Single Exposures to Antiproliferatives
Long-Term Effects on Ocular Fibroblast Wound-Healing Behavior


Purpose. To determine the long-term effects of single, 5-minute exposures to 5-fluorouracil (5FU) and mitomycin-C (MMC) on Tenon's capsule fibroblast migration, growth factor production, growth factor receptor expression, and extracellular matrix (ECM) production.

Methods. Monolayer cultures and the overlying growth medium of Tenon's capsule fibroblasts exposed to 5FU (0.25 to 25 mg/ml) or MMC (0.001 to 0.1 mg/ml) were harvested up to 48 days after treatment. The expression of growth factors and growth factor receptors, including transforming growth factor β (TGF/β), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), and ECM molecules (collagen type I, collagen type III, and fibronectin) were quantitated at the mRNA and protein levels. The ability of fibroblasts exposed to 5FU and MMC to migrate to fetal calf serum was also investigated up to 48 days after treatment.

Results. Control cultures were found to produce the growth factors TGF/β and bFGF but not EGF. Exposure to 5FU or MMC resulted in an initial significant increase ($P < 0.05$) in the production of TGF/β and bFGF, with levels then decreasing toward those of controls. Cells exposed to 5FU or MMC exhibited an initial significant decrease ($P < 0.05$) in the number of TGF/β, bFGF, and EGF growth factor receptors, with subsequent recovery toward control levels by day 48 after treatment. Both 5FU and MMC caused a significant reduction ($P < 0.05$) in collagen type I and fibronectin production compared to controls throughout the 48-day culture period. The production of collagen type III was initially elevated ($P < 0.05$) compared to controls after exposure to 5FU or MMC, production then decreasing toward control levels over the remainder of the 48-day culture period. The migration of cells exposed to 5FU or MMC was significantly reduced ($P < 0.05$) compared to controls up to 48 days after treatment; these cells exhibited a partial recovery of migratory ability throughout this period.

Conclusions. Fibroblasts whose growth was arrested using single, short exposures to 5FU or MMC appear to be capable of performing several crucial aspects of wound healing, including the expression of growth factors and receptors and ECM molecules and the ability to migrate. These findings may help explain why in some patients treated with antiproliferatives, glaucoma filtration surgery fails because of scarring. Invest Ophthalmol Vis Sci. 1997;38:1998-2007.

The major determinant of successful intraocular pressure control after glaucoma filtration surgery is the degree of postoperative scarring. Several studies have shown that exposure to antiproliferatives, whether administered as subconjunctival injections of 5-fluorouracil (5FU) or as single intraoperative applications of mitomycin-C (MMC), increased the success of filtration surgery by reducing postoperative scarring.1-3 The fibroblast is the key player in this scarring response; among its crucial functions are proliferation, extracellular matrix (ECM) contraction, migration, and ECM production. Many of these functions are under the control of growth factors and the specific receptors through which they elicit their effects.4,5

We previously demonstrated that short, single exposures to the antiproliferatives 5FU and MMC resulted in the long-term growth arrest of fibroblasts in vitro6,7 and in vivo.8 However, the overall effects of these treatments on fibroblast functions other than...
proliferation are unclear. This is important, because single intraoperative exposures to 5FU and MMC are used in the treatment of many patients undergoing glaucoma filtration surgery. Also, high-risk patients can still fail glaucoma filtration surgery due to scarring despite treatment with these agents. The reasons for this are unclear, but it may be that although growth-arrested, these fibroblasts still can contribute to a scarring response.

In a previous study, we reported that fibroblasts could partially contract the ECM before the inhibitory effects of single exposure to antiproliferatives became apparent. Previous reports suggested that cellular functions such as migration and attachment may or may not be affected by these agents. Also, feeder layers of growth-arrested fibroblasts have been used for more than 20 years to support the long-term serial cultivation of skin epithelial cells, the mechanisms underlying this process are unknown. Finally, injecting high concentrations of growth factor such as transforming growth factor β1 (TGFβ1) may reverse the effects of MMC in an animal model. What these data suggest, in conjunction with clinical findings, is that growth-arrested fibroblasts may still be able to perform functions that contribute to a scarring response sufficient to result in the failure of filtration surgery. It is therefore important to understand what functions, and to what degree, these cells are capable of performing so that treatment can be adjusted appropriately.

