COLLAGEN TYPE I AND III SYNTHESIS BY TENON'S CAPSULE FIBROBLASTS IN CULTURE: INDIVIDUAL PATIENT CHARACTERISTICS AND RESPONSE TO MITOMYCIN C, 5-FLUOROURACIL, AND ASCORBIC ACID*

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ABSTRACT

Purpose: This study was performed to better understand the differences between patients in specific components of wound healing as it may pertain to glaucoma filtration surgery, including the use of antimetabolites.

Methods: Human Tenon's capsule fibroblasts were obtained at the time of glaucoma filtering surgery and established in individual cell cultures from 35 glaucoma patients. The dose-response to 5-fluorouracil (5FU) and mitomycin C (MMC) was determined. The individual cell lines were exposed to the antimetabolites and ascorbic acid with measurement of collagen type I and III production by an ELISA-type dot blot assay. These results were then statistically compared to the individual patient characteristics including age, race, previous surgery and medications, and type of glaucoma.

Results: 5-FU had little effect on collagen type I and III production or protein synthesis. MMC had an inhibitory effect on collagen secretion and total protein synthesis with increasing concentration. Photomicrographs of the cells after each treatment condition revealed characteristic morphologic changes when compared to controls. There was a large range of collagen type I and III production with correlation between the amounts of each collagen type secreted in response to the antimetabolites. However, there was no correlation with accepted risk factors for filtration failure.

Conclusion: These antimetabolites act similarly on different cell lines in a nonspecific manner. The results suggest that the increased risk of filtration failure due to age, race, diagnosis, and previous conjunctival surgery

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is not due to differences in secretion of collagen types I and III by Tenon's capsule fibroblasts.

INTRODUCTION

There are over 5,000 new cases of glaucoma-induced legal blindness each year in the United States. Many of these patients received incisional glaucoma filtration surgery that failed because of excessive wound healing, resulting in scar formation and occluding the filtration site. Clinically, wound healing varies greatly among individual patients. The basis for this difference is unknown. There are some generally accepted risk factors that increase the chance of failure of glaucoma filtering surgery. These include race, age, previous incisional surgery involving the conjunctiva, previous use of antiglaucomatous medications, and many secondary forms of glaucoma. However, even taking these factors into account, there is enormous variability in the response of apparently similar individual patients to the same surgical procedure. Therefore, the wound-healing response of a single patient cannot be predicted preoperatively, and we are left with the dilemma of using potentially dangerous agents to inhibit healing without the ability to predict their actual effect.

In this study, the variability among patients in one specific area of wound healing was examined—collagen-type secretion in cultured fibroblasts from individual human Tenon's capsule. The influence of agents clinically used to modify healing, 5-fluorouracil (5-FU) and mitomycin C (MMC) was also studied. Additionally, because of its unique presence in aqueous humor and because it has been implicated to play a role in wound healing, the effect of ascorbic acid (vitamin C) was also studied. The hypothesis of this study is that fibroblasts in cell culture derived from individual glaucoma patients demonstrate different amounts of type I and type III collagen synthesis. Additionally, there is a difference in response to antifibrotic agents. These findings were correlated with the accepted risk factors for filtration failure. It is hoped that this may begin to address the underlying causes for the variability in clinical response to glaucoma filtration surgery among patients.

WOUND HEALING

Wound healing is the reparation of damaged tissue with return to normal architecture and function, whereas scarring is tissue restoration without the preservation of normal architecture or function. The response to surgical injury involves a complex cascade of events characterized by coagulation, inflammation, epithelialization, angiogenesis, fibroplasia, extracellular matrix deposition, and remodeling.[1] Many types of cells are involved in this process, including platelets, granulocytes, macrophages, endothelial cells,

lymphocytes, and fibroblasts.[1,2] Increased presence of local growth factors stimulates fibroblast proliferation. The fibroblasts contain protein-synthesizing endoplasmic reticulum that produces collagen and other extracellular matrix proteins. Fibroblasts then secrete collagen, fibronectin, and glycosaminoglycans from the cell to form an interstitial matrix.[3] This then forms granulation tissue that remodels over time with collagen synthesis, lysis, cross-linking, contraction, and condensation.[4] The chemical composition of a scar is similar to normal tissue, but it differs in organization, specifically the inability to reassemble collagen into normal patterns.[5]

Wound healing following glaucoma filtering surgery is similar to that following injury to most other sites.[6] Many factors contribute to failure of filtration, but 3 types of excessive wound healing have been described: (1) immediate closure due to granulation tissue, (2) loculation of aqueous within an encapsulated bleb, and (3) failure of the bleb resulting from scarring in the episcleral tissue.[6] Fibroblasts play an important role in each of these aspects of wound healing. In animal models, fibroblasts are attracted to the site of surgery from Tenon's capsule in the subconjunctival and episcleral tissues.[7-9]

COLLAGEN TYPES I AND III IN WOUND HEALING

Type I and type III collagen are both involved in wound healing and scar formation.[10] During wound healing, type III collagen levels are elevated early and are then gradually replaced by type I collagen during the remodeling phase.[11,12] Synthesis of type III collagen may begin as early as 10 hours after insult,[13] and it predominates during the first 3 weeks, while gradual replacement with type I collagen begins the first week.[14-18] An increase of type III collagen facilitates myofibroblast migration and scar proliferation.[19] Additionally, in human dermal fibroblasts, fibroblasts from keloids and hypertrophic scars continue to produce elevated mRNA levels for type I and type III collagen compared to normal fibroblasts.[20,21] On a molecular level, scarring may be distinguished from normal healing by the relative amounts of different collagen types deposited.[17] In scars, the ratio of type I to type III is reduced from that found in normal tissue; thus there is a relative excess of type III in scarred tissue. Thus, agents that modify the production of these collagen types might be particularly useful in optimizing the outcome of filtration surgery by reducing excessive scar formation while permitting and/or encouraging normal healing.

Fibroblasts from different species, and even from different tissues of the same animal, produce markedly different ratios of collagens and respond uniquely to the influences of various cellular factors. Thus, human Tenon's capsule fibroblasts from glaucoma patients were used in this study.

Unfortunately, human Tenon's capsule fibroblasts in culture, mirroring

the in vivo situation, stop producing type III collagen after 5 to 7 passages.[22-24] Attempts to immortalize these cells while maintaining type III collagen production have been unsuccessful. Therefore, only early primary cultures of Tenon's fibroblasts mimic the early scarring process.

EXPERIMENTAL MODULATION OF WOUND HEALING

Scarring and wound healing play a key role in determining the outcome of glaucoma filtration surgery. Many agents have been studied to assess their effects on fibroblasts and collagen production with the purpose of making the surgery more successful, safer, and/or more predictable. Aqueous humor has been shown to selectively stimulate discrete protein synthesis and act as a chemo-attractant for Tenon's capsule fibroblasts.[25,26] Tenon's capsule fibroblast proliferation is nonspecifically inhibited by numerous agents, including beta irradiation,[27] vitamin E,[28] interferon alpha-2b,[29] cyclooxygenase inhibitors,[30] and corticosteroids.[31] The methylxanthine derivatives pentoxifylline and pentifylline have been shown to also decrease collagen production.[32] The inhibition of collagen types I and III synthesis has been shown with gamma-interferon,[33] fibrostatin C,[34] and decorin and suramin.[35] Decorin was shown to have differential effects on the two collagen types with more effective inhibition of type III than type I synthesis. Theoretically, a profile such as this may be particularly desirable.

