitate comparability, sections from the control vehicle-treated and PG-treated eye of each monkey were immunostained at the same time.[99,100] To serve as controls for nonspecific staining, sections from each eye were simultaneously processed by the same protocol but without the primary antibody.

Densitometric Analysis

Immunohistochemical staining intensity was directly measured with a high-resolution imaging densitometer.[94] Measurements from multiple sections stained at the same time facilitated assessment of measurement precision and permitted statistical comparison of differences among control and experimental eyes. Immunostained sections were scanned by placing the slides directly on the platen of an imaging densitometer (model GS-700, Bio-Rad, Hercules, Calif). Resolution of the scans was set to 1,200 dpi (50 μm-wide pixels), and the scanning mode was set to transillumination. The optical density measurements of the immunostained sections all were less than 1.00 optical density units. Because the densitometer can accurately measure optical densities greater than 3.0 units (Bio-Rad

specifications), these measurements were well within the appropriate range for accurate determinations. The scanned digital data were displayed in a masked fashion and analyzed by using an image analysis program (Molecular Analyst : version 2.1, Bio-Rad). The optical density along 2 line segments positioned over the sclera was measured in each section by using two-dimensional imaging densitometry. A similar line segment was positioned perpendicular to the sclera adjacent to the ciliary body to assess background optical density.

Mean optical density scores were determined from the optical density volume scores (optical density x mm) and the corresponding line segments (mm).[94] For each eye, 10 scores were obtained from 5 midsagittal sections. Background optical density was defined as baseline and subtracted from the original optical density scores. The specific optical density scores along each line segment over the sclera were calculated by dividing the optical density area score (optical density (mm, provided by the densitometer) by the length of the line segment (mm) for that score. Mean specific optical density scores from the PG-treated eye of each monkey were compared to corresponding scores from the contralateral control eyes using the paired Student's *t* test. The unpaired Student's *t* test was used to compare the mean of mean optical density scores of all PG-treated and all control eyes. In each case, a *P* value less than 0.05 was considered significant.

2. FP RECEPTOR GENE TRANSCRIPTION AND PROTEIN EXPRESSION IN NORMAL HUMAN SCLERA

These experiments were undertaken to determine whether the FP receptor is expressed in normal human sclera.

Tissue Preparation

Postmortem human eyes from a 76-year-old donor were obtained from within 24 hours of death. Eyes were placed in chilled Hepes-buffered saline solution (HBSS) and maintained on ice. Eyes were surgically cleaned of connective tissue, blood vessels, muscle, and conjunctiva and rinsed once in HBSS. The anterior chamber was removed by a circumferential incision approximately 4 to 5 mm behind the limbus and snap-frozen, as described below. A circumferential incision was made in front of the optic nerve head, and ocular contents, including retina and choroid, were removed. The sclera was cut into 10 mm-square pieces. Residual pigmented tissue was removed with a cotton-tipped applicator. Sclera tissue was further cut into 2 mm squares and placed in a sterile 50 mL conical tube on ice.

Isolation of Total RNA

The scleral tissue squares were homogenized in 8 mL of TRIzol reagent (Gibco, BRL, Life Technologies, Grand Island, NY) using a homogenizer (Polytron P-10; Brinkmann, NY). Homogenized sample was transferred to sterile 1.5-mL tubes in 1-mL aliquots and incubated for 5 minutes at 25°C. Chloroform (200 µL) was added to each tube and mixed by brief vortex and incubated for 3 minutes at 25°C. Samples were centrifuged (12,000 $\times$ 3) for 15 minutes at 4°C. The aqueous phase was transferred to fresh sterile 1.5 mL tubes. Isopropanol (500 µL/tube) was added and allowed to incubate for 10 minutes at 25°C. Samples were centrifuged (12,000 ($\times$ g) for 10 minutes at 4°C. Supernatant was removed, and the RNA pellet was washed with 75% ethanol/diethylpyrocarbonate water and air dried. RNA was resuspended in a total volume of 50 µL diethylpyrocarbonate water, and quality was checked by gel electrophoresis.

Reverse Transcription-Polymerase Chain Reaction (PCR)

Primers were chosen to amplify 1,186-nucleotides of the human FP receptor. The sense primer (nucleotides 170 to 193) corresponds to a position 61 nucleotides upstream of the translation start site, and the antisense primer (nucleotides 1333 to 1356) corresponds to a position 39 nucleotides downstream of the stop codon in the human FP sequence. Both PCR primers were 100% homologous with the reported cloned sequence of the human FP receptor. The sense and antisense primers were used for reverse transcription (RT)-PCR as previously described with total RNA isolated from human sclera tissue.[101] The PCR (final volume, 50 µL) contained 5 µL of the RT reaction, 5 µL of 10X PCR buffer, 1 µL of 10 mM dNTP mixture, 1.5 µL of 50 mM $MgCl_2$, 2.5 µL of the sense and antisense primers (20 µM), and 0.5 µL taq polymerase (all reagents from Gibco BRL, Grand Island, NY). The PCR program consisted of an initial step at 95°C for 3 minutes, followed by 30 cycles at 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final step at 72°C for 7 minutes. Products were analyzed by electrophoresis in a 1% agarose gel.

FP Receptor Protein Localization in Sclera

Antibodies to the human FP receptor were generated in rabbits by using a recombinant fusion protein consisting of glutathione-S-transferase and a portion of the carboxyl terminus of the receptor consisting of amino acids 317 to 362. Preparations of the fusion protein and antibody purification were done as described.[102] Initial characterization of the antibodies was done as previously described[103] using COS-7 (African green monkey kidney) cells transfected with plasmid DNA encoding the human FP receptor. For labeling of human tissues, pieces of sclera (8 to 10 mm square) were snap-frozen in embedding medium (OCT, Tissue-Tek, Miles Inc, Elkhart, Indiana), sectioned on a

cyrostat (8 to 10-μm sections), mounted on glass cover-slips, and postfixed in 4% paraformaldehyde. Tissue sections were washed twice with phosphate buffered saline (PBS) and then placed in 30 mM sodium chloride/300 mM sodium citrate for 20 minutes. Sections were then incubated in 100 mM glycine solution for 20 minutes to block nonspecific binding, and then washed twice in sodium citrate buffer for 10 minutes. Sections were permeabilized with 30 mM sodium chloride/300 mM sodium citrate containing 0.1% triton-X100 for 1 hour. After an overnight incubation at 4°C with the primary antibody (0.5 to 1.0 μg/mL), the cells were washed with sodium citrate containing 0.05% triton-X100 and incubated for 1 hour at room temperature with secondary antibody (rhodamine red-goat-anti-rabbit, Molecular Probes, Eugene, Ore) at a dilution of 1:200. Coverslips were washed and mounted on glass slides for viewing.

3. MEASUREMENT OF HUMAN SCLERAL PERMEABILITY AND MMPS WITH PROSTAGLANDIN EXPOSURE

If there is increased MMP-1, MMP-2, and MMP-3 immunoreactivity in the sclera of monkey eyes that have received topical $PGF_{2\alpha}$-IE treatment, it would be unclear whether it is a direct response, reflecting increased production within sclera, or an indirect consequence of increased MMP release into the suprachoroidal space of the uveoscleral outflow pathway by ciliary muscle cells. The following experiments were undertaken to investigate this question by determining whether exposure of organ cultures of human sclera to various PGs increases scleral permeability and whether this is associated with increased release of MMPs.

Human Scleral Organ Cultures

Twenty-three pairs of human eyes from donors 45 to 80 years old were obtained within 24 hours after death. Enucleation was completed within 6 hours postmortem, and the eyes were stored in a moist chamber at 4°C for less than 24 hours prior to generation of the organ cultures. Donors had no known history of glaucoma or other eye disease. The eyes were placed in Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture (DMEM-F12) medium containing 50 U/mL penicillin and 50 μg/mL streptomycin for 15 minutes. This was repeated twice prior to dissection. Tenon's capsule and episclera were removed from the surface of the sclera using a sterile cotton-tip applicator. Curved scissors were used to excise circular pieces of sclera. The chosen areas were selected to avoid the perforating anterior ciliary vessels and the vortex veins. The uveal tissues and retina were gently removed from the vitreous side of the sclera with a cotton-tipped applicator. The circular pieces of scleral tissue were placed into 12-well culture plates containing DMEM-F12

supplemented with 1% fetal bovine serum and 1 ng/mL recombinant human FGF-2. As serum contains agents known to stimulate MMP biosynthesis,[104] low serum concentration was used to minimize nonspecific induction of MMPs. The cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Prostaglandin Treatments

The culture medium was changed to fresh medium supplemented with $PGF_{2\alpha}$, 17-phenyltrinor-$PGF_{2\alpha}$, PhXA85 (latanoprost acid) (Cayman Chemical Co, Grand Rapids, Mich), or vehicle control. 17-phenyltrinor-$PGF_{2\alpha}$ and PhXA85 bind with greater specificity to the FP receptor (the endogenous PG-receptor that preferentially recognizes F-type prostaglandins) than $PGF_{2\alpha}$. Each PG was tested at concentrations of 100 nM, 200 nM, and 500 nM. PG concentrations were chosen on the basis of their receptor binding profiles as well as the observation that the peak concentration of PhXA85 observed in aqueous humor following topical application of a clinical dose of latanoprost to human eyes is approximately 100 nM.[105,106] Exposure durations of 24, 48, and 72 hours were chosen on the basis of previous experiments that found increased MMPs in ciliary smooth muscle cells exposed to PGs for 24 hours to 72 hours.[107,108] Experimental treatment was initiated by addition of the test PGs prepared from 10 mM stock solutions in ethanol and appropriately diluted with DMEM-12 nutrient mixture.

Permeability Analysis

Following 1- to 3-day incubation with test PG or vehicle control, the scleral tissue was clamped into the in vitro perfusion apparatus (Ussing apparatus, model CHM2; World Precision Instruments Inc, Sarasota, Fla). The 2 chambers, each with a 9 mm-diameter opening, sandwiched a 14 mm-diameter piece of scleral tissue. This assembly was held together with a clamp. Each unstirred chamber contained 0.75 mL and could be filled, drained, and purged through 3 ports. Three rhodamine-dextran polymers (Molecular Probes) (MW, 10,000, 40,000, and 70,000 kDa) were diluted in phenol red-free HBSS (250 μg/mL). The "uveal-side" chamber was filled with phenol-free HBSS, and the "orbital-side" chamber was filled with rhodamine-dextran diluted in phenol red-free HBSS. Permeability was assessed in this direction because the orbital side was smoother than the uveal side, and thus the potential for measurement-altering small leaks around the edge of the tissue piece was less. Solutions were freshly prepared and warmed to 37°C prior to use. After assembly and filling, the system was placed in the 37°C incubator. The apparatus was checked after 30 minutes to verify that no leaks were present. Any leaks of the dextran solution were readily apparent owing to the dark red color

of the solution Leaks of the phenol red-free Hanks from the uveal-side chamber were recognized by reduction of the level of the fluid visible through the clear walls of the chamber. Four hours later, a 750 µL sample was removed through a valved port connected to the uveal-side chamber and stored at -80⁰C. Samples were protected from light at all times before fluorescence measurement.

Scleral Permeability Coefficient
Diffusion from the "orbital" chamber to the "uveal" chamber was characterized by means of a permeability coefficient (Pc), which is the ratio of steady-state flux (the mass of solute crossing a planar unit surface normal to the direction of transport per unit time) to the concentration gradient.[64] In this study, the concentration of "uveal-side" chamber, C_U, was less than 1% of the concentration in the "orbital" chamber, C_o which did not change measurably over the course of the experiment. Hence, the permeability coefficient was calculated as follows:

$$Pc \text{ (cm/sec)} = (C_{Ut}-C_{U0.5})V \ / \ AtC_o$$

where $C_{U0.5}$ and C_{Ut} are the concentration in the "uveal" chamber at 0.5 hour and at t hours, respectively. C_o is the initial drug concentration (0.25 mg/mL), A is the surface area of exposed sclera (0.65 cm²). V is volume of the each chamber (0.75 ml), and t is duration of steady-state flux converted from hours to seconds. The term (C_{Ut} - $C_{U0.5}$) / t is the permeation rate of dextran across each excised scleral piece (µg/hr).

The fluorescence of rhodamine-dextran was measured with a spectrofluorimeter at room temperature. The excitation and emission wavelengths were 550 and 580 nm, respectively. Standard curves of fluorescence versus concentration were obtained by serial dilution of rhodamine-dextran dissolved in diffusion medium (phenol red–free Hank's buffered saline solution).

Viability After Prolonged Exposure to Prostaglandins
To assess viability in vitro, scleral organ cultures were incubated with 500 nM of each PG, the highest dose in this study, for 1, 2, or 3 days. Ethidium-homodimer was then added to the cultures to a final concentration of 1 µM and the cultures were returned to the incubator for 30 minutes (Molecular Probes). As this dye cannot penetrate living cells, it is only bound to the DNA of dead cells in the cultures. The scleral cultures were rinsed with phosphate buffered saline without phenol red and then exposed to 2% paraformaldehyde in phosphate buffered saline for 10 minutes. The cultures were then permeabilized by passage through graded methanols (50%, 70%, 90%, 95%, and 100%), rehydrated, rinsed in phosphate buffered saline, and exposed to 5 mM Sytox green for 15

minutes. This stain cannot pass through the plasma membrane of living cells but readily stains DNA within dead cells. The cultures were rinsed twice with (PBS). The cultures were then homogenized in PBS using a homogenizer. The homogenates were centrifuged and the supernatants were collected.