We investigated the long-term expression of several growth factors, growth factor receptors, and ECM molecules, which have been implicated as crucial components of the scarring response, by growth-arrested ocular fibroblasts. We also investigated the migratory ability of growth-arrested fibroblasts up to 48 days after treatment.

MATERIALS AND METHODS

Cell Culture

Human ocular (Tenon’s capsule) fibroblasts were propagated from a 4-year-old male donor eye (Moorfields Eye Hospital Eye Bank, London, UK), as previously described. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), amphotericin B (Fungizone; 0.25 µg/ml), and glutamine (2 mM) and supplemented with 10% (vol/vol) newborn calf serum (all Gibco Life Technologies, Paisley, UK). Cultures were used between passages 3 to 7 for experimentation. The tenets of the Declaration of Helsinki were followed and institutional human experimentation committee approval was granted.

Treatment of Monolayers With 5-Fluorouracil and Mitomycin-C

Cells were seeded into 150 cm² tissue culture flasks at 10,000 cells/cm² and allowed to settle overnight. The overlying medium was aspirated and the monolayers were exposed to 5FU (0.25, 2.5, and 25 mg/ml), MMC (0.001, 0.01, and 0.1 mg/ml), or phosphate-buffered saline (PBS; controls) for 5 minutes. Higher, directly relevant clinical concentrations of MMC (0.2 to 0.5 mg/ml) were not used because too much cell death occurred to be able to carry out experiments. The monolayers were then washed with 5 × 50 ml of PBS, ensuring that no liquid remained after each of the five washes. The cultures were then overlaid with 50 ml of serum-containing medium.

Conditioned Medium Production

At 3, 7, 14, 24, 36, and 48 days after treatment, serum-containing medium was aspirated from the tissue culture flasks. The monolayers were then overlaid with 20 ml of DMEM containing 1% (wt/vol) bovine serum albumin (BSA; Sigma, Dorset, UK) and incubated at 37°C twice (4 hours each incubation) to ensure residual serum depletion. The monolayers were then incubated for 24 hours in 20 ml of DMEM containing 1% (wt/vol) BSA. This conditioned medium was then collected, filter-sterilized through a 0.2-µm filter (Sartorius, Surrey, UK) to remove debris and insoluble material, and stored in aliquots at −70°C until use.

After the collection of conditioned medium, the cell monolayers were trypsinized and centrifuged and total cell numbers were determined using a Coulter counter (Coulter Electronics, Bedfordshire, UK). The viability of these cells was confirmed using a trypan blue dye exclusion test. Samples of conditioned medium were then analyzed for growth factors and ECM molecules as described in the following sections.

Quantitation of Growth Factors

Several growth factors have been implicated as playing crucial roles in the scarring process, including members of the TGFβ, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) families. Levels of TGFβ1, bFGF, and EGF mRNA were analyzed using the quantitative competitive reverse transcriptase polymerase chain reaction (QCRT-PCR) technique described by Tarnuzzer et al. Fixed quantities of this RNA (1 µg), in conjunction with known copy numbers of synthetic RNA templates, were then subjected to QCRT-PCR using specific primers, and the copies of each mRNA in the cell culture samples were calculated. The results were expressed as the number of mRNA copies per 100 cells.
The levels of TGFβ1, bFGF, and EGF protein were quantitated in samples of conditioned medium from growth-arrested and control cultures up to 48 days after treatment. Briefly, samples of conditioned medium were analyzed using commercially available enzyme-linked immunosorbent assays (ELISAs) obtained from R and D Systems, Oxfordshire, United Kingdom. Results were expressed as the mean quantity of growth factor (pg) from triplicate wells per 10^5 cells. The data were statistically analyzed using computer software (SPSS for Windows; SPSS, Chicago, IL) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. P < 0.05 was considered significant.