In contrast, it has been shown that growth factors, including transforming growth factor beta-1, beta-2, and tumor necrosis factor can stimulate Tenon's capsule fibroblast proliferation and increase synthesis of collagen types I and III.[36-38] At times, studies have demonstrated results that have contradicted generally accepted principles. As an example, medications commonly used to treat glaucoma have been studied. It has been suggested that long-term use of topical antiglaucoma medications may have a detrimental effect on glaucoma-filtering surgery. Numerous classes of medications have been tested, including beta-adrenergic blockers, cholinergic drugs, and adrenergic drugs, all of which actually inhibited fibroblast proliferation and were toxic to human Tenon's capsule fibroblasts in cell culture.[39-41]

Therefore, although many different kinds of agents have been studied, these effects have been described only in these specific models, and their possible clinical application is largely unknown.

RISK FACTORS FOR FILTRATION FAILURE

Glaucoma filtering surgery has evolved with techniques to improve success and minimize severe complications. These have included guarded filtration with suture lysis or releasable sutures, use of antimetabolites, and

postoperative use of corticosteroids. However, probably the most important factor(s) in determining the long-term success of glaucoma filtration surgery are the unique characteristics of the actual individual patient.

Several studies have identified young patient age as a risk factor for filtration failure.[42] In one large series, successful filters were achieved in only 56% of patients aged 50 or less, a rate lower than would be expected in older patients.[43] Although African American patients are not a single homogeneous group, filtration surgery as a whole is less successful in these patients than in white patients.[44-48] Most feel that this is likely not just a result of socioeconomic factors but is actually largely pathophysiologic in origin. In fact, histologically, differences have been reported in the conjunctiva of black patients compared to white patients. There are an increased number of macrophages and fibroblasts in black patients.[49,50] This is consistent with other studies that have demonstrated glaucoma, specifically primary open-angle glaucoma, exhibits different characteristics in African Americans than in other races. This includes earlier age at onset of disease, a fourfold to fivefold increased prevalence, and a 4 to 8 times greater occurrence of blindness.[51] Patients with secondary forms of glaucoma also tend to have less successful filtration. These types of glaucoma include neovascular,[52, 53] uveitic,[54] and aphakic or pseudophahic.[55,56] Conjunctival scarring also has been shown to increase the risk of filtration failure.[57] This is the case following previous conjunctival surgery in the area that filtration is to be performed.

The previous use of topical antiglaucoma medications has also been implicated as a risk factor for filtration failure. It has been shown that there is an increase in the number of tissue inflammatory cells including macrophages, lymphocytes, mast cells, and fibroblasts in Tenon's capsule and conjunctiva of patients receiving at least two types of topical medications for at least 1 year.[58] This has been confirmed in several additional studies that also implicate the preservatives in commercial preparations as exacerbating this finding.[59-61] Among the classes of medications, miotics and sympathomimetic agents have shown the greatest effect, possibly related to their propensity to increased tissue inflammation.[62,63] This implied negative effect has been confirmed clinically with eyes having received chronic topical medications preoperatively, resulting in lower success of filtration surgery.[63] In that study, the control group underwent primary glaucoma filtration surgery. These types of studies have provided further support to substantiate the advantages of earlier and, in some cases, initial filtration surgery in glaucoma patients.[64]

ANTIMETABOLITE USE IN GLAUCOMA FILTRATION SURGERY

As a result of the decreased rate of successful filtration in patients, partic-

ularly those with one or more risk factors, the antimetabolites 5-FU and MMC have been used clinically in glaucoma filtration surgery to increase success, but they have also resulted in an increase in severe complications.[65] These agents interrupt not only scarring but also normal wound healing and cell turnover, accounting for adverse effects, including corneal epithelial toxicity, conjunctival bleb breakdown, chronic wound leaks, hypotony, and endophthalmitis.[66]

5-FU inhibits thymidylate synthetase and selectively acts on the S phase of the cell cycle, resulting in decreased fibroblast proliferation.[4] Numerous clinical studies have demonstrated improved control of intraocular pressure following intraoperative use[67-69] and with multiple postoperative subconjunctival injections of 5-FU.[70-72] MMC is an antitumor antibiotic isolated from *Streptomyces caespitosus*.[73] It inhibits DNA synthesis by acting as an alkylating agent and also results in generation of reactive free radicals. The effects of MMC do not depend on the phase of the cell cycle. Many studies have demonstrated the effectiveness of adjunctive intraoperative MMC use with filtration surgery.[74-78] This is particularly true for types of glaucoma, such as developmental glaucomas, in which historically filtration surgery success is limited.[79,80]

Unfortunately, the exact mechanism(s) by which the clinical effects of these antimetabolites are determined is not known. In filtration surgery, these agents appear to be nonspecific in their targets, such that along with the increased ability to obtain filtration is a concomitant increase in serious complications. These complications include hypotony maculopathy, bleb leaks, infection, and scleral necrosis.[65,74,81-83] When these serious side effects occur, they can be quite difficult to treat, with complications arising from treatment.[84,85] Additional ocular effects of MMC, including corneal endothelial cell loss[86] and decreased aqueous humor production, also have been described and can be attributed to the nonspecific effects of this agent.[87]

These two agents were chosen for inclusion in this study because they are in current clinical use. However, despite the increase in success, the unavoidable increase in complications as well has resulted in attempts to modify the method of application by changing concentration, duration of application, delivery devices, and timing of drug delivery.[88-94] Unfortunately, the optimal parameters for drug delivery are not known. It is critical to better understand the effects of these agents on the actual tissues involved, if there is a means to better predict their effects in individual patients.

It has been shown that short-term exposure to antiproliferative agents such as 5-FU and MMC can result in long-term effects on human Tenon's capsule fibroblasts in culture.[95,96] MMC does not immediately result in

cell death but rather almost completely inhibits proliferation.[97,98] On a cellular basis, exposure to 5-FU or MMC resulted in an increase in growth factors, including TGF-β and bFGF, while causing a reduction in type I collagen. Type III collagen production was initially elevated, then decreased toward control levels over time.[99] The mechanism by which this occurs is not understood; however, this decrease in collagen production may well play a role in the histopathologic evidence that human scleral tissue exposed to MMC results in disruption of collagen fibrils with loss of proteoglycan cross-links and organization.[100] Scar tissue obtained from failed trabeculectomies without MMC demonstrated parallel-oriented collagen fibers, whereas in the tissue from eyes exposed to MMC, collagen fibers were arranged randomly with less ground substance.[101]

There is little information as to how this information applies to individual patients. In one study, a correlation was demonstrated between human Tenon's capsule fibroblast "sensitivity" or "resistance" to MMC and clinical outcome in 17 cases.[102] Collagen production, particularly that of types I and III, may well play a vital role in the mechanism of action of these antimetabolites. Therefore, it is imperative to better understand these antiproliferative agents to allow more precise clinical usage to maximize success while minimizing complications.