Cell viability in these samples was determined by first measuring Sytox green fluorescence using a spectrofluorimeter (model SFM 25, Kontron, Zürich, Switzerland) with the excitation and emission wavelengths set at 500 and 525 nm, respectively. The amount of ethidium homodimer was then measured using a 550 nm excitation wavelength. This wavelength excited ethidium homodimer at 83% of maximal efficiency, but minimally excited Sytox green. The emission wavelength analyzed was 650 nm because it retained 71% of maximal efficiency for ethidium homodimer and eliminated greater than 99% of the cross-talk signal coming from Sytox green. The photomultiplier voltage was optimized to 480 V to obtain all readings on 1 setting. The signals from the ethidium homodimer were normalized with signals from the Sytox green by dividing the ethidium homodimer results by the Sytox green results. Positive (live) controls were fresh cultures not exposed to any treatment, and negative (dead) controls were cultures first treated with 2% paraformaldehyde for 10 minutes and permeabilized with graded methanols before evaluation. The viability of each sample was determined by interpolation from a standard curve that was generated by plotting positive and negative control values.

Scleral Hydration Analysis
Thirty scleral specimens were obtained from human eye bank eyes for the determination of scleral hydration. These studies were performed to ensure that maintaining sclera in the Ussing perfusion system did not hydrate the sclera, which may alter scleral permeability. Ten circular scleral pieces from 3-day-old preparations were incubated in DMEM/F-12 media only, or with media for 3 days followed by HBSS for an additional 4 hours. The preparations were then weighed by using an analytical balance (accuracy, 0.0001 g, Mettler, Geissen, Germany), dried to constant weight at 100°C for 24 hours, placed immediately in a tissue desiccator to cool for 30 minutes, and reweighed. Another 20 circular scleral pieces from fresh and 3-day-old moist chamber-stored globes perfused with HBSS and without perfusion were used to evaluate potential effects of storage. The level of hydration in each piece of sclera was calculated by the following equation:

$$mg \ H_2O \ / \ mg \ tissue = (wet \ weight - dry \ weight) \ / \ dry \ weight.$$

MMP Immunosorbant Assays
At the conclusion of the 1- to 3-day incubations with PGs

or vehicle, media samples were collected from the scleral cultures for enzyme-linked immunosorbant assay (ELISA) analysis. Measurements of MMP-1, -2, and -3 concentration were performed with commercially available ELISA kits (Biotrak, Amersham Pharmacia Biotech Inc, Piscataway, NJ). These assays are based on a two-site ELISA "sandwich" format, and detected both latent and active MMPs. For the MMP-1 assay, purified MMP standards and samples were incubated in microtiter wells precoated with anti-MMP-1 antibody. The wells were then washed, incubated with second polyclonal antibody to MMP-1, washed, incubated with anti-rabbit horseradish peroxidase, washed, and developed by tetramethyl benzidine. After development at room temperature, the absorbency was measured at 630 nm using a microtiter plate reader (SpectraMax 250, Molecular Devices, Sunnyvale, Calif.). The procedures for the MMP-2 and MMP-3 assays were the same except the antibodies were to MMP-2 and MMP-3, respectively.

Statistical Evaluation

Experimental differences between control culture results and a single treatment group were evaluated using the Student *t* test. When results from several treatment groups were compared to those of a single control, significance was evaluated using analysis of variance and the Student Newman Keuls test. A *P* value less than .05 was considered statistically significant.

4. MEASUREMENT OF FIBROBLAST GROWTH FACTOR-2 PERMEATION THROUGH HUMAN SCLERA WITH PROSTAGLANDIN EXPOSURE

These experiments were undertaken to determine whether exposure of scleral explants to the PG analogue latanoprost acid increases permeability to fibroblast growth factor-2 (FGF-2) (also known as basic fibroblast growth factor).

Human Scleral Tissue Explant

Eight pairs of human eyes from donors were obtained from the San Diego Eye Bank. Donors had no history of glaucoma or other ocular diseases. The mean age was 70 ± 6 (mean ± SD) years old. Each pair of eyes was enucleated within 5 hours after death and immediately preserved in a moist chamber at 4°C. Apparently intact eyes were selected and any eye showing scleral damage or thin sclera (posterior staphyloma) was not used. Within 24 hours after preservation, the sclera was dissected and placed into organ culture. Briefly, after incubation in HBSS medium containing 50 U/mL of penicillin and 50 U/mL of streptomycin for 30 minutes, residual extraocular muscles and orbital connective tissues were removed. Sclera was dissected into 4 pieces to exclude the long ciliary

nerve and artery, insertion of muscles, or vortex veins in each center area. Uveal tissue and retina were gently removed with a cotton-tipped applicator. Scleral pieces were placed into 12-well plates containing DMEM/F-12 supplemented with 1% fetal calf serum and 1 ng/mL human recombinant FGF-2. The low concentration of serum was used to minimize nonspecific increase of MMP because serum contains various stimulating factors of MMP synthesis. The explants were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Latanoprost Acid Pretreatment

To investigate the effect of latanoprost acid on the scleral permeability, the culture medium was changed to fresh medium supplemented with latanoprost acid (Cayman Chemical Co). Tested concentrations included 50, 100, and 200 nM, because the peak concentration in human aqueous humor following topical application of a clinical dose is ~100 nM.[106] After 3 days exposure, the permeability assay was performed.

Scleral Permeability Analysis

After 3 days' incubation, the scleral tissue was clamped into the in vitro Ussing perfusion apparatus. Each chamber contained 0.75 mL of fluid. The 2 chambers were facing an opening of 9 mm in diameter and were held together by a screw clamp. The scleral tissue was washed twice in phenol red-free HBSS to remove culture medium and carefully sandwiched to avoid vortex veins between both chambers. Both chambers were tightly clamped to avoid leakage of the medium. Each chamber had 3 ports to fill and drain samples. Tested molecules included human recombinant FGF-2 (16kD, R & D Systems, Minneapolis, Minn) and 10 kDa rhodamine-dextran polymer (Molecular Probes). This dextran was included in the analysis because it is stable in tissue, it has no physiologic activity, and its transscleral movement has been previously characterized in normal sclera.[64] Rhodamine-dextran or FGF-2 was diluted in phenol red-free HBSS and applied in the orbital side chamber. After checking that there were no leaks in the uveal side chamber, phenol red-free HBSS was filled in this side. After assembly of Ussing chambers, the system was incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The assay of FGF-2 and dextran was dependently performed in the same scleral tissue. After the intended time, each sample was drained from the uveal side chamber and stored in a light-protected box.

Measurement of Dextran

Rhodamine-dextran concentration in the HBSS collected from the uveal side chambers was determined using a spectrofluorimeter. The excitation and emission

wavelengths were 550 and 580 nm, respectively. Standard curves of fluorescence versus concentrations were obtained by serial dilution of rhodamine-dextran dissolved in phenol red-free HBSS. Each sample was immediately measured 8 times, and the measurements were averaged.

Measurement of FGF-2 Concentration
FGF-2 concentration in the medium collected from the Ussing chamber was measured using a sandwich enzyme immunosorbent assay (R & D Systems). Optical density was measured at 450 nm and 540 nm using a microtiter plate reader (SpectraMax 250, Molecular Devices). To correct for nonspecific variation, the absorbence value at 540 nm was subtracted from that of 450 nm. Standard curves of absorbency versus concentrations were obtained by serial dilution of standard purified FGF-2.

Permeability Coefficient Determination
Diffusion from the orbital chamber to the uveal chamber was characterized by determination of a permeability coefficient (Pc), which is the ratio of steady-state flux to the concentration gradient.[64] In this study, the concentration of agents in the uveal side chamber, C_U, was less than 1% of it in the orbital chamber, C_o, thus the change of C_o was assumed to be under the limit of detection. Hence, the permeability coefficient was calculated as follows:

$$Pc\ (cm/sec) = (C_{Ut}-C_{U0})V/C_otS$$

where C_{U0} and C_{Ut} are the concentration in the Ussing chamber at 0 hour and at t hours, respectively. C_o is the initial drug concentration in the orbital chamber. V is a volume of each chamber (0.75 mL), and t is a duration time of steady-state flux converted the unit from hour to second. S is the surface area of exposed sclera (0.65 cm^2).

Statistical Evaluation
At least 7 experiments were performed on FGF-2 and dextran at each concentration of latanoprost acid. Each group was compared by using a Student's *t* test. A *P* value less than 0.05 was considered statistically significant. All data are presented as mean ± SD.

RESULTS

1. MEASUREMENT OF SCLERAL METALLOPROTEINASES (MMPS) AFTER TOPICAL PROSTAGLANDIN
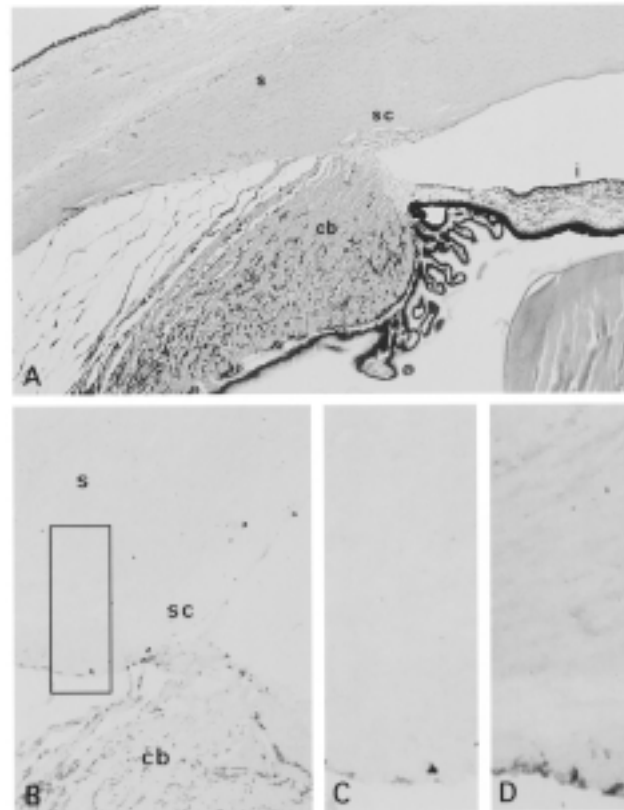
Immunohistochemistry
In the vehicle-treated monkey eyes, moderate immunoreactivity for MMP-1 was observed in the sclera (Fig 1). The distribution of MMP-2 immunoreactivity in the vehicle-treated eyes was similar with diffuse light staining

(Fig 1). Minimal MMP-3 immunoreactivity was present in the vehicle-treated eyes.

In the eyes treated with $PGF_{2\alpha}$-IE, there was increased MMP-1 and MMP-2 immunoreactivity in the sclera when compared with the corresponding vehicle-treated eye (Fig 1). Compared to the vehicle-treated eyes, moderate MMP-3 staining also was observed in sclera of the treated eyes.

Densitometric Analysis
The intensity of immunostaining was assessed along 2 lines placed over the image of the sclera observed with an imaging densitometer (Fig 2). Compared with the vehicle-treated eyes, there was increased MMP-1 immunoreactivity in all treated eyes (Table I). Table II shows the combined scores for MMP-1, MMP-2, and MMP-3. Overall, the optical density score for MMP-1 in the sclera in the treated eyes was increased by 63 ± 35% (mean ± SD). Similarly, the optical density score for MMP-2 was increased by 267 ± 210%, and the MMP-3 optical density score in the treated eyes was increased by 726 ± 500%. In

FIGURE 1
Anterior-segment tissues comparing hematoxylin and eosin staining (A) with MMP-1 immunoreactivity (B) containing sclera (s), ciliary body (cb), Schlemm's canal (sc), and iris (i). Dark areas in panel B indicate MMP-1 immunoreactivity. Box in panel B indicates scleral region shown at high magnification in panel C. D, Comparable area of sclera from eye that received topical $PGF_{2\alpha}$-IE shows increased MMP-1 immunoreactivity (magnification: A × 30; B × 39; C and D × 116).

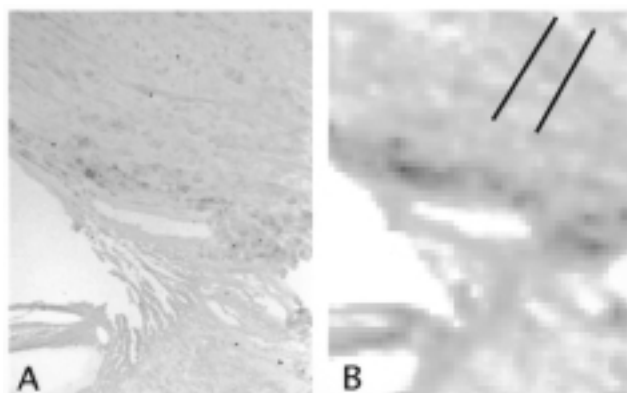*Enhancement of Scleral Macromolecular Permeability with Prostaglandins*

FIGURE 2

Comparison of bright field image of anterior segment (A) with densitometry image (B) showing placement of 2 measurement lines over sclera. Optical density along these lines was integrated and then mean optical density was determined by dividing the integrated score by the length of the measurement line (magnification $\times$ 56).