Quantitation of Extracellular Matrix Molecules

Total RNA was isolated from monolayer cultures up to 48 days after treatment and was quantitated for collagen types I and III and fibronectin mRNA levels as described for growth factors. Samples of BSA-free conditioned medium were analyzed for collagen types I and III protein, and samples of conditioned medium containing BSA were analyzed for fibronectin protein levels up to 48 days after treatment using ELISAs.

Collagen type I or III standards (EuroGenetics, Middlesex, UK, were diluted in PBS at concentrations of 0 to 10 μg/100 μl) or 100 μl per well of conditioned medium and bound to the plates at 4°C overnight. Nonspecific binding sites were then blocked by adding 300 μl per well of 1% (wt/vol) BSA in PBS and incubating for 1 hour at room temperature. The plates were then aspirated, and the primary polyclonal antibody (goat anti-human collagen type I or III; EuroGenetics) was added at a dilution of 1:2000 (100 μl per well) in PBS and incubating for 1 hour at room temperature. The plates were then aspirated, and the primary polyclonal antibody (goat anti-human collagen type I or III; EuroGenetics) was added at a dilution of 1:2000 (100 μl per well) and incubated for 1 hour. The plates were then washed with a total of three times with PBS and 1% (wt/vol) BSA (300 μl per well). A horseradish peroxidase conjugated rabbit anti-sheep antibody (EuroGenetics) diluted at 1:8000 was then added (100 μl per well), followed by incubation for 1 hour at room temperature. The plates were then washed a total of three times with PBS (300 μl per well) before the addition of 100 μl per well of o-phenylamine substrate (SigmaFast tablets; Sigma). Plates were then developed for 30 minutes, the reaction was stopped by the addition of 50 μl per well of 3 M hydrochloric acid, and the optical density of each well was determined using a Titertek Plus MS2 microplate reader (ICN Flow).

Standard curves for collagen types I and III were expressed as the means of triplicate wells (±SE), and an equation for this standard curve was determined using a polynomial trendline fit generated using computer software (Microsoft Excel, Seattle, WA). The levels of collagen types I and III in conditioned medium samples were calculated from this standard curve equation and expressed as the mean of triplicate wells (±SE). Because the dilution of the conditioned medium sample and the number of cells these samples were generated from were known, this allowed the results to be expressed as the quantity of collagen types I or III produced (μg) per 10^5 cells. Although this assay showed no cross-reactivity with fibronectin, there was cross-reactivity of the anti-collagen type I antibody with purified collagen type III and vice versa. The cross-reactivity of these antibodies and antigens was taken into account for data analysis. Data were statistically analyzed using a one-way ANOVA; P < 0.05 was considered significant.

The levels of fibronectin produced by growth-arrested fibroblasts in conditioned medium containing 1% (wt/vol) BSA were quantitated using a double-sandwich ELISA. First, 96-well microtiter plates were coated at 4°C overnight with 100 μl per well of a monoclonal anti-human fibronectin antibody (Dako, Buckinghamshire, UK) diluted 1:1000 in PBS. The wells were then aspirated, and excess protein-binding sites were blocked by adding 300 μl per well of 1% (wt/vol) BSA in PBS and incubated for 1 hour at room temperature. Plates were then aspirated, and 100 μl per well of fibronectin standards (0 to 10 μg/ml; Sigma) or samples of conditioned medium from growth-arrested fibroblasts were added, followed by incubation for 2 hours at room temperature. The wells were then aspirated and washed a total of three times with PBS and 1% (wt/vol) BSA (300 μl per well). A second anti-human fibronectin monoclonal antibody (Dako), which was horseradish peroxidase conjugated, was then added to the plates (100 μl per well) at a dilution of 1:4000 in PBS and 1% (wt/vol) BSA, followed by incubation for 1 hour at room temperature. The plates were then washed a total of three times with PBS (300 μl per well) before the addition of 100 μl per well of o-phenylamine substrate. Plates were then developed for 45 minutes at room temperature, the reactions were stopped by adding 50 μl per well of 3 M hydrochloric acid, and the optical density of each well was determined using a Titertek Plus MS2 microplate reader (ICN Flow).