ASCORBIC ACID (VITAMIN C)

Ascorbic acid, or vitamin C, was chosen for inclusion, since in earlier investigations it was shown to have an unparalleled stimulatory effect on collagen-type synthesis.[103] Ascorbic acid is an important modulator of collagen production, acting as a cofactor for the hydroxylation of proline and lysine residues in procollagen. Hydroxyproline stabilizes the collagen triple-helix structure, a requirement for the secretion of procollagen and its processing to collagen. There is a uniquely elevated concentration of ascorbate in aqueous humor of 1.1mM, a 25-fold increase compared to serum.[104] This concentration results from active transport from plasma into the posterior chamber. The reason for this particular increase is not known, but it is thought to be related to its ability to protect the eye from ultraviolet light damage and thus reduce free radicals.[105,106] However, this may be additionally important in eyes undergoing glaucoma filtration surgery because the Tenon's capsule fibroblasts are being constantly bathed in aqueous humor, which has been implicated in playing a role in wound healing.[25,26] Ascorbic acid at 100 μM increases mRNA levels for both type I and type III collagen in tissue culture.[107,108] Ascorbic acid has been shown to stimulate secretion of type I and type III collagen in human dermal fibroblasts.[109,110] In ocular tissues, the role of ascorbic acid in collagen type I gene expression by cells from the trabecular meshwork has been

described.[111] Ascorbic acid was also found to enhance the production of type I and type III collagen peptides in cultured rabbit keratocytes.[112] Similar findings have been reported in human Tenon's capsule fibroblasts in which maximum stimulation of collagen type I and type III production was obtained with ascorbic acid concentrations of 100 mg/mL (0.57 mM), approximately half of the level normally found in aqueous humor.[103] In that study, no decrease in protein synthesis, cell density, or cell morphology consistent with toxicity was observed at up to 300 μg/mL. In contrast, at higher concentrations, at and above the level of ascorbate in aqueous, decreased plating efficiency of human Tenon's capsule fibroblasts has been reported.[104] However, in 169 patients there was no correlation between primary aqueous humor concentration of ascorbic acid and outcome of trabeculectomy.[113] Therefore, given the unique concentration of ascorbate in aqueous, the variability of stimulation by ascorbate remains a potentially important unanswered question concerning the wound healing response in filtration surgery.

IMPROVING GLAUCOMA FILTRATION SURGERY

It will be through greater understanding of the wound-healing process that we will be able to improve the care of glaucoma patients. Reported rates of success of filtration at 10 years following initially successful surgery is 73%, but it is reduced to 42% by 15 years. This leaves much room for improvement.[114] Currently, it is not possible to predict the outcome of glaucoma filtration surgery in a specific individual. The same surgery has vastly different outcomes when performed in different patients. Even patients with similar accepted risk factors for failure of filtration may have markedly different results.[115] These risk factors usually include previous surgery with a conjunctival incision, use of topical medications, age, race, and type of glaucoma. These are the cases in which antimetabolites such as 5-FU and MMC are most commonly used. However, some studies do not support these risk factors nor the additional efficacy of antimetabolite use.[116,117] This study addresses the range of response of fibroblasts derived from glaucoma patients to three agents that have been shown to, or appear to, modify the natural outcome of filtration surgery. With greater understanding of the potential differences in response and correlating that with risk factors for excessive wound healing, greater understanding and more prudent application in surgical therapy become possible.

MATERIALS AND METHODS

Numerous methods have been used to model the complex wound-healing response. Most commonly, these have included in vitro tissue culture and

in vivo animal models. The activities of fibroblasts are essential in understanding the wound-healing process.[2] Tissue culture models of fibroblasts allow the study of isolated cellular functions, such as attachment, migration, proliferation, extracellular protein synthesis, and contraction under controlled conditions. These conditions may be modified to facilitate the study of the effects of different agents on specific areas of cellular function. Tissue cultures allow consistent drug delivery to the cells.[2] However, there are limitations to the information obtained, because the cell culture is not equivalent to the complex cellular environment in vivo.

The assay used in this study to measure collagen types offers four significant advantages over previous systems.[103] First, exported collagen is measured directly rather than an intracellular form of the protein or procollagen mRNA. Second, this assay differentiates type I collagen from type III collagen. Third, the assay is performed on cells in serum-free media, much closer in composition to primary aqueous humor (ie, the protein levels in aqueous humor are approximately 0.02 g/dL, whereas media containing 10% fetal calf serum contains approximately 0.6 g/dL of protein). Fourth, the use of serum-free media allows the factors to be tested at their true concentrations and without interference from serum components.

REAGENTS

Monoclonal mouse IgG against human type I collagen was obtained from Calbiochem (San Diego, Calif), monoclonal mouse IgG to human type III collagen from Gibco-Life Sciences (Gaithersburg, Md). Goat anti-mouse IgG was from Biorad (Richmond, Calif), and donkey anti-mouse IgG from Biodesign (Kennebunk, Me). Serum-free Fibroblast Growth Media (FGM) was obtained from Clonetics (San Diego, Calif). All other tissue culture reagents were obtained from Gibco-Life Sciences. For chemiluminescence detection, SuperSignal CL-HRP Substrate System was purchased from Pierce (Rockford, Ill). MMC (0.5 mg/mL) was obtained from Bristol-Myers (Princeton, NJ), 5-FU (Adrucil, 50 mg/mL) from Pharmacia (Kalamazoo, Mich), and vitamin C from Fluka (Ronkonkoma, NY). For total protein determinations, (2,3,4,5 ^{3}H) L-leucine was purchased from ICN (Costa Mesa, Calif).

EXPLANT CULTURE

After informed consent and approval by the Baylor College of Medicine Institutional Review Board, fibroblast cell cultures were established from human Tenon's capsule specimens obtained during trabeculectomy surgery using the method previously described.[103] This was a consecutive series of patients who underwent glaucoma filtration surgery between December 1997 and May 1998. Topical antiglaucoma medications were

discontinued not more than 24 hours prior to surgery. The eyes were treated with a prophylactic antibiotic, ofloxacin (Allergan Pharmaceuticals, Irvine, Calif), within the 24 hours preoperatively. After opening the conjunctiva to form a limbus-based conjunctival flap, a small piece of Tenon's capsule measuring approximately 2 x 2 mm was removed prior to the application of any intraoperative antimetabolite. The tissue was placed immediately in tissue culture media on ice and then stored at 4°C. Within 24 hours, the tissue sample was cut into in small pieces and cultured in growth medium containing minimal essential medium (MEM) containing 1% L-glutamine, 1% antibiotic solution (penicillin, streptomycin, and fungizone), and 15% fetal bovine serum and incubated at 37°C in 5% CO_2-95% air. Fibroblasts appeared after 4 days and were passaged after 13 days. First-passage cells were diluted and seeded into multiple 100 mm dishes (Corning) at densities of 250 to 500 cells per cm2 and frozen when they reached 75% confluence. It has been reported that fibroblastic components of explanted scar specimens retain at least the histologic characteristics of original human scar specimens.[118]

CELL CULTURE

Cells were thawed, diluted with growth medium (MEM containing 1% L-glutamine, 1% antibiotic solution [penicillin, streptomycin, and fungizone], and 15% fetal bovine serum) and transferred into 100 mm dishes. These cultures were incubated in 5% CO2-95% air at 37°C.94 The cultures were grown until 85% confluence was obtained. At this point, the cultures were trypsinized and aliquotted into 35 mm dishes at 20% confluence (1,000 cells/cm^2) in growth medium. After an additional 5 days of culture, the cells were ready for treatment. Collagen samples were obtained from the culture media by rinsing the cells 5 times in 10 mM phosphate buffered saline (PBS) solution (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) and incubating for 24 hours in 1.0 mL of serum-free Fibroblast Growth Media with 100 µg/mL ascorbic acid.[103] Reports have previously shown that conditioned media from fibroblasts yielded collagen signals that were linear with respect to both media volume and serial dilutions of media when reacted with antibody.[103] It has been shown that Tenon's capsule fibroblasts, while not proliferating, remain viable in serum-free medium for at least 7 days.[119]

Initially, to determine the concentration of 5-FU and MMC to be used for the evaluation of in vitro effects on fibroblasts from individuals, a range of concentrations of the antifibrotic agents to be evaluated were tested on a single representative cell line. The agents at varying concentrations were mixed with the serum-free growth media immediately before addition to the fibroblast cultures containing serum-free Fibroblast Growth Media

with 100 μg/mL ascorbic acid. In the case of 5-FU, the pH of the media was adjusted to 7.2 with 6N HCl. Deionized water was added to serum-free media as the control. After 24 hours, the media was removed and cells rinsed with 0.2 LBS. The media and PBS rinse were combined into one sample. Photomicrographs of the cells taken at 24 hours under each treatment condition were made by draining the media and placing a coverslip over the cells. Digital-phase images were collected with a Hamamatsu chilled CCD camera (C5810) on a Zeiss Axiophot microscope, with PaintShop Pro 4.0 as the software acquisition interface.