TABLE I: INCREASE OF MMP-1 IMMUNOREACTIVITY IN SCLERA OF 4 MONKEYS FOLLOWING 5 DAYS OF TOPICAL PGF$_{2\alpha}$-IE TREATMENT

MONKEY	CONTROL EYE*	TREATED EYE*	% INCREASE
1	0.0193	0.0240	24
2	0.0219	0.0458	109
3	0.0151	0.0230	51
4	0.0229	0.0386	68
Mean $\pm$ SD	0.0198 $\pm$ 0.0034	0.0328 $\pm$ 0.0112	63 $\pm$ 35

IE, isopropyl ester.
*Mean optical density.

TABLE II: MEAN INCREASE OF MMP IMMUNOREACTIVITY IN MONKEY SCLERA FOLLOWING 5 DAYS OF TOPICAL PGF$_{2\alpha}$-IE TREATMENT

MMP TYPE	ALTERNATE NAME	% INCREASE ($\pm$ SD)	P VALUE*
MMP-1	Interstitial collagenase	63 $\pm$ 35	0.01
MMP-2	Gelatinase A	267 $\pm$ 210	0.005
MMP-3	Stromelysin-1	729 $\pm$ 500	0.02

IE, isopropyl ester.
* P value by Student's t test; N=4.

each case, the increases in the treated eyes were statistically significant when compared to the vehicle-treated eyes (Table II).

2.   FP RECEPTOR GENE TRANSCRIPTION AND PROTEIN EXPRESSION IN HUMAN SCLERA

FP Receptor Transcripts in Human Sclera

To confirm that human sclera tissue contained mRNA that encodes the prostanoid FP receptor, RT-PCR was performed with primers that were predicted to yield a

FP-specific product of 1,186 base pair (bp). Figure 3 shows an ethidium-stained agarose gel with the PCR products obtained from cDNA prepared using 3 different primer
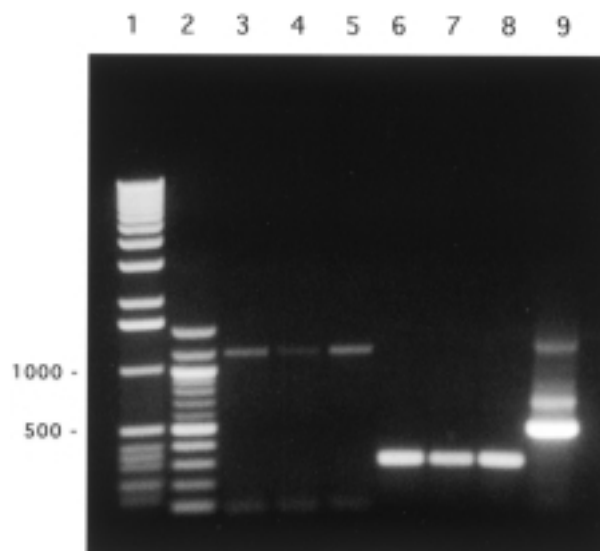

FIGURE 3

Reverse transcription–polymerase chain reaction (RT-PCR) of total RNA isolated from human sclera tissue and amplified with specific primers for human prostanoid FP receptor and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The products were separated by electrophoresis on a 1% DNA agarose gel. Standards (lanes 1 and 2) are 1 kilobase (kb) and 100 base pair (bp) DNA ladder (Gibco, BRL), respectively. Each reaction condition is represented. Lanes 3 (oligo DT alone), 4 (random primer alone), and 5 (oligo DT+random primer) yielded the predicted product (1,186 bp) after RT-PCR using total RNA isolated from donor sclera tissue, respectively. Lanes 6 (oligo DT alone), 7 (random primer alone), and 8 (oligo DT+random primer) represent the products obtained using specific primers for GAPDH from the identical RNA sample. The predicted product (299 bp) was obtained in each condition. The control (Gibco, BRL) for the cDNA synthesis reaction also yielded the predicted product size (500 bp, lane 9).

conditions from total RNA isolated from a single donor eye (lanes 1 through 3).   Additionally, PCR products using primers specific for GAPDH that were predicted to yield a product of 299 bp were analyzed from the same cDNA samples used for amplification of the FP receptor transcript (lanes 4 through 6).   The RT-PCR kit control was in vitro transcribed RNA from the chloramphenicol acetyltransferase (CAT) gene that was engineered to contain a 3′ poly(A) tail. Gene-specific primers used were predicted to yield a CAT-specific product of 500 bp (lane 9). As shown, products of the expected sizes were obtained in all conditions with the RNA isolated from the donor sclera. Because the primers used for the amplification of the FP receptor mRNA were chosen to span an intron within the FP receptor gene, the PCR products did not result from the amplification of genomic DNA and are consistent with the presence of mRNA encoding a human prostanoid FP receptor.

FP Receptor Protein in Human Sclera

Immunoreactivity for the FP receptor was observed within the cytoplasm of the scleral fibroblasts (Fig 4). The intensity of this granular staining was similar throughout the fibroblast processes that extended between the scleral collagen bundles. No staining of these collagen bundles was observed.

3. MEASUREMENT OF HUMAN SCLERAL PERMEABILITY AND MMPS WITH PROSTAGLANDIN EXPOSURE

Scleral Permeability

Scleral permeability was measured by assessing the flux of labeled dextrans across the scleral cultures in a Ussing chamber. Dextrans of different sizes were evaluated to model the potential differences among aqueous proteins of different sizes. As shown in Fig 5, flux across the scleral cultures incubated without PGs was 1.5×10^{-6} cm/second for 10 kDa dextran, 0.7×10^{-6} cm/second for 40 kDa dextran, and 0.4×10^{-6} cm/second for 70 kDa dextran. Moreover, these fluxes did not change among cultures incubated without PGs for 1, 2, or 3 days. In contrast, incubation with $PGF_{2\alpha}$ significantly increased the flux of the 10 kDa tracer. These increases ranged from 21% to 124%, were dose-dependent, became larger as exposure time increased up to 3 days, and were significant for all concentration and tested time points ($P<.05$). The flux of 40 kDa dextran also increased with increasing $PGF_{2\alpha}$ and

FIGURE 4
FP receptor immunoreactivity within human scleral fibroblasts (A, B) and staining control (C, D). For each specimen, the bright-field image (A, C) and the fluorescence image (B, D) are shown. Staining was similar throughout fibroblast processes that extended between the scleral collagen bundles. Control sections were processed without exposure to primary antibody (magnification, x280).

exposure time; however, these increases ranged from 7% to 21%. These permeability increases were statistically significant only on day 3 in the case of 100 nM $PGF_{2\alpha}$, but were significant for 200 nM or 500 nM $PGF_{2\alpha}$ on all 3 days. Similar to the 40 kDa dextran, the flux of 70 kDa dextran increased with $PGF_{2\alpha}$ dose and exposure time by 5% to 28%. These increases were significant with longer treatments at 100 nM or 200 nM, and were significant with 500 nM $PGF_{2\alpha}$ on all 3 days.

Incubation of scleral cultures with 17-phenyltrinor-$PGF_{2\alpha}$ also increased permeability of the scleral organ cultures to the labeled dextrans. Permeability to the 10 kDa tracer increased in a dose-dependent and time-dependent manner from 5% to 183% (Fig 6). These increases were significant for all conditions except 100 nM 17-phenyltrinor-$PGF_{2\alpha}$ exposure for 1 day. Permeability to the 40 kDa tracer increased in a dose- and time-dependent manner from 4% to 31%. These increases were significant at all concentrations tested on days 2 and 3. Permeability to the 70 kDa tracer increased from 9% to 24%. These increases were significant at all concentrations and times measured. Overall, the increases observed with 17-phenyltrinor-$PGF_{2\alpha}$ were similar to the increases observed with $PGF_{2\alpha}$. The exception to this was the larger permeability increase observed at 3 days with 100 nM 17-phenyltrinor-$PGF_{2\alpha}$ than with 100 nM $PGF_{2\alpha}$. PhXA85 generally induced moderately larger increases in scleral permeability than $PGF_{2\alpha}$ or 17-phenyltrinor-$PGF_{2\alpha}$ (Fig 7). Flux of the 10 kDa tracer was increased by 45% to 213%. These increases were dose-dependent, became larger as exposure time increased up to 3 days, and were significant for all conditions. The flux of 40 kDa dextran also increased with increasing PhXA85 and exposure time; however, these increases ranged from 6% to 41%. These increases were significant for all conditions except 100 nM PhXA85 exposure for 1 day. Similar to 40 kDa dextran, the flux of 70 kDa dextran increased with PhXA85 dose and exposure time by 13% to 48%. Also, these increases were significant for all conditions except 100 nM PhXA85 exposure for 1 day.
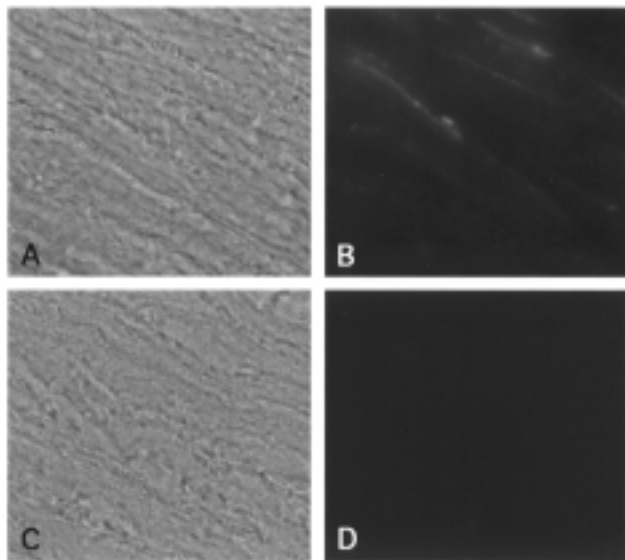
Viability of Sclera

Survival of cells in the organ culture was assessed by measuring the exclusion of ethidium homodimer, a vital stain that binds to DNA. The standard for maximal viability was freshly obtained donor sclera, and the standard for complete loss of viability was donor sclera that had been exposed to 2% paraformaldehyde prior to ethidium homodimer exposure. As shown in Fig 8, viability for all cultures was about 83% on day 1, 81% on day 2, and 80% on day 3. Differences of viability among cultures exposed to 500 nM $PGF_{2\alpha}$, or 17-phenyltrinor-$PGF_{2\alpha}$ were less than 1% on all 3 days. This suggests that incubation with

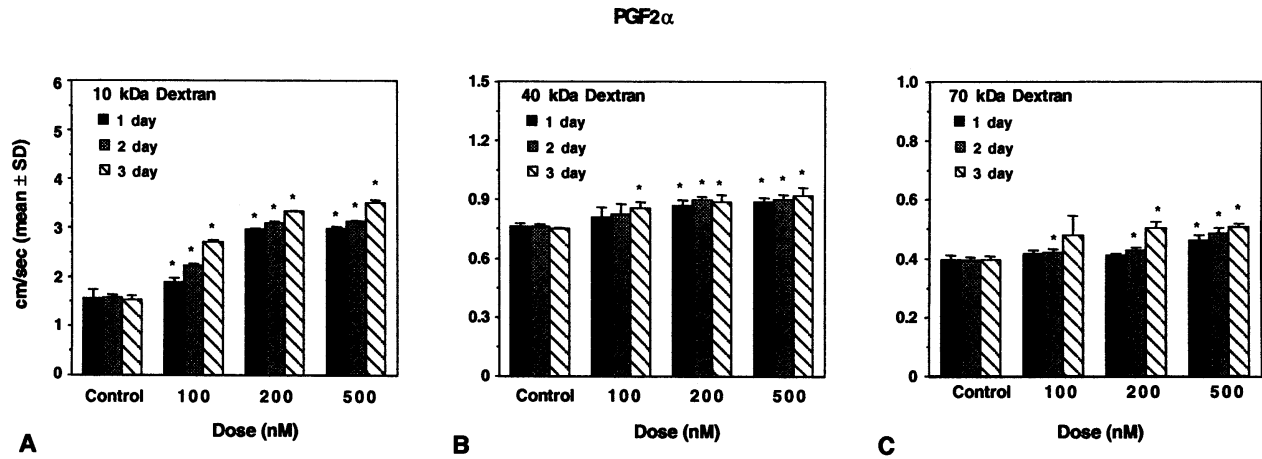*Enhancement of Scleral Macromolecular Permeability with Prostaglandins*