Standard fibronectin curve results were expressed as the means of triplicate wells (±SE), and an equation for this standard curve was determined using a polynomial trendline fit generated using computer software (Microsoft Excel). The levels of fibronectin in conditioned medium samples were calculated from this standard curve equation and expressed as the mean of triplicate wells (±SE). Because the dilution of the conditioned medium sample and the number of cells these samples were generated from were known, this allowed the results to be expressed as the quantity of...
fibronecandin produced (µg) per 10^5 cells. This assay showed no detectable cross-reactivity with collagen types I or III. Data were statistically analyzed using a one-way ANOVA, and P < 0.05 was considered significant.

Growth Factor Receptor Quantitation

Samples of total RNA were analyzed for growth factor receptor mRNA level up to 48 days after treatment by QRT-PCR as described above for growth factors. The method used to quantitate TGFβ, bFGF, and EGF cell surface receptors was that described by Massague. Briefly, human ocular fibroblasts were seeded at a density of 10,000/cm² into 48-well tissue culture plates and allowed to settle overnight. The medium was then aspirated from each well and the cells were exposed in sextuplicate for 5 minutes to 5FU (0.25, 2.5, and 25 mg/ml), MMC (0.001, 0.01, and 0.1 mg/ml), or PBS (controls). The wells were then washed a total of five times with PBS (500 µl per well each wash) and exposed to serum-containing medium. Growth factor receptors were then quantitated at days 7, 24, and 48 after treatment as described below.

Plates were washed with PBS (5 × 500 µl per well) before incubation in serum-free medium for 24 hours (500 µl per well). Each well was then aspirated and either was counted for cell numbers (triplicate wells) or had 50 µl per well of binding buffer (DMEM and 0.1% [wt/vol] BSA and 25 mM Hepes, pH 7.4) added (triplicate wells). Varying concentrations (0, 10^-12 M, 10^-11 M, 10^-10 M, 10^-9 M, 10^-8 M, and 10^-7 M) of each of the unlabeled growth factors TGFβ, bFGF, or EGF (all Sigma) were added to triplicate wells in a volume of 100 µl. To each well, 100 µl of a [125I]-labeled growth factor (Amerham, Buckinghamshire, UK) was then added (100,000 counts per minute per well), and the plates were incubated at 4°C overnight to avoid receptor internalization. The plates were then aspirated and each well was washed a total of four times (500 µl per well) with binding buffer (DMEM and 0.1% [wt/vol] BSA and 25 mM Hepes, pH 7.4). Cell monolayers were then solubilized by adding 500 µl per well of solubilization buffer (1% [vol/vol] Triton X-100, 10% [vol/vol] glycerol, and 0.01% [wt/vol] BSA, pH 7.4). Solubilization of the cells was then checked microscopically before counting 450 µl of each extract using a gamma counter (Wil National, Kent, UK). Receptor numbers were then calculated via Scatchard analysis and expressed as the number of receptors per cell. The data were statistically analyzed using computer software (SPSS for Windows; SPSS) at individual time points using a one-way ANOVA. The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. P < 0.05 was considered as indicating significance.