To evaluate the variability of effect of these agents on fibroblasts from different individuals, the cells from each patient were maintained separately throughout the cell culture process. For each individual cell line, 4 conditions were examined: serum-free media with (1) deionized water as control, (2) 100 μg/mL ascorbic acid, (3) 100 μg/mL ascorbic acid and 5-FU 10 μg/mL, and (4) 100 μg/mL ascorbic acid with MMC 10 μg/mL. The concentration of ascorbate was derived from earlier findings as the level that maximally stimulates collagen-type production.[103] The concentration of 5-FU is similar to the levels found in tissue after clinical intraoperative application.[94] The MMC concentration was found to be near the midpoint of the range of effectiveness (2.5 to 40 μg/mL) determined in this study and was similar to concentrations used previously in other studies.[99] All determinations were performed in duplicate. After 24 hours, the media was removed and the cells rinsed with 0.2 mL of PBS. The media and PBS rinse were then combined into one sample. The samples were frozen at –20°C.

To make sure that differences in collagen production were not attributable to differences in cell number or survival, a random sampling of 10 cell lines was then trypsinized and counted via hemocytometer to ensure equivalent growth rate and cell number among cultures.

Cultures of all cell lines were then treated with 0.5 mL of 1.0N NaOH and frozen for subsequent Lowry total protein determination.

DOT BLOT DETERMINATIONS

Secreted collagen was measured using a dot blot assay.[103] Briefly, media samples were added directly to nitrocellulose filter paper using a dot blot vacuum filtration apparatus. Collagen-type standards were included in every assay. The nitrocellulose filters were incubated in a blocking solution containing 10 mM PBS and 2.5% nonfat dry milk for 2 hours at 37°C. The filters were then rinsed with PBS, and then fresh PBS/milk containing the primary antibodies was added and incubated at 37°C for 1 hour. For type I collagen, monoclonal mouse IgG against human type I collagen was added at a 1:20,000 dilution. For type III collagen, monoclonal mouse IgG to human type III collagen was used at a 1:10,000 dilution. Cross-

reactivity between the anti–collagen type I antibody and the anti–collagen type III antibody was determined and found to be less than 2%. After rinsing, the blots were incubated for 1 hour at 37°C in PBS/milk containing the secondary antibodies: goat anti-mouse IgG at a 1:20,000 dilution for type I and type III collagen. The secondary antibody was conjugated to horseradish peroxidase. Dots were visualized with chemiluminescence on autoradiography film and quantitated using densitometry. Results from the collagen standards were used to create a standard curve, and sample results were fit to the curve to obtain the total amount of collagen in nanograms (ng). The limit of sensitivity of the dot blot was 0.5 ng for both collagen types. The curves show a linear range with no signal below the limit of detection and with the upper limit between 6 and 8 densitometric units where the film was too black for further detection.[103] The arithmetic mean of the two determinations was used for analysis. For values of collagen types that were measured to be zero, these dot blots were repeated.

PROTEIN SYNTHESIS

As part of the dose-response curves of the antimetabolites, tritiated leucine incorporation into total protein was used to test cell viability.[103] A range of concentrations of the agents to be evaluated was added to 1 mL FGM in combination with 4 μCi/mL (2,3,4,5 -3H) L-leucine (ICN) and incubated for 24 hours. Samples containing media, cells, and rinse were precipitated with trichloroacetic acid (TCA), and after rinsing, pellets were counted in a liquid scintillation counter.

CLINICAL PATIENT DATA

The clinical data from each patient were obtained from the patient record. Data entered on the data collection sheet included patient demographic information: age, race, and sex. The type of glaucoma, previous surgery in that eye, and previous use of antiglaucoma medication within 1 month of surgery were entered. The preoperative Snellen visual acuity and Goldmann applanation intraocular pressure on the two visits prior to surgery were collected. The preoperative intraocular pressure was the mean of the two measurements. Details of the surgical procedure included type of antimetabolite used and date of surgery.

DATA ANALYSIS

All data were entered into a database. Data were analyzed using STATA 1999 (Stata Corp, Stata Statistical Software: Release 6.0. College Station, Tex) (Cary, NC). Fifteen bivariate correlations were computed in order to determine any possible corelations between the variables measured. To control Type I error, an adjusted P of 05/187 = .0003 was used. Bivariate

analysis was chosen as an initial method to assess possible correlations that may be present.

RESULTS

ANTIMETABOLITE DOSE RESPONSE AND HISTOPATHOLOGIC EFFECTS

The effects of 5-FU and MMC on the synthesis and secretion of collagen types I and III and overall protein synthesis are shown in Figs 1 and 2. 5-FU had no effect on the synthesis of types I and III collagen in concentrations up to 5 mg/mL (Fig 1). However, a slight inhibitory effect on protein synthesis was noticed, resulting in a 15% inhibition at the highest concentration tested (5 mg/mL).

MMC had no effect on the production of either collagen type at concentrations up to 2.5 μg/mL, but decreased production of both types in a dose-dependent manner from 2.5 μg/mL to 40 μg/mL (Fig 2). MMC inhibits both collagen types to a similar degree. Overall protein synthesis, as measured by 3H-leucine incorporation into TCA precipitable protein, shows a gradual decrease beginning at 1 μg/mL with an eventual 80% reduction at 40 μg/mL.

The two antimetabolites produced characteristic morphologic changes in the cells after exposure to the highest concentration of each drug (Fig 3). Controls, not exposed to antimetabolite, at 24 hours in serum-free media show a healthy population of fibroblasts with round nuclei and distinct nucleoli that spread out over the entire plate. The cytoplasm is appropriately vacuolated for actively secreting fibroblasts. There is a

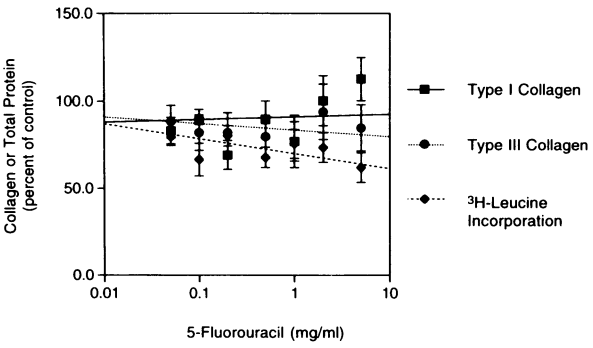

FIGURE 1

Effects of 5-FU on type I and type III collagen secretion and total protein synthesis. Increasing concentrations of 5-FU show little effect on levels of type I collagen (-■-), type III collagen (-●-), and a slight decrease in total protein (- → -). Error bars represent SEM.