FIGURE 5
Scleral permeability after $PGF_{2\alpha}$ exposure. Permeability determined by the transscleral movement of 10 kDa (A), 40 kDa (B), or 70 kDa (C) dextrans across treated sclera. Values represent mean ((10-6 cm/sec) ± SD. Asterisk indicates $P<.05$ by the Student Newman Keuls test (N=4).
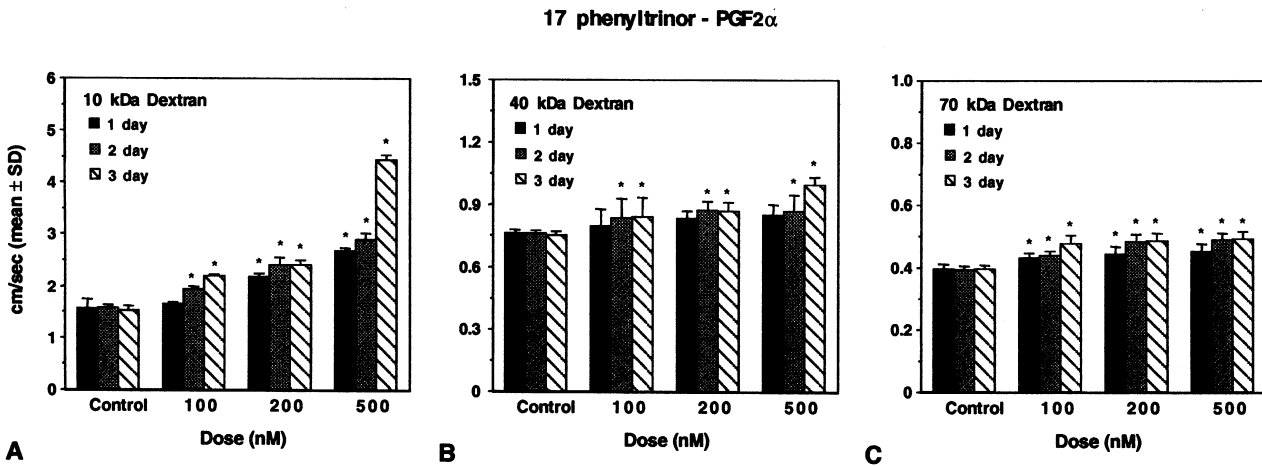

FIGURE 6
Scleral permeability after 17-phenyltrinor-$PGF_{2\alpha}$ exposure. Permeability determined by the transscleral movement of 10 kDa (A), 40 kDa (B), or 70 kDa (C) dextrans across treated sclera. Values represent mean ((10-6 cm/sec) ± SD. Asterisk indicates $P<.05$ by the Student Newman Keuls test (N=4).
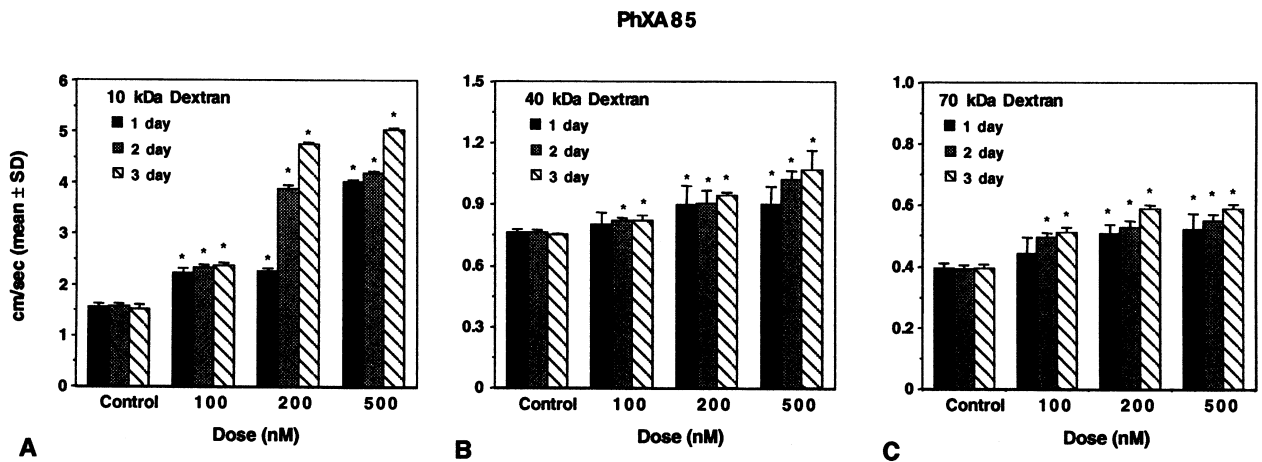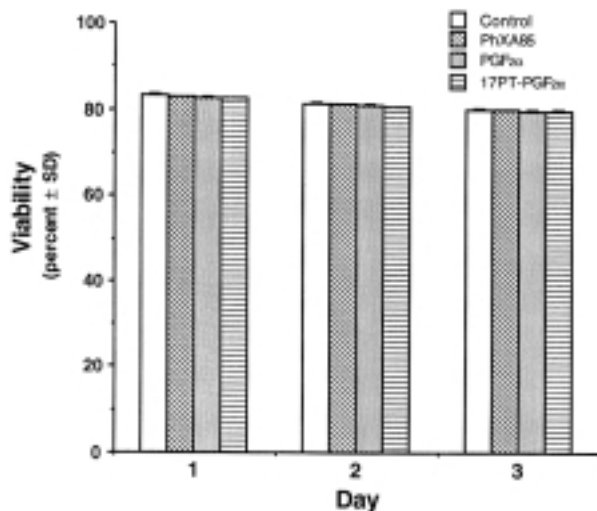

FIGURE 7
Scleral permeability after PhXA85 exposure. Permeability determined by transscleral movement of 10 kDa (A), 40 kDa (B), or 70 kDa (C) dextrans across treated sclera. Values represent mean ((10-6 cm/sec) ± SD. Asterisk indicates $P<.05$ by the Student Newman Keuls test (N=4).

FIGURE 8
Cell viability within organ-cultured human sclera incubated for 3 days with vehicle, $PGF_{2\alpha}$, 17-phenyltrinor-$PGF_{2\alpha}$, or PhXA85. Concentration of each prostaglandin was 500 nM. Values represent mean ± SD (N=4).

these PGs for 3 days had minimal influence on cell survival in the scleral cultures.

Scleral Hydration

To evaluate whether changes in scleral hydration occur with the culture conditions, the water content in the scleral cultures was determined in scleral cultures exposed to HBSS for 4 hours at room temperature, to complete culture medium for 3 days at 37°C, or to complete culture medium for 3 days followed by 4 hours in HBSS. The mean water content, or scleral hydration, of fresh sclera was 3.05 ± 0.11 mg water/mg dry weight (N=5). As shown in Fig 9, scleral cultures incubated 4 hours in HBSS alone, in medium for 3 days, or in medium for 3 days followed by 4 hours in HBSS were insignificantly different from the fresh cultures (*P*<.05, Student Newman Keuls test). This indicated that these culture conditions did not alter hydration within the scleral cultures.

MMP Release Induced by Prostaglandin Treatments

One possible explanation for the observed increases in scleral permeability following exposure to the PGs is reduction in collagen content by MMPs. Hence, the media of scleral cultures incubated with $PGF_{2\alpha}$, 17-phenyltrinor-$PGF_{2\alpha}$ or PhXA85 were assayed for changes in the concentration of MMP-1, MMP-2, and MMP-3. Among cultures incubated in control medium for 1, 2, or 3 days, there were no significant changes in the concentration of MMP-1, MMP-2, or MMP-3 (Figs 10 through 12).

Evaluation of MMP-1 in the media of the treated cultures showed moderate increases in cultures exposed to $PGF_{2\alpha}$, 17-phenyltrinor-$PGF_{2\alpha}$ or PhXA85 (Fig 10). These increases ranged up to 37%, increased with time of

exposure, and were significant only for the higher concentrations and longer incubation times examined. Overall, there were slight increases of MMP-1 with increasing dose, and the effects of the different PGs tested were similar.

In contrast to MMP-1, increases in MMP-2 were much larger and ranged from 124% to 267% (Fig 11). These increases were significant in every condition examined and showed marked increases with increasing time of exposure. Overall, there were slight increases of MMP-2 with increasing PG concentration. The magnitude of the effects was least with 17-phenyltrinor-$PGF_{2\alpha}$, intermediate with $PGF_{2\alpha}$, and greatest with PhXA85.

MMP-3 concentration also increased in the media of cultures exposed to $PGF_{2\alpha}$, 17-phenyltrinor-$PGF_{2\alpha}$ or PhXA85 (Fig 12). These increases ranged up to 96% and were larger than seen with MMP-1 but smaller than seen with MMP-2. These increases were clearly time-dependent, being generally insignificant on day 1 and significant on days 2 and 3. Dose dependence was clearly present with PhXA85 at every time point and less clear with $PGF_{2\alpha}$ or 17-phenyltrinor-$PGF_{2\alpha}$.

4. MEASUREMENT OF FIBROBLAST GROWTH FACTOR-2 PERMEATION THROUGH HUMAN SCLERA WITH PROSTAGLANDIN EXPOSURE

Time Course Analysis

The time course of FGF-2 penetration of sclera within the Ussing chamber was assessed by withdrawing a 40 μL sample from the test side at 30-minute intervals. As shown in Fig 13, the concentration increased linearly for the duration of the experiment.
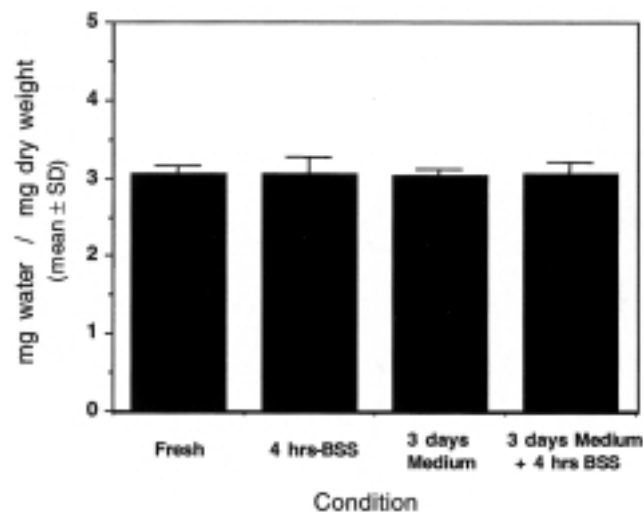

FIGURE 9
Evaluation of scleral hydration in fresh sclera, scleral organ cultures following incubation with Hank's balanced salt solution (BSS) for 4 hours (4hrs-BSS), tissue culture medium for 3 days (3days-Medium), or in culture incubated in medium for 3 days followed by 4 hours in Hank's balanced salt solution (3 days Medium + 4 hrs BSS). Values represent mean ± SD (N=5).

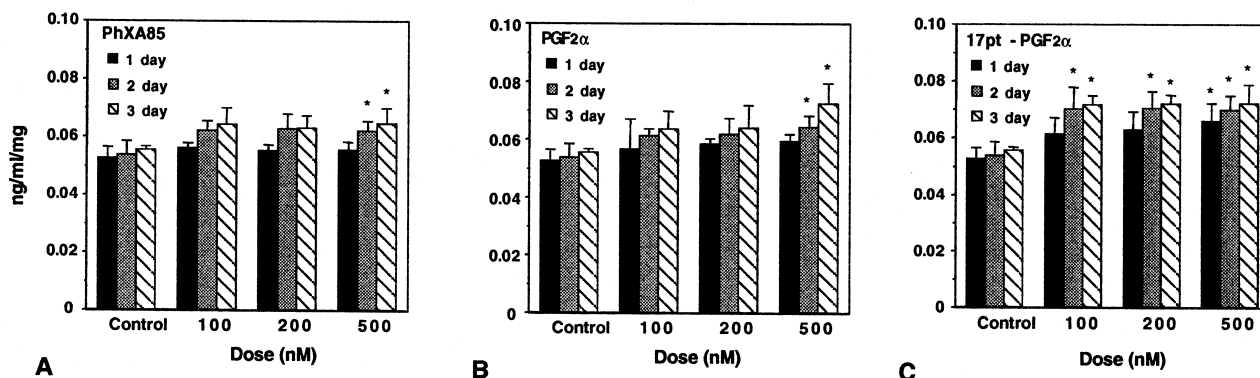*Enhancement of Scleral Macromolecular Permeability with Prostaglandins*

MMP-1

FIGURE 10

Concentration of MMP-1 in the medium of scleral organ culture exposed to PhXA85 (A), $PGF_{2\alpha}$ (B), or 17-phenyltrinor- $PGF_{2\alpha}$ (C) for 1 to 3 days as determined by ELISA. Cultures analyzed were from cultures generated from a 76-year-old male donor, a 66-year-old male donor, and a 45-year-old female donor (N=3). Values represent mean ± SD. Asterisk indicates *P*<.05 by the Student Newman Keuls test (N=4).
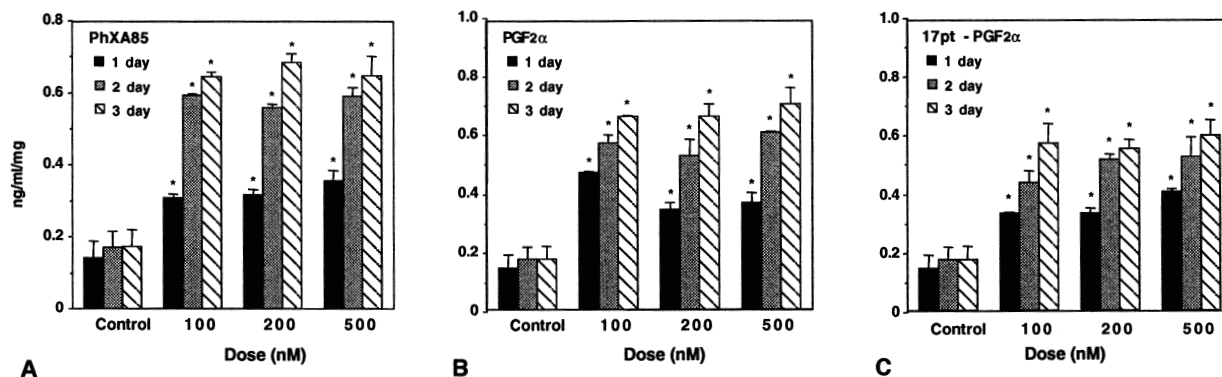
MMP-2