Migration Assay

The migration assay involved the use of 24-well-plate transwell tissue culture inserts (Costar, Buckinghamshire, UK). Preliminary experiments performed to standardize the assay included checkerboard analysis and determination of the optimal concentration of chemoattractant (fetal calf serum; FCS) and optimal assay time. For the migration assay, cultures of ocular fibroblasts were seeded at a density of 10,000 cells/cm² into six-well tissue culture plates and allowed to settle overnight. The cells were then exposed to antiproliferatives as described above and were harvested by trypsinization 3, 7, 14, 24, 36, and 48 days after treatment. Total and viable cell numbers were determined on a hemocytometer using a trypan blue dye exclusion test. Either serum-free medium (control) or medium containing a predetermined optimal stimulatory concentration of 20% (vol/vol) FCS (Gibco Life Technologies) was added to triplicate wells (600 µl per well) of a 24-well plate (Marathon, London, UK). Transwell tissue culture inserts were then placed into each well, and 100 µl of serum-free medium containing 8000 viable cells from each growth-arrested sample was added to the top chamber. Cells were allowed to settle and migrate through the polycarbonate membrane in the transwell (8-µm pores) over a period of 16 hours (determined in preliminary experiments as the optimal migration period) at 37°C in 5% CO₂ in air. After this migration period, medium was flicked off the transwells, which were then washed in PBS, followed by fixation in 400 µl of 100% ethanol (5 minutes). The fixative was then removed and each transwell was immersed in hematoxylin for 5 minutes; excess dye was removed and blueing of hematoxylin was achieved by rinsing the transwell in tap water.

For settled cell number determinations, the bottom of the membrane of the transwell (migrated side) was cleared of cells using a cotton swab. For migrated cell number, the tops of the transwell membranes were cleared of cells. The membranes were then cut out of the transwells using a scalpel blade and mounted on glass slides with an aqueous mountant (Dako). Sets of triplicate membranes were then counted for total cell numbers settled and migrated either to chemoattractant (chemotaxis) or to serum-free medium (chemokinesis). For settled cell numbers, due to the high seeding density (8000 cells) per well, 10 high-power fields (magnification ×400) were counted on each of the triplicate membranes. These results were then pooled and analyzed as described below. For migrated cells, the whole of the membrane was counted because the maximal number of cells was never particularly high (usually 300 to 400 cells per membrane). Results were expressed as the mean percentage of cells (settled or migrated; ±SE) compared
FIGURE 1. Effects of 5FU (A,C) and MMC (B,D) on growth factor production. Cells were exposed to 5FU at 25 (●), 2.5 (△), 0.25 mg/ml (▼), PBS (○), or MMC at 0.1 (■), 0.01 (▲), 0.001 mg/ml (●), PBS (○). Levels of TGFβ (A,B) and bFGF (C,D) were quantitated up to 48 days after treatment.

The data were statistically analyzed using computer software (SPSS for Windows; SPSS) at individual time points using a one-way ANOVA. The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. P < 0.05 was considered statistically significant.

RESULTS

Effects of 5-Fluorouracil and Mitomycin-C on Long-Term Growth Factor Production

Cells exposed to PBS (controls) exhibited a decreased production of TGFβ1 and bFGF protein, and EGF was not detectable, over the 48-day assay period. Exposure to 5FU at all concentrations tested resulted in an initially significantly increased (P < 0.05) production of TGFβ1 and bFGF protein compared to controls (Figs. 1A and 1C). TGFβ1 and bFGF production by cells exposed to lower concentrations of 5FU (0.25 and 2.5 mg/ml) decreased toward control levels after this initial increase; production of these growth factors by cells exposed to high concentrations of 5FU (25 mg/ml) continued to increase throughout the 48-day assay period (see Figs. 1A and 1C). As for control cultures, EGF was not detectable in conditioned medium from cells exposed to 5FU, at all concentrations tested, throughout the 48-day period.

Exposure to MMC elicited similar results to those seen with 5FU, with cells initially producing significantly increased (P < 0.05) levels of both TGFβ1 and bFGF proteins compared to controls (Figs. 1B and 1D). Unlike cells exposed to 5FU, the production of TGFβ1 and bFGF by cells exposed to all concentrations of MMC decreased toward control levels throughout the 48-day assay period. EGF production was not detectable by cells exposed to MMC throughout the 48-day period. The expression of growth factors detected at the protein level was confirmed by the presence of specific growth factor mRNA by QRT-PCR in both control and growth-arrested cultures (data not shown).