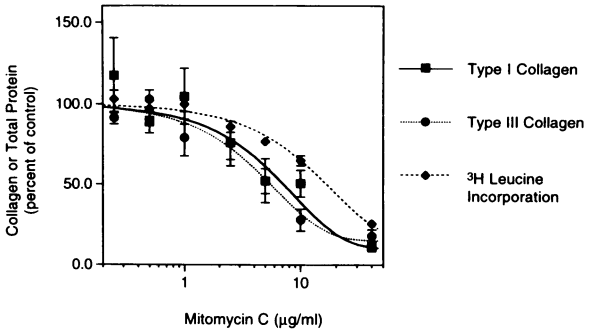

FIGURE 2

Effects of MMC on type I and type III collagen secretion and total protein synthesis. Fibroblasts were incubated for 24 hours in FGM containing increasing concentrations of MMC. Conditioned media assayed using dot blot assay demonstrate decreasing levels of type I collagen (-■-) and type III collagen (-●-). Tritiated leucine measured in cells and media also show decrease in total protein (-→-). Error bars represent SEM.

scarcity of wide pseudopods extending from the plump cell bodies (Fig 3A).

Fibroblasts treated for 24 hours with 5-FU at 5 mg/mL appear pycnotic with bloated cytoplasm, poorly defined nuclei, and sprouting pseudopods. The cytoplasm has a reduced, vacuolated appearance compared to the control panel (Fig 3B). Finally, MMC-treated fibroblasts (40 µg/mL) display extensive bloating with cytoplasmic fragmentation. There is a proliferation of lacy, thin pseudopodia from the perinuclear cytoplasm (Fig 3C). After exposure to the antimetabolites, the cell bodies became a darker gray with reduced size and exhibited a reduced growth rate.

PATIENT CHARACTERISTICS

Between December 1997 and April 1998, 53 guarded glaucoma filtration procedures in 53 eyes were performed. Of these, 35 individual cell lines of Tenon's capsule fibroblasts were established in cell culture. The remaining patients' tissues were not able to be included because there was inadequate Tenon's capsule, the removal of which at surgery could present possible difficulty in wound closure (11 cases); the surgeon inadvertently forgot to harvest Tenon's capsule prior to application of antimetabolite (4 cases), and tissue sample for unknown reason did not grow in cell culture or became contaminated (3 cases).

The baseline demographic characteristics of the patients are presented in Table I. The average age was 63.6 years, with a range of 6 months to 92 years. The majority of samples were obtained from right eyes (63%) and women (60%). There was a preponderance of white patients (68%),

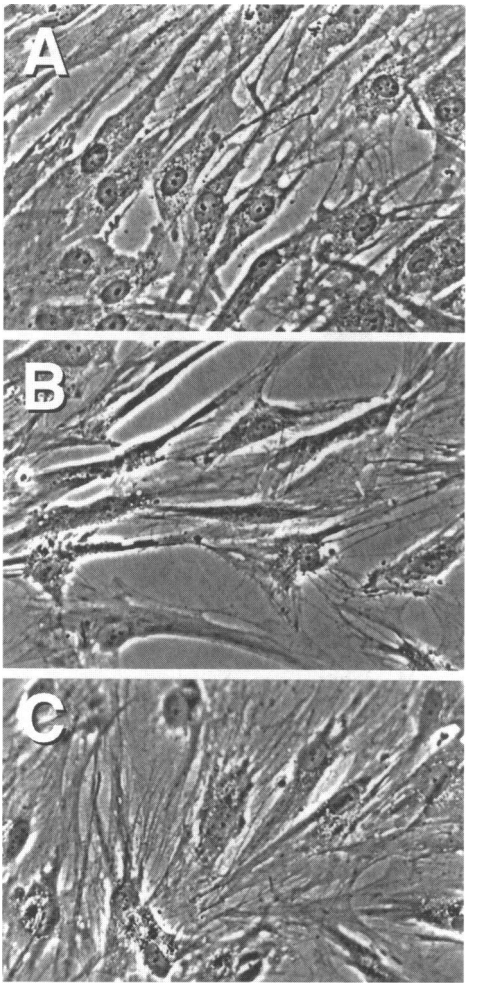

FIGURE 3
Photomicrographs of Tenon's capsule fibroblasts after 24 hours exposure to 5-FU (B), or MMC (C). A, Controls show cells with plump cell bodies and distinct nuclei characteristic of actively secreting fibroblasts. B, Cells exposed to 5-FU at 5 mg/mL have a pycnotic appearance, an absence of vacuoles in the cytoplasm, and retracted pseudopods. C, Cells treated with MMC at 40 μg/mL show a proliferation of lacy pseudopodia from the bloated cell bodies. The cells show extensive cytoplasmic fragmentation. (bar in C = 50 μm)

with 14% Hispanics, 11% blacks, and 6% Asians. The only statistically significant difference among the patients was that the black patients were younger than patients of other races (P=.0001). Nine of the eyes had

undergone previous cataract extraction, and in a similar number, unsuccessful glaucoma filtration surgery had been previously performed. Of those, 4 patients had both procedures. Twelve eyes had previously been

TABLE I: BASELINE PATIENT CHARACTERISTICS

CELL LINE	AGE (YR)	EYE	SEX	RACE	CAT EXT	TRAB	LAS	DX	PRE IOP
1	55	L	F	Black		X		POAG	55
2	73	L	F	Hispanic			X	POAG	22
3	43	R	M	Black		X	X	POAG	26
4	78	L	M	White				NVG	32
5	80	R	F	White	X	X		POAG	37
6	18	L	M	White				UG	33
7	89	L	F	Hispanic	X			POAG	24
8	74	R	F	White	X	X		POAG	31
9	67	L	F	White	X		X	LTG	15
10	6	R	M	Black				UG	22
11	0.5	R	M	Black		X		CONG	26
12	69	L	M	White	X	X		POAG	29
13	66	R	M	White				NVG	52
14	79	R	F	White	X			POAG	26
15	75	R	M	White			X	POAG	28
16	85	R	M	White	X			POAG	45
17	62	R	F	Asian				POAG	22
18	44	R	F	Hispanic				POAG	39
19	61	R	M	White			X	LTG	19
20	74	R	F	White			X	POAG	24
21	59	R	F	White			X	PXFG	34
22	71	R	M	White				POAG	21
23	71	L	F	Hispanic			X	POAG	28
24	92	R	F	White				POAG	31
25	56	R	F	Asian			X	POAG	17
26	81	L	F	White			X	POAG	32
27	76	L	F	White	X	X		ACG	52
28	78	R	M	White			X	POAG	31
29	74	L	F	White				LTG	15
30	77	R	F	White				POAG	26
31	82	R	F	White		X		POAG	18
32	14	R	F	White	X		X	CONG	38
33	72	R	F	White		X		POAG	14
34	63	L	M	White				POAG	21
35	62	L	M	Hispanic				NVG	40

ACG, angle-closure glaucoma; Cat ext, previous cataract extraction; CONG, congenital or developmental glaucoma; Dx, diagnosis; G, uveitic glaucoma; Las, previous glaucoma laser including argon laser trabeculoplasty or laser iridotomy; LTG, low-tension glaucoma; NVG, neovascular glaucoma; POAG, primary open-angle glaucoma; PreIOP, preoperative intraocular pressure (mm Hg); Trab, previous trabeculectomy superiorly.

treated with intraocular laser, 1 peripheral iridotomy and the remainder argon laser trabeculoplasty. The most common type of glaucoma was primary open-angle (66%), followed by low-tension or normal-tension glaucoma (9%), and neovascular (9%), uveitic and congenital (6% each), and pseudoexfoliative and angle-closure (3% each). The mean preoperative IOP was 29.3 mm Hg, with a range of 15 mm Hg to 55 mm Hg.