FIGURE 11

Concentration of MMP-2 in the medium of scleral organ culture exposed to PhXA85 (A), $PGF_{2\alpha}$ (B), or 17-phenyltrinor-$PGF_{2\alpha}$ (C) for 1 to 3 days as determined by ELISA. Cultures analyzed were from cultures generated from a 76-year-old male donor, an 80-year-old male donor, and a 66-year-old male donor Values represent mean ± SD. Asterisk indicates *P*<.05 by the Student Newman Keuls test (N=3).
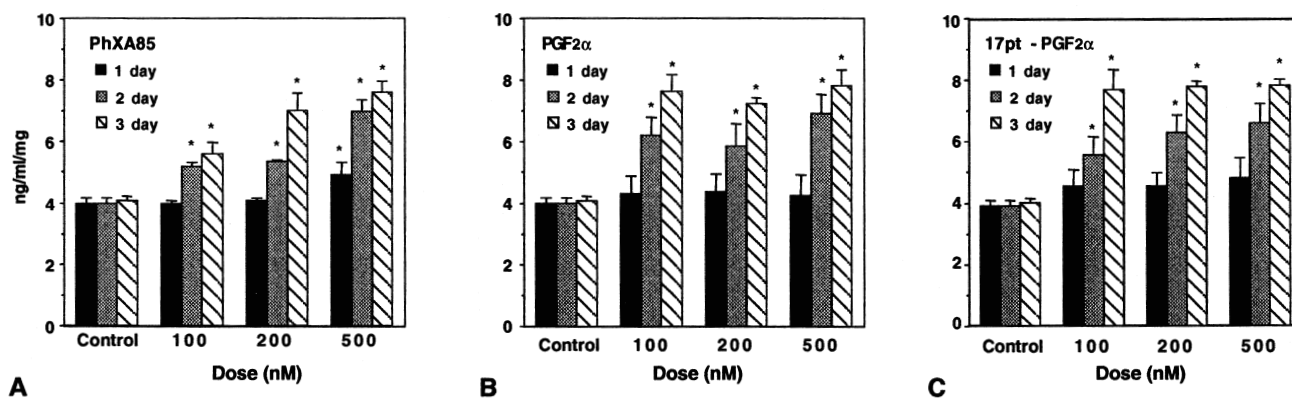
MMP-3

FIGURE 12

Concentration of MMP-3 in the medium of scleral organ culture exposed to PhXA85 (A), $PGF_{2\alpha}$ (B), or 17-phenyltrinor- $PGF_{2\alpha}$ (C) for 1 to 3 days as determined by ELISA. Cultures analyzed were from cultures generated from a 76-year-old male donor, a 66-year-old male donor, and a 45-year-old female donor. Values represent mean ± SD. Asterisk indicates *P*<.05 by the Student Newman Keuls test (N=3).
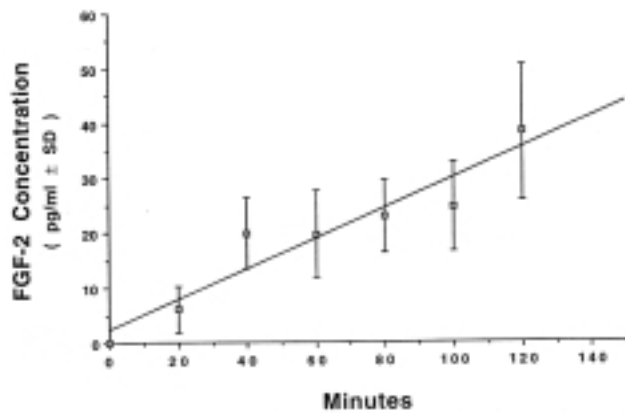
FIGURE 13
Time course of FGF-2 concentration in receiving Ussing chamber fitted with human sclera previously cultured for 3 days in control medium. Data presented as mean ± SD ((10-8 cm/sec). Increase in concentration with time was linear (R^2=0.91). Based on this, permeability was determined to be 1.68 x 10^{-8} cm/sec. N=4 pairs of donor eyes. Mean age of donors was 67.5 ± 2.9 (SD) years.
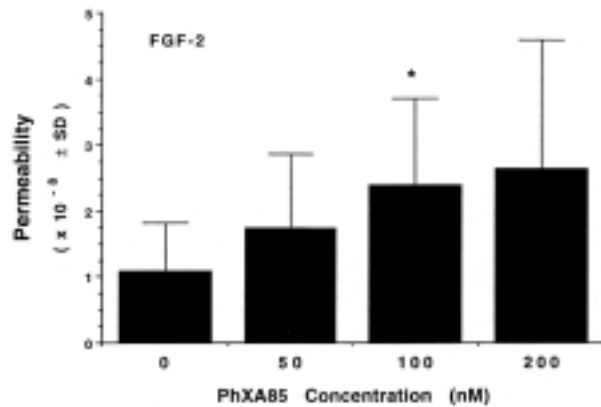

FIGURE 14
Scleral permeability to FGF-2 after exposure to various concentrations of PhXA85 for 3 days. Data presented as mean ± SD (x 10^{-8} cm/sec). Asterisk indicates *P*<.05 by the Student Newman Keuls test. N=8 pairs of donor eyes. Mean age of donors was 71.8 ± 6.9 (SD) years.

Dose Response Analysis

Increasing the concentration of latanoprost acid in scleral explant cultures maintained for 3 days increased the permeability of both FGF-2 and 10 kDa dextran (Figs 14 and 15). FGF-2 permeability following 50 or 100 nM latanoprost acid was increased by an average of 56 ± 77% and 126 ± 120%, respectively (mean, ± SD, N=8). FGF-2 permeability in sclera incubated with 200 nM latanoprost acid was similar to sclera incubated with 100 nM latanoprost acid. In contrast, 10-kDa dextran permeability following 50, 100, or 200 nM latanoprost acid was increased by an average of 50 ± 24%, 39 ± 19%, and 48 ± 24%, respectively. The ratio of FGF-2 to 10 kDa dextran permeability ranged from 40-fold to 90-fold; however, there was no clear relationship between the magnitude of the ratio and the latanoprost acid dose.

DISCUSSION

The limits of the possible are enlarged.
                                        Ralph Waldo Emerson

In contrast to the sclera being "inert and purely supportive in function,"[54] these studies clearly demonstrate that it has the potential to be metabolically active, to be pharmacologically responsive, and to have other functions in addition to structural support. Moreover, PGs can directly induce sclera to undergo structural modifications that enhance transscleral permeability, a response that is likely mediated by FP receptors on scleral fibroblasts. These results may have important implications for the facilitation of macromolecule delivery to posterior segment tissues.

1. TOPICAL PROSTAGLANDIN ADMINISTRATION REDUCES SCLERAL COLLAGEN BY INCREASING SCLERAL METALLO-PROTEINASES

The quantitative immunohistochemistry results show that topical treatment of monkey eyes with $PGF_{2\alpha}$-IE increased expression of MMP-1, MMP-2, and MMP-3 in the sclera adjacent to the ciliary muscle. Increased MMP biosynthesis could then reduce scleral collagens and other extracellular matrix molecules after treatment with topical $PGF_{2\alpha}$-IE. This reduction has been previously confirmed for collagen type I, collagen type III, and collagen type IV.[82] MMP-1 is known to hydrolyze a specific site found in collagen types I and III.[109,110] Likewise, MMP-2 is known to hydrolyze specific sites found in collagen type IV as well as in fibronectin. MMP-3 is known to hydrolyze specific sites found in collagen types III and IV, as well as
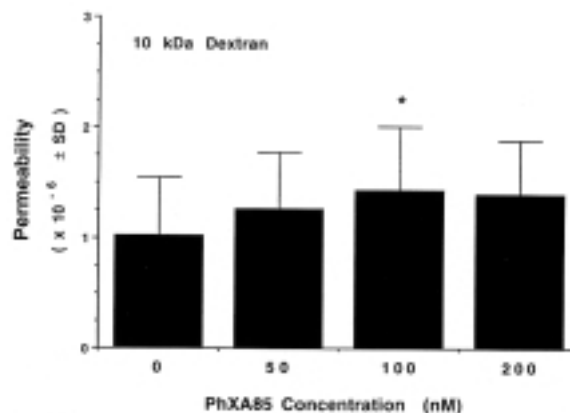

FIGURE 15
Scleral permeability to 10 kDa dextran after exposure to various concentrations of PhXA85 for 3 days. Data presented as mean ± SD (x 10^{-6} cm/sec). Asterisk indicates *P*<.05 by the Student Newman Keuls test. N=8 pairs of donor eyes. Mean age of donors was 71.8 ± 6.9 (SD) years.

in fibronectin and laminin. Hence, the observed increases in MMPs-1, -2, and -3 suggest a concerted response leading to reduced scleral collagen. Other extracellular hydrolases also are likely to participate in the reduction of extracellular matrix. It should be noted that MMPs are secreted as inactive pro-enzymes that are subsequently activated by proteolytic truncation.[111,112] Also, MMP activity is regulated by the presence of tissue inhibitor of matrix metalloproteinases (TIMPs).[113,114] Each of the antibodies used can recognize both the proenzyme and the active enzyme. Thus, the magnitude of the increased MMP activity may be less than the magnitude of the increased immunoreactivity.

2. FP RECEPTOR GENE TRANSCRIPTS AND PROTEIN ARE PRESENT IN HUMAN SCLERA

As the biologic activity of a drug is often mediated by a specific receptor, the observation of FP receptor transcripts and protein within human sclera suggests that FP receptor agonists can directly activate these receptors and initiate physiologic and pharmacologic responses. The predominant cell type in the sclera is the scleral fibroblast. Also present are vascular endothelial cells within the penetrating blood vessels. Scleral fibroblasts are interspersed among the collagen layers that make up the scleral stroma and biosynthesize scleral collagen. The immunohistochemical results in these studies confirm that FP receptor in sclera is present on the scleral fibroblasts. Cultured fibroblasts from other tissues are known to increase their production of MMPs following stimulation with certain peptides.[115-117] Hence, it is highly plausible that exposure of scleral fibroblasts to FP agonists may promote their biosynthesis of MMPs. The second cell type is the vascular endothelial cell of blood vessels penetrating the sclera. FP receptors have been detected in association with other ocular blood vessels.[90] However, previous studies indicate that specific FP receptor agonists have minimal effects on the permeability of intraocular blood vessels.[118,119]

3. PROSTAGLANDIN EXPOSURE INCREASES HUMAN SCLERAL PERMEABILITY AND MMPS

Pharmacologic considerations of the permeability and MMP changes observed with the tested PGs further support involvement of FP receptor activation. The concentrations of $PGF_{2\alpha}$ and 17-phenyltrinor-$PGF_{2\alpha}$ tested were greater than the EC_{50} for activation of the FP receptor.[105] It is possible that, if present, EP1 receptors also may have been activated by the $PGF_{2\alpha}$ or 17-phenyltrinor-$PGF_{2\alpha}$ treatments in this study as EC_{50}'s for these agonists are 320 nM and 650 nM, respectively.[105] However, the response to PhXA85 is likely to reflect FP receptor activation, for

which the EC_{50} is 100 nM, and not activation of EP1 or other PG receptors. The EC_{50} concentrations for PhXA85 activation of PG receptors other than the FP receptor are at least tenfold higher than the highest PhXA85 concentration tested.[105] Hence, it is likely that the increased MMPs observed in the PG-treated scleral cultures were released by FP-receptor-mediated activation of scleral fibroblasts. These MMPs would be well positioned to initiate collagen remodeling within the scleral stroma that enlarged intrascleral supramolecular passages and thereby facilitated transscleral protein permeability. As the MMPs in the present experiments could accumulate in the closed culture system, whereas they might dissipate upon secretion in situ, the concentration of the MMPs measured may be greater than the concentrations that might occur in scleral interstitial fluid in situ.

The studies of dextran permeability indicate that PGs directly increase the permeability of human sclera in organ culture. This increase in permeability is accompanied by increased release of MMPs from scleral tissue. These changes are consistent with the reduced collagens observed in monkey sclera following topical PG treatment and suggest that remodeling of the scleral extracellular matrix may explain the increased permeability. Hydration analysis indicates that this response does not reflect any alteration of scleral hydration. Viability analysis indicates that this response is not associated with altered cell survival in the experimental system, nor is there any evidence of toxicity due to the PG treatments. These permeability changes are likely to be normal physiologic responses, as they are both dose- and time-dependent. That the PG treatments also increased release of MMP-1, MMP-2, and MMP-3 in these cultures suggests that the permeability changes may reflect a direct response of scleral tissue to PG exposure and that the mechanism of increased transscleral permeability likely reflects intrascleral collagen remodeling. This proposed model is well supported by the findings of Dan and Yaron,[76] who observed increased flow of saline across bovine sclera and thinning of rabbit sclera in response to focal application of clostridial collagenase, an MMP-1 analogue.