Effects of 5-Fluorouracil and Mitomycin-C on Long-Term Growth Factor Receptor Expression

Control cultures (exposed to PBS) expressed decreasing numbers of TGFβ1, bFGF, and EGF receptors throughout the 48-day assay period (Fig. 2). Exposure to the PBS-treated controls (which were taken as 100% settlement or migration).

The data were statistically analyzed using computer software (SPSS for Windows; SPSS) at individual time points using a one-way ANOVA. The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. P < 0.05 was considered statistically significant.

FIGURE 2. Effects of 5FU (A,C,E) and MMC (B,D,F) on growth factor receptor expression. Cells were exposed to 5FU at 25 (□), 2.5 (△), 0.25 mg/ml (▼), PBS (○), or MMC at 0.1 (■), 0.01 (▲), 0.001 mg/ml (●), PBS (○). Levels of TGFβ (A,B), bFGF (C,D) and EGF receptors (E,F) were quantitated up to 48 days after treatment.
Effects of 5-Fluorouracil and Mitomycin-C on Long-Term Extracellular Matrix Molecule Production

Control cultures produced increasing quantities of collagen type I and fibronectin throughout the 48-day assay period, whereas the levels of collagen type III production decreased (Fig. 3). Exposure to 5FU at all concentrations tested significantly reduced \((P < 0.05)\) the levels of collagen type I and fibronectin production throughout the 48-day period compared to controls (Figs. 3A and 3E). Collagen type III production was initially significantly increased \((P < 0.05)\) after exposure to 5FU, but the levels of production then fell toward control levels throughout the remainder of the 48-day period (Fig. 3C). Effects similar to those of 5FU were obtained after exposure to MMC (Figs. 3B, 3D, 3F). The expression of ECM molecules detected at the protein level was confirmed by the presence of specific ECM molecule mRNA by qRT-PCR in both control and growth-arrested cultures (data not shown).

Effects of 5-Fluorouracil and Mitomycin-C on Long-Term Fibroblast Migration

Using a Zigmund Hirsch checkerboard analysis,\(^1^8\) the migration of fibroblasts up to 20\% (vol/vol) FCS was found to be primarily a chemotactic response (Table

### Table 1. Checkerboard Analysis of Human Tenon’s Capsule Fibroblast Migration to Different Concentration of Fetal Calf Serum

<table>
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<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
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<td>Lower chamber (number of cells; ± SE)</td>
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<td>21 ± 2</td>
<td>38 ± 4</td>
<td>67 ± 7</td>
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<td>191 ± 18</td>
<td>132 ± 4</td>
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<td>45 ± 7</td>
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FIGURE 4. Effects of antiproliferatives on long-term fibroblast migration. Cells were exposed to 5FU (A) at 25 (●), 2.5 (△), 0.25 mg/ml (▲), PBS (○), or MMC (B) at 0.1 (■), 0.01 (▲), 0.001 mg/ml (▼), PBS (●).

1). Exposure to 5FU or MMC at all concentrations tested did not significantly (P > 0.05) affect the settlement of cells during the migration assay compared to controls (data not shown).

The migration of cells exposed to 5FU was significantly reduced (P < 0.05) up to 48 days after treatment compared to controls for all concentrations of 5FU tested (Fig. 4A). However, it appeared that cells exposed to 5FU partially recovered their ability to migrate toward FCS (80% for 0.25 and 2.5 mg/ml, and 40% for 25 mg/ml) over the 48-day period. The inhibitory effects of MMC on migration were less marked than those of 5FU (Fig. 4B), although still significantly reduced (P < 0.05) compared to controls at day 48.