The patients' exposure to antiglaucomatous medications is presented in Table II. All but one patient had been treated with medications preoperatively. The most common class of medications used was the beta-adrenergic blocking agents (91%). This was followed by the prostaglandins (71%), alpha-adrenergic agonists (66%), topical carbonic anhydrase inhibitors (49%), miotics (43%), and (least used) the oral carbonic anhydrase inhibitors (14%). The patients in this study were taking an average of 3.34 ± 1.1 glaucoma medications (range, 0-5), with an average of 3.2 topical medications (range, 0-5). Within this range, 1 patient was using no topical medications, 1 used one type of medication, 4 patients used two types, 12 patients used three types, 14 used four types, and 3 used five classes of medications within the month prior to filtration surgery.

TYPE I AND TYPE III COLLAGEN PRODUCTION

There was a large variation among patients in the production of both type I and type III collagen (Table III). The levels of type I and type III collagen measured were similar following exposure to ascorbic acid and 5-FU, confirming the finding that 5-FU does not greatly impact collagen production by Tenon's capsule fibroblasts in cell culture. Also, as expected, MMC exposure did result in decreased collagen type I and III measurements in essentially all cell lines studied. There was only a single cell line in which detectable levels of type I or type III collagen could be measured in the water-added controls without ascorbic acid (cell line 25). The amount measured was very small, 15 ng/culture dish of type III collagen. Therefore serum-free Fibroblast Growth Media with 100 µg/mL ascorbic acid was used in all determinations of antimetabolite effect.

The differences in collagen production were not attributable to differences in cell number or survival. The random sampling of 10 cell lines that were counted showed less than a 10% difference among the different agents added to the culture when performed in triplicate, ensuring an equivalent growth rate and cell number among cultures.

CORRELATIONS WITH COLLAGEN TYPES I AND III PRODUCTION

The results of the statistical analysis between collagen type I and type III production and the factors examined are presented in Table IV. The risk of failure variables including age, race, diagnosis, previous surgery, or previous

TABLE II: PATIENTS' GLAUCOMA MEDICATION EXPOSURE

Cell Line	Age	Race	Dx	BB	AA	TCAI	OCAI	M	Pr
1	55	Black	POAG	X	X	X		X	X
2	73	Hispanic	POAG	X			X	X	X
3	43	Black	POAG	X			X	X	X
4	78	White	NVG	X	X	X			X
5	80	White	POAG	X	X				X
6	18	White	UG	X	X	X			
7	89	Hispanic	POAG	X	X	X			X
8	74	White	POAG	X	X	X			X
9	67	White	LTG	X					X
10	6	Black	UG	X	X	X			X
11	0.5	Black	CONG	X					
12	69	White	POAG	X	X	X			X
13	66	White	NVG	X	X	X			
14	79	White	POAG	X	X	X		X	
15	75	White	POAG	X	X			X	X
16	85	White	POAG	X	X	X		X	X
17	62	Asian	POAG	X		X		X	X
18	44	Hispanic	POAG	X	X			X	
19	61	White	LTG	X	X			X	
20	74	White	POAG	X	X	X		X	X
21	59	White	PXFG	X		X		X	X
22	71	White	POAG	X	X				X
23	71	Hispanic	POAG	X			X		X
24	92	White	POAG		X	X			
25	56	Asian	POAG	X	X		X		X
26	81	White	POAG	X	X			X	
27	76	White	ACG	X	X		X		X
28	78	White	POAG	X				X	X
29	74	White	LTG	X	X	X			
30	77	White	POAG	X	X	X			X
31	82	White	POAG	X				X	X
32	14	White	CONG		X				X
33	72	White	POAG						
34	63	White	POAG	X		X			
35	62	Hispanic	NVG	X	X	X			X

AA, previous use of alpha-adrenergic agonist; ACG, angle-closure glaucoma; BB, previous use of beta-adrenergic blocking agent; CONG, congenital or developmental glaucoma; Dx, diagnosis; LTG, low-tension glaucoma; M, previous use of miotic; NVG, neovascular glaucoma; OCAI, previous use of oral carbonic anhydrase inhibitor; POAG, primary open-angle glaucoma; Pr, previous use of prostaglandin; TCAI, previous use of topical carbonic anhydrase ; UG, uveitic glaucoma;

medication use showed no statistical correlation ($P = .0003$). Similar results were found when the ratio of type III:type I was used for comparison.

The correlations that were statistically relevant included the production of each collagen type by the individual patient's fibroblast (Table V). Thus, there was correlation of type I production in the presence of ascorbic acid with the production of type I in the presence of 5-FU or MMC. Similar results were present with type III collagen measured.

TABLE III : COLLAGEN TYPES I AND III PRODUCTION BY INDIVIDUAL CELL LINES

CELL LINE	AGE	RACE	DX	I VIT C	I 5FU	I MMC	III VIT C	III 5FU	III MMC
1	55	Black	POAG	10930	6045	3146	60	48	0
2	73	Hispanic	POAG	17968	16809	6127	159	87	45
3	43	Black	POAG	5465	5299	2815	0	0	0
4	78	White	NVG	6459	10361	6714	0	0	0
5	80	White	POAG	25089	23929	10267	99	42	12
6	18	White	UG	2410	2708	360	426	411	42
7	89	Hispanic	POAG	1031	1466	435	123	72	54
8	74	White	POAG	2832	3018	882	165	249	258
9	67	White	LTG	397	199	99	264	90	45
10	6	Black	UG	1639	4372	198	426	447	174
11	0.5	Black	CONG	3279	3353	1366	417	405	87
12	69	White	POAG	795	273	149	66	111	27
13	66	White	NVG	4036	4529	104	672	612	234
14	79	White	POAG	20700	13745	6624	156	82	66
15	75	White	POAG	5714	3830	62	600	438	183
16	85	White	POAG	1221	807	41	123	99	33
17	62	Asian	POAG	5010	4986	766	519	372	156
18	44	Hispanic	POAG	2881	3292	1284	177	246	141
19	61	White	LTG	2770	2993	87	213	60	33
20	74	White	POAG	994	932	75	27	33	0
21	59	White	PXFG	1106	683	75	24	24	0
22	71	White	POAG	745	521	373	177	222	117
23	71	Hispanic	POAG	3651	3279	1192	486	468	378
24	92	White	POAG	770	348	199	756	420	351
25	56	Asian	POAG	7974	7924	2881	963	885	885
26	81	White	POAG	348	348	75	108	84	24
27	76	White	ACG	397	1540	174	210	150	21
28	78	White	POAG	621	174	149	30	24	30
29	62	White	LTG	3105	1614	149	162	138	48
30	77	White	POAG	1366	4670	99	87	111	45
31	82	White	POAG	3636	1118	248	0	0	0
32	14	White	CONG	1266	1714	199	531	705	177
33	72	White	POAG	2832	1639	132	75	33	30
34	63	White	POAG	472	447	49	129	72	12
35	62	Hispanic	NVG	373	1739	99	57	85	75