The permeability relationships of the various sizes of labeled dextran observed in the present control scleral cultures is similar to the permeability relationships[64] of these tracers previously observed in sclera freshly dissected from donor eyes. For example, the present study found that permeability of the 40 kDa dextran through the scleral organ cultures was 1.7-fold less than that of 10 kDa dextran. This is similar to the previous observation that 40 kDa dextran permeability is 1.4-fold to 3.8-fold less than 10 kDa dextran in freshly dissected sclera.64 Likewise, the present observation that permeability of 70 kDa dextran in the scleral organ cultures was 3.7-fold less than

that of 10 kDa dextran is similar to the previous observation that 70 kDa dextran permeability was 2.6-fold to 4.2-fold less than 10 kDa dextran permeability in freshly dissected donor eye sclera.[64] These similarities suggest that hydrodynamic constraints to macromolecule movement through the scleral organ cultures were similar to freshly dissected donor sclera. Hence, the scleral cultures represent a reasonable model system in which to study modulation of transscleral macromolecule movement by PGs.

The greater increase in 10 kDa dextran permeability through PG-treated scleral cultures than was observed with 40 kDa or 70 kDa dextran suggests that PGs may alter the size of intrascleral supramolecular passages. Scleral collagen fibrils are organized into bundles that vary in their organization according to position near the outer or inner wall of the sclera.[50-52] Overall, the bundles vary in width and thickness, often give off branches, and intertwine with each other. At the outermost layers, there is substantial irregular intermingling of collagen fibrils in adjacent bundles. Like sclera, synthetic hydrogels contain substantial water content and long polymer units characterized by chemical cross-links and polymer entanglements.[120,121] Within pH-sensitive hydrogels, lower pH increases the size of pore channels through the matrix, while higher pH causes the gel network to swell with a resulting increase in the size of pore channels. Analysis of a pH-sensitive hydrogel confirmed that protein permeability is enhanced under conditions that increase the size of the pore channels.[121] Moreover, the magnitude of the permeability increase was greater with lower-molecular-weight proteins than with higher-molecular-weight proteins. This relationship among protein size, macromolecule permeability, and pore size also has been seen in hydrogels in which pore size was altered by changing the size of polymer subunits used to synthesize the hydrogel.[121] Hence, the greater permeability increases with the smaller dextran tracers that was observed in the PG-treated scleral cultures is consistent with enlargement of the intrascleral supramolecular passages.

The mechanism of increased permeability within the PG-treated scleral cultures is suggested by the increased amounts of MMP-1, MMP-2, and MMP-3 detected within the medium of the treated scleral cultures. Sclera contains collagen types I, III, VI, VIII, XII, possibly a small amount of collagen type V, as well as fibronectin.[56,122-126] Of these extracellular matrix components, MMP-1, MMP-2, and MMP-3 are known to cleave sites within collagen types I, III, V, and fibronectin.[85,127] Increased MMP-1, MMP-2, MMP-3, and MMP-9 has been found in cultures of human ciliary smooth muscle cells exposed to $PGF_{2\alpha}$, 17-phenyltrinor-$PGF_{2\alpha}$, and PhXA85.[107,108] These treatments also induce reorganization of collagen type I, collagen type III, laminin, and collagen type IV within the

human ciliary muscle cultures.[128,129] Hence, increased MMPs within sclera are likely to mediate reduction of scleral collagens.

4. TRANSSCLERAL PERMEATION OF FIBROBLAST GROWTH FACTOR-2 IS INCREASED WITH PROSTAGLANDIN EXPOSURE

The present results also indicate that exposure of human sclera to latanoprost acid, a prostaglandin analogue, increases FGF-2 permeability. This increase is dose dependent and increases with increasing exposure times. It is likely to reflect increased general permeability as it parallels increased permeability to 10 kDa rhodamine-labeled dextrans.

The greater permeability of 10 kDa dextran may be related to binding of FGF-2 to molecules within the sclera. These molecules include collagen types I, III, V, VI, and VIII and the glycosaminoglycans (GAGs) chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and hyaluronan.[57,58] Much of the chondroitin sulfate, dermatan sulfate, and keratan sulfate may be covalently linked to the proteoglycans decorin, biglycan, and aggrecan.[58] Immunoreactivity of each of these proteoglycans is distributed throughout the thickness of sclera.[58] It is well established that FGF-2 strongly binds to heparan sulfate (Kd=0.34 μM).[130] Recently, it has been shown that FGF-2 also can bind to dermatan sulfate (Kd=2.5 μM).[131] As each of these GAGs is present within sclera, it is possible that binding of FGF-2 to these extracellular matrix components could impede the movement of FGF-2 through the sclera.

Increased transscleral permeability to FGF-2 following PG treatments suggests that cotreatment with PGs could facilitate the use of FGF-2 to enhance survival of retinal neurons in glaucoma and other eye diseases. Previous studies have shown that FGF-2 can promote neuronal survival in vitro and in vivo.[132,133] Beneficial effects were observed with concentrations as low as 20 pg/mL. Moreover, intraventricular infusion of FGF-2 can promote neuronal survival following experimental axotomy, ischemia, neurotoxin treatment, or contusion of brain or spinal cord tissue.[134-136] However, infusion of FGF-2 can stimulate responses in many other tissues besides neural tissues that may be either beneficial or detrimental to the desired neural tissue response. There also may be specific requirements for additional factors in the case of retinal ganglion cells.[137] Further, systemic infusion of FGF-2 also can stimulate responses in many nonneural tissues that may be either beneficial or detrimental to the desired neural tissue response.[138] Hence, the ability to enhance scleral penetration of FGF-2 using PGs may allow a smaller concentration of FGF-2 to be delivered directly to the eye with consequent reduction of systemic absorption.

Enhancement of Scleral Macromolecular Permeability with Prostaglandins

SIGNIFICANCE OF INCREASED PERMEABILITY FOR INTRAOCULAR DRUG DELIVERY

Assessment of drug diffusion in vitro permeability studies is a useful approach in the field of ocular pharmacokinetics to estimate drug movement for in vivo conditions. Intraocular absorption of a drug is directly related to the transport characteristics of absorptive tissues of the eye, such as the sclera. The increased scleral permeability following PG exposure may have implications for facilitating delivery of therapeutics to the posterior segment of the eye. For example, growth factors that may facilitate retinal neuron survival range from 10 kDa to 40 kDa (Table III).[1,16,139] Because of their size, these molecules cannot readily cross the cornea. Hence, a noncorneal absorption route through sclera may facilitate usefulness of such therapeutics.

It may not be sufficient only to increase scleral permeability to large molecules, as there also may be other limiting factors for drug absorption in the posterior seg-

TABLE III: MOLECULAR WEIGHT AND SIZE OF SEVERAL NEUROTROPHIC PROTEINS

MOLECULE	MOLECULAR WEIGHT (DA)	MOLECULAR RADIUS (NM)*
Fibroblast growth factor–2 (FGF-2)	18,000	2.1
Ciliary neurotrophic factor	22,800	2.3
Nerve growth factor-β	26,000	2.5
Brain-derived neurotrophic factor (dimer)	27,200	2.5
Neurotrophin-3 (dimer)	27,200	2.5
Neurotrophin-4 (dimer)	28,000	2.5

*Estimated.

ment. Orbital clearance, intraocular pressure, uveoscleral outflow, and choroidal blood flow might each limit drug access to the target tissues. Blood-ocular barriers, especially the blood-retinal barrier, also may be important.[140-144] However, the integrity of the blood-ocular barriers can be disrupted under certain conditions.[17,145,146] As an example, fluorescein (MW, 376) particles that are not bound to albumin can pass through the spaces between the endothelial cells of the capillaries of the choriocapillaris, but normally they cannot leak through the retinal pigment epithelium and zonula occludens between adjacent retinal pigment epithelial cells. Fluorescein in the choroid cannot enter the neurosensory retina unless there is a defect in the retinal pigment epithelium. Therefore, if the tight junctions of the blood-retinal barrier preclude retinal drug absorption of a transsclerally delivered drug, it may be possible to minimally damage retinal pigment epithe-

lial cells and transiently facilitate macromolecular movement from the suprachoroidal space to the retina.[17,145]

While the blood vessels in the optic nerve head, by virtue of tight junctions, have a blood-optic nerve barrier, it is significant that the optic nerve head itself is not thought to possess a blood-ocular barrier.[147,148] The border tissue of Elschnig (separating the peripapillary choroid and optic nerve head) allows choroidal interstitial tissue fluid to leak into the optic nerve head from the peripapillary choroid. Horseradish peroxidase (42 kDa) can enter the monkey optic nerve head from blood.[148] Glial cells at the edge of the optic disc form a barrier that prevents the spread of peroxidase into the retina.

Despite these possible limitations, the prospect of increased transscleral permeability by PG cotreatment may allow sufficient transscleral transport to provide delivery of therapeutics to posterior segment tissues in concentrations not otherwise possible. This may be particularly important for glaucomatous eyes, as elevated intraocular pressure may reduce scleral permeability, particularly for macromolecules.[81] Besides being important for macromolecule delivery to the posterior segment, transscleral fluid movement through scleral stroma may be important for uveoscleral outflow. Further study is needed to determine if PG-induced increases in transscleral permeability contribute to increased uveoscleral outflow facility and decreased intraocular pressure observed following topical PG treatments.

CONCLUSION

Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

Winston Churchill

The sclera is not inert, but metabolically active. PG treatment can increase scleral MMPs and facilitate transscleral permeability. This can promote the transscleral movement of molecules as large as 70 kDa and may enhance transscleral delivery of a number of different ocular drugs.

Several findings reported here provide support for a series of cellular and molecular events that could explain the mechanism of this response. These findings include observation of a concomitant increase in the amount of MMPs within the sclera of PG-treated monkey eyes, the presence of FP receptor and protein within human sclera, increased MMPs within the medium of human scleral cultures incubated with PGs, and increases in the amount of FGF-2 permeation within human scleral cultures exposed to increasing concentrations of latanoprost acid. Decreased collagen type I and III has been observed previously in monkey sclera and ciliary muscle following repeated topical PG treatment.[82] Moreover, induction of

c-Fos, MMP synthesis, and collagen reduction in ciliary muscle cell cultures exposed to PGs also have been described.[128,129,149] Together, these observations suggest a cellular mechanism for the increased transscleral permeability occurring with PG exposure. As summarized in Fig 16, a PG diffuses into the scleral stroma following topical or periocular treatment. Within the sclera, it then binds to FP receptors on scleral fibroblasts. This triggers a cascade of molecular events that increase MMP gene tran-
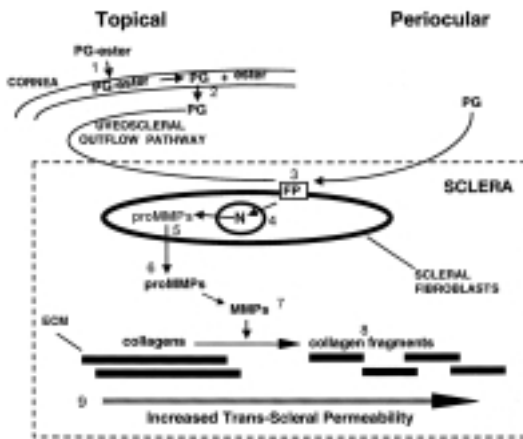

FIGURE 16

Model of FP receptor agonist action on sclera that results in increased transscleral permeability. FP receptor agonists (indicated by PG) can gain access to sclera either via topical drops (in which the PG is linked to an ester group to enhance transcorneal permeability), and then travel to sclera by uveoscleral outflow, or directly by periocular placement, as shown. Topical PG-ester is hydrolyzed as it passes through the cornea (1) into the anterior chamber (2). PG arriving at the sclera binds to FP receptors on scleral fibroblasts (3) and triggers signals that lead to increased MMP gene transcription (4) and proMMP biosynthesis (5) and secretion (6). Upon extracellular activation (7), the MMPs initiate collagen degradation (8) that results in increased transscleral permeability (9).

scription and lead to increased proMMP biosynthesis and secretion. Upon activation, the MMPs alter scleral collagen, which increases scleral permeability.[69] In this way, cotreatment with PGs may induce pharmacologic alterations of the sclera that promote transscleral delivery of peptide therapeutics. This could be useful for ameliorating some diseases of the posterior segment of the eye.