	I VITC	I 5FU	I MMC	III VITC	III 5FU	III MMC
Number	35	35	35	35	35	35
Minimum	348	174	33	0	0	0
Maximum	25089	23929	10267	963	885	885
Mean	4288	4029	1359	248	213	109
SD	5852	5116	2428	238	220	166

ACG, angle-closure glaucoma; CONG, congenital or developmental glaucoma; Dx, diagnosis; LTG, low-tension glaucoma; NVG, neovascular glaucoma; POAG, primary open-angle glaucoma; UG, uveitic glaucoma; I VitC, amount (ng/ culture dish) of type I collagen measured after exposure to 100 μM ascorbic acid; I 5FU, amount (ng/ culture dish) of type I collagen measured after exposure to 10 μg/mL 5-FU; I MMC, amount (ng/ culture dish) of type I collagen measured after exposure to 10 μg/mL MMC; III VitC, amount (ng/ culture dish) of type III collagen measured after exposure to 100 μM ascorbic acid; III 5FU, amount (ng/ culture dish) of type III collagen measured after exposure to 10 μg/mL 5-FU; III MMC, amount (ng/ culture dish) of type III collagen measured after exposure to 10 μg/mL MMC.

TABLE IV: STATISTICAL CORRELATION OF COLLAGEN TYPE I AND III MEASUREMENTS WITH RISK FACTORS

RISK FACTOR			I VIT C	I 5FU	I MMC	III VIT C	III 5FU	III MMC
Age		*r*	-0.1074	0.2884	0.1253	-0.1913	-0.3284	-0.0823
		P	0.5393	0.0929	0.4048	0.2708	0.0541	0.6383
Race	White	*r*	0.2769	-0.4104	-0.5089	0.1451	0.2006	0.2982
		P	0.1073	0.0143	0.0018	0.4056	0.2479	0.0818
	Black	*r*	0.2065	-0.2463	-0.2974	-0.0662	-0.0083	-0.0955
		P	0.2340	0.1538	0.0827	0.7054	0.9622	0.5854
Dx	High	*r*	-0.0206	-0.2539	-0.0972	0.2181	0.3530	0.0115
	Risk	*P*	0.9065	0.1411	0.5785	0.2082	0.0376	0.9479
Previous surgery	Cat ext	*r*	-0.0874	0.0702	0.0705	-0.0087	-0.0376	-0.1179
		P	0.6177	0.6888	0.6875	0.9607	0.8302	0.5000
	Trab	*r*	0.1948	-0.1850	-0.1853	-0.2509	0.2181	-0.2050
		P	0.2620	0.2874	0.2865	0.1460	0.2081	0.2375
	Laser	*r*	-0.0160	0.2138	0.1068	0.0240	-0.0141	0.1553
		P	0.9272	0.2174	0.5413	0.8910	0.9361	0.3731
Meds	BB	*r*	0.1139	-0.1392	-0.0407	-0.2432	-0.2112	-0.1435
		P	0.5146	0.4251	0.8166	0.1592	0.2233	0.4108
	AA	*r*	-0.0424	-0.2303	0.0665	0.1704	0.2289	0.1601
		P	0.8089	0.1832	0.7042	0.3277	0.1860	0.3582
	TCAI	*r*	-0.1130	-0.0788	0.2059	-0.0856	-0.0566	-0.1161
		P	0.5179	0.6525	0.2353	0.6248	0.7469	0.5064
	OCAI	*r*	0.2221	-0.2708	-0.3624	0.1358	0.1311	0.3895
		P	0.1998	0.1156	0.0324	0.4367	0.4529	0.0207
	M	*r*	0.2493	0.0104	0.0445	-0.3337	-0.3738	-0.2936
		P	0.1487	0.9527	0.7995	0.0501	0.0270	0.0869
	Pr	*r*	0.0100	-0.0395	-0.0517	-0.2197	-0.1195	0.0267
		P	0.9547	0.8216	0.7681	0.2048	0.4942	0.8789
	Sum	*r*	0.1555	-0.2842	-0.0021	-0.2456	-0.1757	-0.0276
		P	0.3724	0.0980	0.9904	0.1549	0.3128	0.8751

AA, alpha-adrenergic agonist; BB, beta- adrenergic blocker; Cat Ext, previous cataract extraction; Dx, diagnosis; High, high risk, NVG, UG, CONG; Laser, previous ALT or PI; M, miotic; OCAI, oral carbonic anhydrase inhibitor; Pr, prostaglandin; *r*, correlation coefficient; TCAI, topical carbonic anhydrase inhibitor; Trab, previous trabeculectomy; I VitC, amount (ng/ culture dish) of type I collagen measured after exposure to 100 μM ascorbic acid; I 5FU, amount (ng/ culture dish) of type I collagen measured after exposure to 10 μg/mL 5-FU; I MMC, amount (ng/ culture dish) of type I collagen measured after exposure to 10 μg/mL MMC; III VitC, amount (ng/ culture dish) of type III collagen measured after exposure to 100 μM ascorbic acid; III 5FU, amount (ng/ culture dish) of type III collagen measured after exposure to 10 μg/mL 5-FU; III MMC, amount (ng/ culture dish) of type III collagen mea ured after exposure to 10 μg/mL MMC.

DISCUSSION

Scar formation is a complex process involving the sequential synthesis and secretion of extracellular matrix components involving numerous cell types, and it is therefore very difficult to study in vivo. To better understand the mechanism and regulation of this process, our experimental system uses human Tenon's capsule fibroblasts in tissue culture and models the postsurgical condition by specifically measuring the secretion of 2 collagen types known to be involved in wound healing and scar formation. The proportion of type I to type III collagen production by human Tenon's capsule fibroblasts measured in this study is typical for fibroblasts from other tissues.

TABLE V: CORRELATION OF COLLAGEN TYPES I AND III

	I VIT C	I 5FU	I MMC	III VITC	III 5FU	III MMC
I VitC						
I 5FU	-0.7621°					
I MMC	-0.6719°	0.5348				
III Vit C	0.0860	-0.1252	-0.0485			
III 5FU	0.0615	-0.2005	-0.0920	0.9379°		
III MMC	0.1796	-0.1728	-0.2588	0.7267°	0.7489°	

I VitC, amount (ng/ culture dish) of type I collagen measured after exposure to 100 μM ascorbic acid; I 5FU, amount (ng/ culture dish) of type I collagen measured after exposure to 10 μg/mL 5-FU; I MMC, amount (ng/ culture dish) of type I collagen measured after exposure to 10 μg/mL MMC; III VitC, amount (ng/ culture dish) of type III collagen measured after exposure to 100 μM ascorbic acid; III 5FU, amount (ng/ culture dish) of type III collagen measured after exposure to 10 μg/mL 5-FU; III MMC, amount (ng/ culture dish) of type III collagen measured after exposure to 10 μg/mL MMC.

°Correlation coefficients (r), $P = .0000$; n = 35.

ANTIMETABOLITE DOSE RESPONSE AND HISTOPATHOLOGIC EFFECTS

MMC acts as a selective inhibitor of DNA synthesis and has been shown to decrease protein synthesis and inhibit proliferation of human Tenon's capsule fibroblasts in cell culture.[97,98] In this study, at concentrations above 2.5 μg/mL, it significantly inhibited the secretion of both collagen types. Not surprisingly, in the same concentration range, it inhibited overall protein synthesis by the cultured fibroblasts. However, the type III:type I ratio remained relatively constant at all concentrations tested, indicating that MMC does not appear to selectively modify wound healing by

specifically decreasing type III collagen. This agrees with the clinical finding that although MMC increases success, this occurs because of a nonselective reduction in wound healing that unfortunately is associated with an increase in severe complications.