ACKNOWLEDGEMENTS

REFERENCES

1. Lindsay RM. Therapeutic potential of the neurotrophins and neurotrophin-CNTF combinations in peripheral neuropathies and motor neuron diseases. *Ciba Found Symp* 1996;196:39-48.
2. Horner PJ, Gage FH. Regenerating the damaged central nervous system. *Nature* 2000;407:963-970.
3. Weinreb RN, Levin LA. Is neuroprotection a viable therapy for glaucoma? *Arch Ophthalmol* 1999;117:1540-1544.
4. LaVail MM, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci* 1998;39:592-602.
5. Fontaine V, Kinkl N, Sahel J, et al. Survival of purified rat photoreceptors in vitro is stimulated directly by fibroblast growth factor-2. *J Neurosci* 1998;18:9662-9672.
6. Hicks D, Courtois Y. Fibroblast growth factor stimulates photoreceptor differentiation in vitro. *J Neurosci* 1992;12:2022-2033.
7. Lindsey JD, Weinreb RN. Survival and differentiation of purified retinal ganglion cells in a chemically defined microenvironment. *Invest Ophthalmol Vis Sci* 1994;35:3640-3648.
8. Pease ME, McKinnon SJ, Quigley HA, et al. Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma. *Invest Ophthalmol Vis Sci* 2000;41:764-774.
9. Chong NH, Alexander RA, Waters L, et al. Repeated injections of a ciliary neurotrophic factor analogue leading to long-term photoreceptor survival in hereditary retinal degeneration. *Invest Ophthalmol Vis Sci* 1999;40:1298-1305.
10. Hackett SF, Schoenfeld CL, Freund J, et al. Neurotrophic factors, cytokines and stress increase expression of basic fibroblast growth factor in retinal pigmented epithelial cells. *Exp Eye Res* 1997;64:865-873.
11. Caffe AR, Soderpalm AK, Holmqvist I, et al. A combination of CNTF and BDNF rescues rd photoreceptors but changes rod differentiation in the presence of RPE in retinal explants. *Invest Ophthalmol Vis Sci* 2001;42:275-282.
12. Edelhauser HF, Maren TH. Permeability of human cornea and sclera to sulfonamide carbonic anhydrase inhibitors. *Arch Ophthalmol* 1988;106:1110-1115.
13. Schoenwald RD. Ocular drug delivery. Pharmacokinetic considerations. *Clin Pharmacokinet* 1990;18:255-269.
14. Lee VH, Robinson JR. Topical ocular drug delivery: recent developments and future challenges. *J Ocul Pharmacol* 1986;2:67-108.
15. Schoenwald R. Ocular Pharmacokinetics / Pharmacodynamics. In: Mitra A, ed. *Ophthalmic Drug Delivery Systems*. New York, NY: Marcel Dekker; 1993:83-110.
16. Ip NY. The neurotrophins and neuropoietic cytokines: Two families of growth factors acting on neural and hematopoietic cells. *Ann N Y Acad Sci* 1998;840:97-106.
17. Takeuchi A, Kricorian G, Yao XY, et al. The rate and source of albumin entry into saline-filled experimental retinal detachments. *Invest Ophthalmol Vis Sci* 1994;35:3792-3798.
18. Kamei M, Misono K, Lewis H. A study of the ability of tissue plasminogen activator to diffuse into the subretinal space after intravitreal injection in rabbits. *Am J Ophthalmol* 1999;128:739-746.
19. Velez G, Whitcup SM. New developments in sustained release drug delivery for the treatment of intraocular disease. *Br J Ophthalmol* 1999;83:1225-1229.
20. Smith TJ, Pearson PA, Blandford DL, et al. Intravitreal sustained-release ganciclovir. *Arch Ophthalmol* 1992;110:255-258.

Enhancement of Scleral Macromolecular Permeability with Prostaglandins

21. Martin DF, Parks DJ, Mellow SD, et al. Treatment of cytomegalovirus retinitis with an intraocular sustained- release ganciclovir implant. A randomized controlled clinical trial. *Arch Ophthalmol* 1994;112:1531-1539.

22. Sanborn GE, Anand R, Torti RE, et al. Sustained-release ganciclovir therapy for treatment of cytomegalovirus retinitis. Use of an intravitreal device. *Arch Ophthalmol* 1992;110:188-195.

23. Anand R, Nightingale SD, Fish RH, et al. Control of cytomegalovirus retinitis using sustained release of intraocular ganciclovir. *Arch Ophthalmol* 1993;111:223-227.

24. Pearson PA, Jaffe GJ, Martin DF, et al. Evaluation of a delivery system providing long-term release of cyclosporine. *Arch Ophthalmol* 1996;114:311-317.

25. Jaffe GJ, Yang CS, Wang XC, et al. Intravitreal sustained-release cyclosporine in the treatment of experimental uveitis. *Ophthalmology* 1998;105:46-56.

26. Cheng CK, Berger AS, Pearson PA, et al. Intravitreal sustained-release dexamethasone device in the treatment of experimental uveitis. *Invest Ophthalmol Vis Sci* 1995;36:442-453.

27. Yang CS, Khawly JA, Hainsworth DP, et al. An intravitreal sustained-release triamcinolone and 5-fluorouracil codrug in the treatment of experimental proliferative vitreoretinopathy. *Arch Ophthalmol* 1998;116:69-77.

28. Jaffe GJ, Yang CH, Guo H, et al. Safety and pharmacokinetics of an intraocular fluocinolone acetonide sustained delivery device. *Invest Ophthalmol Vis Sci* 2000;41:3569-3575.

29. Berger AS, Cheng CK, Pearson PA, et al. Intravitreal sustained release corticosteroid-5-fluoruracil conjugate in the treatment of experimental proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci* 1996;37:2318-2325.

30. Zhou T, Lewis H, Foster RE, et al. Development of a multiple-drug delivery implant for intraocular management of proliferative vitreoretinopathy. *J Control Release* 1998;55:281-295.

31. Ahmed I, Patton TF. Importance of the noncorneal absorption route in topical ophthalmic drug delivery. *Invest Ophthalmol Vis Sci* 1985;26:584-587.

32. Ahmed I, Gokhale RD, Shah MV, et al. Physicochemical determinants of drug diffusion across the conjunctiva, sclera, and cornea. *J Pharm Sci* 1987;76:583-586.

33. McCartney H, Drysdale I, Gornall A, et al. An autoradiographic study of the penetration of subconjunctivally injected hydrocortisone into the normal and inflamed rabbit eye. *Invest Ophthalmol* 1965;4:297-299.

34. Barza M, Kane A, Baum J. Intraocular penetration of gentamicin after subconjunctival and retrobulbar injection. *Am J Ophthalmol* 1978;85:541-547.

35. Barza M, Kane A, Baum JL. Regional differences in ocular concentration of gentamicin after subconjunctival and retrobulbar injection in the rabbit. *Am J Ophthalmol* 1977;83:407-413.

36. Lim JI, Maguire AM, John G, et al. Intraocular tissue plasminogen activator concentrations after subconjunctival delivery. *Ophthalmology* 1993;100:373-376.

37. Lincoff H, Stanga P, Movshovich A, et al. Choroidal concentration of interferon after retrobulbar injection. *Invest Ophthalmol Vis Sci* 1996;37:2768-2771.

38. Weijtens O, van der Sluijs FA, Schoemaker RC, et al. Peribulbar corticosteroid injection: vitreal and serum concentrations after dexamethasone disodium phosphate injection. *Am J Ophthalmol* 1997;123:358-363.

39. Weijtens O, Schoemaker RC, Lentjes EG, et al. Dexamethasone concentration in the subretinal fluid after a subconjunctival injection, a peribulbar injection, or an oral dose. *Ophthalmology* 2000;107:1932-1938.

40. Roziere A, Mazuel C, Grove J, et al. Gelrite: A novel, ion-activated, in-situ gelling polymer for ophthalmic vehicles: effect on bioavailability of Timolol. *Int J Pharm* 1989;57:163-168.

41. Miller S, Donovan M. Effect of poloxamer 407 gel on the motic activity of pilocarpine nitrate in rabbits. *Int J Pharm* 1982;12:147-152.

42. Kumar S, Haglund B, Himmelstein K. In situ-forming gels of ophthalmic drug delivery. *J Ocul Pharmacol Ther* 1994;19:47-56.

43. Gurny R, Boye T, Ibrahim H. Ocular therapy with nanoparticulate systems for controlled drug delivery. *J Control Release* 1985;2:353-361.

44. Ahmed I, Patton T. Selective intraocular delivery of liposome encapsulated inulin via the non-corneal absorption route. *Int J Pharm* 1986;34:163-167.

45. Assil KK, Weinreb RN. Multivesicular liposomes. Sustained release of the antimetabolite cytarabine in the eye. *Arch Ophthalmol* 1987;105:400-403.

46. Skuta GL, Assil K, Parrish RK, et al. Filtering surgery in owl monkeys treated with the antimetabolite 5- fluorouridine 5'-monophosphate entrapped in multivesicular liposomes. *Am J Ophthalmol* 1987;103:714-716.

47. Assil KK, Lane J, Weinreb RN. Sustained release of the antimetabolite 5-fluorouridine-5'- monophosphate by multivesicular liposomes. *Ophthalmic Surg* 1988;19:408-413.

48. Assil KK, Hartzer M, Weinreb RN, et al. Liposome suppression of proliferative vitreoretinopathy. Rabbit model using antimetabolite encapsulated liposomes. *Invest Ophthalmol Vis Sci* 1991;32:2891-2897.

49. Assil KK, Frucht-Perry J, Ziegler E, et al. Tobramycin liposomes. Single subconjunctival therapy of pseudomonal keratitis. *Invest Ophthalmol Vis Sci* 1991;32:3216-3220.

50. Komai Y, Ushiki T. The three-dimensional organization of collagen fibrils in the human cornea and sclera. *Invest Ophthalmol Vis Sci* 1991;32:2244-2258.

51. Thale A, Tillmann B, Rochels R. Scanning electron-microscopic studies of the collagen architecture of the human sclera--normal and pathological findings. *Ophthalmologica* 1996;210:137-141.

52. Yamabayashi S, Ohno S, Aguilar RN, et al. Ultrastructural studies of collagen fibers of the cornea and sclera by a quick-freezing and deep-etching method. *Ophthalmic Res* 1991;23:320-329.

53. Thale A, Tillmann B. The collagen architecture of the sclera-SEM and immunohistochemical studies. *Anat Anz* 1993;175:215-220.

54. Duke-Elder S. *System of Ophthalmology*. Vol 8, Part II. London, England: Henry Kimpton; 1963;995.

55. Watson PG, Hazleman BL. The Sclera and Systemic Disorders. Philadelphia, Pa: WB Saunders; 1976;10.

56. Keeley FW, Morin JD, Vesely S. Characterization of collagen from normal human sclera. *Exp Eye Res* 1984;39:533-542.

57. Trier K, Olsen EB, Ammitzboll T. Regional glycosaminoglycans composition of the human sclera. *Acta Ophthalmol* (Copenh) 1990;68:304-306.

58. Rada JA, Achen VR, Perry CA, et al. Proteoglycans in the human sclera. Evidence for the presence of aggrecan. *Invest Ophthalmol Vis Sci* 1997;38:1740-1751.

59. Spitznas M. The fine structure of human scleral collagen. *Am J Ophthalmol* 1971;1:68.

60. Virgilio LA, Williams RJ, Klintworth GK. An unusually large human eye with abnormal scleral collagen. *Arch Ophthalmol* 1976;94:101-105.

61. Shields MB, Shelburne JD, Bell SW. The ultrastructure of human limbal collagen. *Invest Ophthalmol Vis Sci* 1977;16:864-866.

62. Johnston MC, Noden DM, Hazelton RD, et al. Origins of avian ocular and periocular tissues. *Exp Eye Res* 1979;29:27-43.

63. Olsen TW, Aaberg SY, Geroski DH, et al. Human sclera: Thickness and surface area. *Am J Ophthalmol* 1998;125:237-241.

64. Olsen TW, Edelhauser HF, Lim JI, et al. Human scleral permeability. Effects of age, cryotherapy, transscleral diode laser, and surgical thinning. *Invest Ophthalmol Vis Sci* 1995;36:1893-1903.

65. Bill A. Movement of albumin and dextran through the sclera. *Arch Ophthalmol* 1965;74:248-252.

66. Foulds WS. Clinical significance of trans-scleral fluid transfer. *Trans Ophthalmol Soc U K* 1976;96:290-308.

67. Inomata H, Bill A. Exit sites of uveoscleral flow of aqueous humor in cynomolgus monkey eyes. *Exp Eye Res* 1977;25:113-118.

68. Unlu N, Robinson JR. Scleral permeability to hydrocortisone and mannitol in the albino rabbit eye. *J Ocul Pharmacol Ther* 1998;14:273-281.

69. Wood RL, Koseki T, Kelly DE. Uveoscleral permeability to intra-camerally infused ferritin in eyes of rabbits and monkeys. *Cell Tissue Res* 1992;270:559-567.

70. Maurice DM, Polgar J. Diffusion across the sclera. *Exp Eye Res* 1977;25:577-582.

71. Shields MB, Bradbury MJ, Shelburne JD, et al. The permeability of the outer layers of limbus and anterior sclera. *Invest Ophthalmol Vis Sci* 1977;16:866-869.

72. Ahmed I, Patton T. Disposition of timolol and inulin in the rabbit eye following corneal versus non-corneal absorption. *Int J Pharm* 1987;38:9-21.

73. Ambati J, Canakis CS, Miller JW, et al. Diffusion of high-molecu-lar-weight compounds through sclera. *Invest Ophthalmol Vis Sci* 2000;41:1181-1185.

74. Sarraf D, Lee DA. The role of iontophoresis in ocular drug deliv-ery. *J Ocul Pharmacol* 1994;10:69-81.

75. Lam TT, Fu J, Tso MO. A histopathologic study of retinal lesions inflicted by transscleral iontophoresis. *Graefes Arch Clin Exp Ophthalmol* 1991;229:389-394.

76. Dan JA, Yaron A. Focal scleral permeability enhanced by collage-nase digestion. Experimental model of enzymatic sclerostomy. *Ophthalmology* 1994;101:461-472.

77. Trelstad RL, Silbermann NN, Brockhurst RJ. Nanophthalmic scle-ra. Ultrastructural, histochemical, and biochemical observations. *Arch Ophthalmol* 1982;100:1935-1938.

78. Yue BY, Duvall J, Goldberg MF, et al. Nanophthalmic sclera. Morphologic and tissue culture studies. *Ophthalmology* 1986;93:534-541.

79. Gass JD. Uveal effusion syndrome. A new hypothesis concerning pathogenesis and technique of surgical treatment. *Retina* 1983;3:159-163.