5-FU is an analog of thymine and interferes with the production of the normal precursors necessary for DNA synthesis. In this model system, 5-FU had no effect on collagen secretion in concentrations up to 5 mg/mL, the highest concentration we tested. This level corresponds to the concentration range in tissues with intraoperative application.[94] It had a slight inhibitory effect on protein synthesis, probably because of its antimetabolic effects. 5-FU shows no differential effect on the 2 collagen types, which is consistent with the clinical findings of a lesser effect than MMC.

Photomicrographs of the cells taken after 24 hours of exposure to the highest concentration of each drug show a variety of characteristic morphologic changes consistent with the known action of that drug. There was significant cell damage with MMC, a cytocidal agent; whereas 5-FU, a cytostatic agent, had definite but less severe morphologic effects. Longer exposure to these or higher drug concentrations may result in more significant changes in cell morphology.

PATIENT CHARACTERISTICS

The patients included in this study are representative of a typical population of glaucoma patients undergoing filtration surgery in an academic tertiary-referral practice. The number of patients was limited by the long time needed to develop a reliable and reproducible method of directly measuring the collagen-type proteins. Those patients who had filtering surgery during the study but who were not included for various reasons did not exhibit any baseline characteristics different from the study population, which would not be expected to introduce additional bias into this study. They were not included for technical reasons.

The characteristics of the patient population demonstrate no unanticipated findings. It is not surprising that the black patients were younger, as this is to be expected.[51] The finding that nearly all of the patients were receiving medical therapy for glaucoma and that approximately 25% had undergone previous incisional ocular surgery is consistent with this population.

TYPE I AND TYPE III COLLAGEN PRODUCTION

It has been previously reported that the presence of ascorbic acid is essentially obligatory to obtain measurable type III collagen levels in human Tenon's capsule fibroblasts in cell culture.[103] Aqueous humor has been long believed to play a role in the unique ability to maintain a patent fistula in glaucoma filtration into the subconjunctival space. Given the

markedly elevated concentration of ascorbic acid in aqueous, it seems reasonable to hypothesize that ascorbic acid may play a role in modulation of the healing response. This study demonstrated the enormous differences among fibroblasts from different patients in their secretion of collagen in response to ascorbic acid. Conversely, in the absence of ascorbic acid, only one cell line secreted measurable levels of collagen.

The result that collagen-type production following ascorbic acid and 5-FU exposure was similar in nearly all of the cell lines is consistent with the interpretation that 5-FU has little impact on collagen type I or type III production. There was a difference of greater than 10% in approximately one third of the cell lines, but the effect of 5-FU was not consistent. Both type I collagen production and type III collagen production were similarly affected in most cell lines. This adds support to the hypothesis that there is a true difference among fibroblasts derived from different patients.

Exposure of fibroblasts to MMC resulted in a reduction of collagen type I and III production. This study suggests that this effect is at least partially attributable to decreased collagen synthesis. Compared to baseline collagen production, as represented by secretion in the presence of ascorbic acid, there was as little as a less than twofold reduction in type I production to as great as a 40- to 60-fold decrease in some cell lines, with the majority being between these extremes. Interestingly, the relative effect of MMC on type III production was less, with at most a threefold to fourfold decrease, except in a single cell line (6). Thus, this provides further evidence as to the unavoidable increase in complications that accompanies the increase in success that is found with MMC use in filtration surgery. As far as collagen I and III production is concerned, while decreasing both, it appears to potentially exert a preferential effect on type I, thus exhibiting no preferred effect to prevent only excessive wound healing, which would be expected to have an increased type III production. In fact, the data would suggest that there is greater reduction of production of type I collagen, the type that it would theoretically be less desirable to inhibit.

CORRELATIONS WITH COLLAGEN TYPES I AND III PRODUCTION

There are two striking results from this study. First is the lack of correlation between collagen type I or III production and known risk factors for filtration failure. There were some correlations that alone did have statistical validity to the $P = .05$ level. However, in a study such as this, the potential effects of type I error must be taken into account and a higher level of statistical significance must be required to definitively determine correlation. The number of cell lines in the study may also be a limiting factor. Given the exploratory nature of this study, a bivariate analysis was performed. Multivariate regression analysis may be necessary in future

investigations once potential correlations are identified.

Although collagen secretion by fibroblasts plays a key role in wound healing, both normal and excessive, from this study, it appears that the intrinsic ability of the fibroblast to synthesize collagen subtypes is not responsible for the variability among patients. This is not surprising. The generally accepted risk factors for failure of filtration surgery are age, race, diagnosis, previous conjunctival surgery, and previous use of topical antiglaucomatous medications. One would not expect a difference among fibroblasts except possibly due to age or race. These factors may represent true differences among different patient cell lines. There may be different expression of collagen with racial composition or age. Unfortunately, this study does not support that hypothesis. The other risk factors most likely represent secondary effects on the fibroblast that could modify its function. These may include responses to intraocular inflammation or ischemia as one would see in uveitic or neovascular glaucomas and medication-induced inflammation. Although these may well affect collagen production in vivo, it is a limitation of this study that the full cascade of cellular reaction cannot be examined in tissue culture. The factors responsible for the increased healing response are not present in this in vitro model. This study would also support the idea that intrinsic collagen production, while varying greatly among different patients, is not the sole explanation for differences in wound healing attributable to age or race. There must be more complex interactions involving other facets of healing at work.

The second surprising result is the support for the hypothesis that there is substantial variability among patients in their Tenon's capsule fibroblasts production of type I and type III collagen. This study demonstrates an enormous range of up to three orders of magnitude difference. Additionally, there was statistical correlation between the responses to the specific agents, suggesting that the differences are real. This held true for both type I and type III collagen.

One additional obvious question is whether the production of type I and/or type III collagen correlated with clinical outcome. Unfortunately, this study was not designed to answer that query. Since that study would necessitate that all surgical procedures be performed identically, given the wide variation in the characteristics of the patients, that was not felt to be appropriate. In addition, it has been shown that the outcome of filtration surgery does not correlate with levels of ascorbic acid in the primary aqueous.[104] Unfortunately in the clinical circumstance, the bleb contains secondary aqueous during healing and there is no information as to ascorbate levels in secondary aqueous or whether these levels correlate with measured levels in primary aqueous.

SUMMARY

This study has demonstrated that 5-FU does not affect collagen type I or III production, while MMC reduces both, apparently with a preferential effect on type I. This is consistent with the clinical finding that MMC is the more effective inhibitor of healing and that the effect is nonspecific, increasing both success and complications. Collagen type I and III production did not correlate with accepted risk factors for filtration failure. Thus, one can postulate that while patients have excessive healing following filtration surgery, this study does not support that this excessive healing is attributable solely to increased collagen type I or III production.

This study further emphasizes the complex nature of the scarring cascade and reemphasizes the need to continue investigations to better understand wound healing in order to allow modulation. This would be directed toward a means to separate the beneficial and deleterious effects of wound modulators to allow an increase in success without the concomitant increase in potentially sight-threatening complications for the benefit of preserving the sight of our patients.

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