80. Brockhurst RJ. Vortex vein decompression for nanophthalmic uveal effusion. *Arch Ophthalmol* 1980;98:1987-1990.

81. Rudnick DE, Noonan JS, Geroski DH, et al. The effect of intraoc-ular pressure on human and rabbit scleral permeability. *Invest Ophthalmol Vis Sci* 1999;40:3054-3058.

82. Sagara T, Gaton DD, Lindsey JD, et al. Topical prostaglandin F2α treatment reduces collagen types I, III, and IV in the monkey uveoscleral outflow pathway. *Arch Ophthalmol* 1999;117:794-801.

83. Edwards A, Prausnitz M. Fiber matrix model of sclera and corneal stroma for drug delivery to the eye. *Am Inst Chem Eng J* 1998;44:214-225.

84. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491-21494.

85. Nagase H, Fields GB. Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides. *Biopolymers* 1996;40:399-416.

86. Lindsey J, Weinreb R. Effects of prostaglandins on uveoscleral out-flow. In: Alm A, Weinreb R eds. *Uveoscleral Outflow: Biology and Clinical Aspects*. London, England: Mosby-Wolfe; 1998;41-56.

87. Gaton DD, Sagara T, Lindsey JD, et al. Matrix metalloproteinase-1 localization in the normal human uveoscleral outflow pathway. *Invest Ophthalmol Vis Sci* 1999;40:363-369.

88. Abramovitz M, Boie Y, Nguyen T, et al. Cloning and expression of a cDNA for the human prostanoid FP receptor. *J Biol Chem* 1994;269:2632-2636.

89. Pierce KL, Fujino H, Srinivasan D, et al. Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. *J Biol Chem* 1999;274:35944-35949.

90. Ocklind A, Lake S, Wentzel P, et al. Localization of the prostaglandin F2(receptor messenger RNA and protein in the cynomolgus mon-key eye. *Invest Ophthalmol Vis Sci* 1996;37:716-726.

91. Gabelt B, Kaufman P. Normal physiology. In: Alm A, Weinreb R, eds. *Uveoscleral Outflow*. London, England: Mosby-Wolfe; 1998;25-40.

92. Lalani EN, Golding M, Hudson M, et al. Protein extraction and western blotting from methacarn-fixed tissue. *J Pathol* 1995;177:323-328.

93. Shetye JD, Scheynius A, Mellstedt HT, et al. Retrieval of leukocyte antigens in paraffin-embedded rat tissues. *J Histochem Cytochem* 1996;44:767-776.

94. Sagara T, Gaton DD, Lindsey JD, et al. Reduction of collagen type I in the ciliary muscle of inflamed monkey eyes. *Invest Ophthalmol Vis Sci* 1999;40:2568-2576.

95. Raviola G, Freddo TF. A simple staining method for blood vessels in flat preparations of ocular tissues. *Invest Ophthalmol Vis Sci* 1980;19:1518-1521.

96. Alexander RA, Hiscott PS, Hart RL, et al. Effect of melanin bleaching on immunoperoxidase, with reference to ocular tissues and lesions. *Med Lab Sci* 1986;43:121-127.

97. Foss AJ, Alexander RA, Jefferies LW, et al. Immunohistochemical techniques: The effect of melanin bleaching. *Br J Biomed Sci* 1995;52:22-25.

98. Goldberg GI, Strongin A, Collier IE, et al. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collage-nase, and activation of the proenzyme with stromelysin. *J Biol Chem* 1992;267:4583-4591.

99. Gross DS, Rothfeld JM. Quantitative immunocytochemistry of hypothalamic and pituitary hormones: Validation of an automated, computerized image analysis system. *J Histochem Cytochem* 1985;33:11-20.

100. van der Loos CM, Marijianowski MM, Becker AE. Quantification in immunohistochemistry: The measurement of the ratios of colla-gen types I and II. *Histochem J* 1994;26:347-354.

101. Stamer WD, Seftor RE, Snyder RW, et al. Cultured human tra-becular meshwork cells express aquaporin-1 water channels. *Curr Eye Res* 1995;14:1095-1100.

102. Huang Y, Gil DW, Vanscheeuwijck P, et al. Localization of alpha 2-adrenergic receptor subtypes in the anterior segment of the human eye with selective antibodies. *Invest Ophthalmol Vis Sci* 1995;36:2729-2739.

103. Anthony TL, Pierce KL, Stamer WD, et al. Prostaglandin F2α receptors in the human trabecular meshwork. *Invest Ophthalmol Vis Sci* 1998;39:315-321.

104. Parsons SL, Watson SA, Brown PD, et al. Matrix metalloproteinas-es. *Br J Surg* 1997;84:160-166.

105. Stjernschantz J, Selen G, Sjoquist B, et al. Preclinical pharmacolo-gy of latanoprost, a phenyl-substituted $PGF_{2\alpha}$ analogue. *Adv Prostaglandin Thromboxane Leukotriene Res* 1995;23:513-518.

106. Sjöquist B, Almegard B, Khalifeh V, et al. The bioavailability of Xalatan in the human eye. *Invest Ophthalmol Vis Sci* 1997;38:S248.

107. Lindsey JD, Kashiwagi K, Boyle D, et al. Prostaglandins increase proMMP-1 and proMMP-3 secretion by human ciliary smooth muscle cells. *Curr Eye Res* 1996;15:869-875.

108. Weinreb RN, Kashiwagi K, Kashiwagi F, et al. Prostaglandins increase matrix metalloproteinase release from human ciliary smooth muscle cells. *Invest Ophthalmol Vis Sci* 1997;38:2772-2780.

109. Nagase H, Okada Y. Proteinases and matrix degradation. In: Kelly J, Ruddy S, Sledge C, eds. *Textbook of Rheumatology*. Philadelphia, Pa: WB Saunders; 1997;323-341.

110. Woessner JF Jr. The family of matrix metalloproteinases. *Ann N Y Acad Sci* 1994;732:11-21.

111. Nagase H, Suzuki K, Enghild JJ, et al. Stepwise activation mechanisms of the precursors of matrix metalloproteinases 1 (tissue collagenase) and 3 (stromelysin). *Biomed Biochim Acta* 1991;50:749-754.

112. Vincenti MP, White LA, Schroen DJ, et al. Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): Mechanisms that control enzyme activity, transcription, and mRNA stability. *Crit Rev Eukaryot Gene Expr* 1996;6:391-411.

113. Denhardt DT, Feng B, Edwards DR, et al. Tissue inhibitor of metalloproteinases (TIMP, aka EPA): Structure, control of expression and biological functions. *Pharmacol Ther* 1993;59:329-341.

114. Bode W, Fernandez-Catalan C, Grams F, et al. Insights into MMP-TIMP interactions. *Ann N Y Acad Sci* 1999;878:73-91.

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

116. Cook H, Davies KJ, Harding KG, et al. Defective extracellular matrix reorganization by chronic wound fibroblasts is associated with alterations in TIMP-1, TIMP-2, and MMP-2 activity. *J Invest Dermatol* 2000;115:225-233.

117. Westermarck J, Holmstrom T, Ahonen M, et al. Enhancement of fibroblast collagenase-1 (MMP-1) gene expression by tumor promoter okadaic acid is mediated by stress-activated protein kinases Jun N-terminal kinase and p38. *Matrix Biol* 1998;17:547-557.

118. Astin M. Effects of prostaglandin E2, F2alpha, and latanoprost acid on isolated ocular blood vessels in vitro. *J Ocul Pharmacol Ther* 1998;14:119-128.

119. Stjernschantz J, Selen G, Astin M, et al. Microvascular effects of selective prostaglandin analogues in the eye with special reference to latanoprost and glaucoma treatment. *Prog Retin Eye Res* 2000;19:459-496.

120. Brannon-Peppas L, Peppas NA. Dynamic and equilibrium swelling behaviour of pH-sensitive hydrogels containing 2-hydroxyethyl methacrylate. *Biomaterials* 1990;11:635-644.

121. Bell CL, Peppas NA. Water, solute and protein diffusion in physiologicly responsive hydrogels of poly (methacrylic acid-g-ethylene glycol). *Biomaterials* 1996;17:1203-1218.

122. Chapman SA, Ayad S, O'Donoghue E, et al. Glycoproteins of trabecular meshwork, cornea and sclera. *Eye* 1998;12:440-448.

123. White J, Werkmeister JA, Ramshaw JA, et al. Organization of fibrillar collagen in the human and bovine cornea: Collagen types V and III. *Connect Tissue Res* 1997;36:165-174.

124. Kimura S, Kobayashi M, Nakamura M, et al. Immunoelectron microscopic localization of decorin in aged human corneal and scleral stroma. *J Electron Microsc* (Tokyo) 1995;44:445-449.

125. Wessel H, Anderson S, Fite D, et al. Type XII collagen contributes to diversities in human corneal and limbal extracellular matrices. *Invest Ophthalmol Vis Sci* 1997;38:2408-2422.

126. Shuttleworth CA. Type VIII collagen. *Int J Biochem Cell Biol* 1997;29:1145-1148.

127. Matrisian LM. The matrix-degrading metalloproteinases. *Bioessays* 1992;14:455-463.

128. Lindsey JD, Kashiwagi K, Kashiwagi F, et al. Prostaglandin action on ciliary smooth muscle extracellular matrix metabolism: implications for uveoscleral outflow. *Surv Ophthalmol* 1997;41 Suppl 2:S53-59.

129. Lindsey JD, Kashiwagi K, Kashiwagi F, et al. Prostaglandins alter extracellular matrix adjacent to human ciliary muscle cells in vitro. *Invest Ophthalmol Vis Sci* 1997;38:2214-2223.

130. Bikfalvi A, Klein S, Pintucci G, et al. Biological roles of fibroblast growth factor-2. *Endocrinol Rev* 1997;18:26-45.

131. Penc SF, Pomahac B, Winkler T, et al. Dermatan sulfate released after injury is a potent promoter of fibroblast growth factor-2 function. *J Biol Chem* 1998;273:28116-28121.

132. Walicke P, Cowan WM, Ueno N, et al. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc Natl Acad Sci USA* 1986;83:3012-3016.

133. Dreyfus H, Sahel J, Heidinger V, et al. Gangliosides and neurotrophic growth factors in the retina. Molecular interactions and applications as neuroprotective agents. *Ann N Y Acad Sci* 1998;845:240-252.

134. Cummings BJ, Yee GJ, Cotman CW. FGF-2 promotes the survival of entorhinal layer II neurons after perforant path axotomy. *Brain Res* 1992;591:271-276.

135. Nakata N, Kato H, Kogure K. Protective effects of basic fibroblast growth factor against hippocampal neuronal damage following cerebral ischemia in the gerbil. *Brain Res* 1993;605:354-356.

136. Rabchevsky AG, Fugaccia I, Fletcher-Turner A, et al. Basic fibroblast growth factor (FGF-2) enhances tissue sparing and functional recovery following moderate spinal cord injury. *J Neurotrauma* 1999;16:817-830.

137. Lipton SA, Wagner JA, Madison RD, et al. Acidic fibroblast growth factor enhances regeneration of processes by postnatal mammalian retinal ganglion cells in culture. *Proc Natl Acad Sci USA* 1988;85:2388-2392.

138. Pearce RK, Collins P, Jenner P, et al. Intraventricular infusion of basic fibroblast growth factor (FGF-2) in the MPTP-treated common marmoset. *Synapse* 1996;23:192-200.

139. Connor B, Dragunow M. The role of neuronal growth factors in neurodegenerative disorders of the human brain. *Brain Res Brain Res Rev* 1998;27:1-39.

140. Cunha-Vaz JG, Shakib M, Ashton N. Studies on the permeability of the blood-retinal barrier: I. On the existence, development and site of a blood-retinal barrier. *Br J Ophthalmol* 1966;50:441-453.

141. Lee VH, Carson LW, Kashi SD, et al. Metabolic and permeation barriers to the ocular absorption of topically applied enkephalins in albino rabbits. *J Ocul Pharmacol* 1986;2:345-352.

142. Pino RM. Restriction to endogenous plasma proteins by a fenestrated capillary endothelium: An ultrastructural immunocytochemical study of the choriocapillaris endothelium. *Am J Anat* 1985;172:279-289.

143. Toris CB, Pederson JE. Experimental retinal detachment. VII. Intravenous horseradish peroxidase diffusion across the blood-retinal barrier. *Arch Ophthalmol* 1984;102:752-756.

144. Smith RS, Rudt LA. Ocular vascular and epithelial barriers to microperoxidase. *Invest Ophthalmol* 1975;14:556-560.

145. Laties AM, Rapoport S. The blood-ocular barriers under osmotic stress. Studies on the freeze-dried eye. *Arch Ophthalmol* 1976;94:1086-1091.

146. Peyman GA, Bok D. Peroxidase diffusion in the normal and laser-coagulated primate retina. *Invest Ophthalmol* 1972;11:35-45.

147. Grayson MC, Laties AM. Ocular localization of sodium fluorescein: Effects of administration in rabbit and monkey. *Arch Ophthalmol* 1971;85:600-609.

148. Tso MOM, Shih C-Y, McLean IW. Is there a blood-brain barrier at the optic nerve head? *Arch Ophthalmol* 1975;93:815-825.

149. Lindsey JD, To HD, Weinreb RN. Induction of c-fos by prostaglandin $F_{2\alpha}$ in human ciliary smooth muscle cells. *Invest Ophthalmol Vis Sci* 1994;35:242-250.