DISCUSSION

The use of antiproliferative agents in glaucoma filtration surgery has increased the overall success of this procedure. Some high-risk patients still fail surgery even though they received antiproliferatives, suggesting that fibroblasts may still be able to contribute to a scarring response although they cannot proliferate. Whether this is true is unknown. However, if the overall effects of single applications of antiproliferatives on cellular behavior were known, this may allow appropriate adjustment of treatment. The results of this study have demonstrated that fibroblasts, although unable to proliferate, still can perform several crucial aspects of wound-healing behavior, including the expression of growth factors, growth factor receptors, and ECM molecules and the ability to migrate toward a chemotactic stimulus (which is associated with contraction of the ECM).

As illustrated in Figure 5, fibroblast wound-healing behavior is regulated by the actions of external stimuli that include growth factors. Both TGF/β and bFGF were shown in this study to be produced by growth-arrested fibroblasts. Although the reasons for the production of these growth factors are unclear, the production of growth regulatory molecules by control cultures and growth-arrested fibroblasts seen in this study is not an isolated one. The decreasing production of eicosanoids by control cultures of 3T3 fibroblasts as they reached confluence, compared to the increased production of these molecules by MMC growth-arrested 3T3 fibroblasts, has been reported. How 5FU and MMC elicit these effects is unclear, but both agents have been shown to cause DNA damage by inducing single- or double-strand breaks in DNA,
in addition to their other effects.\textsuperscript{20,21} Therefore, a possible mechanism of stimulating growth factor production after exposure to antiproliferatives may involve a sublethal cellular injury or cellular stress response.

Several reports in the literature have suggested that an increase in bFGF production by cells occurs as a result of exposure to various forms of stress, including wounding, hypoxia, and ionizing radiation.\textsuperscript{23–26} Also, bFGF has been shown to be secreted from cells after sublethal injury.\textsuperscript{25} The role of bFGF in this stress response is unclear, although it has been implicated in the protection of cells from hypoxic injury.\textsuperscript{23} A similar role for bFGF in this study is suggested by the upregulation of its production at the earliest time point studied (day 3), followed by a sustained decrease toward control levels. Like bFGF, TGF/β production was upregulated in all groups of growth-arrested cells compared to control cultures, the effects appearing concentration-dependent. These increases in production of TGF/β, like bFGF, may have occurred as a result of sublethal cell injury or stress.

EGF was not detectable at either the mRNA or protein levels in all cultures of Tenon's capsule fibroblasts in this study. The lack of EGF production by these cells may not be surprising because other workers have reported the lack of production of the EGF-related growth factor TGFα by ocular fibroblasts.\textsuperscript{20}

Both TGF/β and bFGF have been shown to stimulate various fibroblast functions, including proliferation, migration, ECM contraction, and ECM synthesis.\textsuperscript{27–30} They also have effects on other cell types involved in the wound-healing process, including endothelial and epithelial cells. TGF/β has also been shown to elicit an autoinductive effect on fibroblasts, resulting in increased production of this factor.\textsuperscript{31} This suggests that growth-arrested fibroblasts may also be able to modulate their own behavior (e.g., stimulation of a number of functions, including growth factor production, migration, proliferation, ECM contraction, and ECM synthesis), as well as that of other cells involved in the healing process. This hypothesis is supported by the fact that addition of exogenous TGF/β has been shown to stimulate the production of TGF/β and ECM RNA by growth-arrested fibroblasts in vitro, as well as reversing the inhibition of scarring in vivo due to MMC after experimental filtration surgery.\textsuperscript{14}

The secretion of growth factors by cells growth-arrested using single exposures to antimetabolites may also stimulate cells on the periphery of the treated area. We know from previous studies that the effects of single sponge treatments are focal, affecting only the fibroblasts in the treated area.\textsuperscript{8} As such, the surrounding peripheral fibroblasts may still be stimulated to carry out cellular activities leading to fibrosis. This is clinically important because some glaucoma filtration blebs treated with single exposures to antiproliferatives have a peripheral ring of scar tissue surrounding the treated area, ultimately resulting in a rise in intraocular pressure despite a lack of scarring in the central treated area.

The expression of growth factor receptors, both at the mRNA and protein levels, appeared to be reduced on exposure to both 5FU and MMC. Although the exact mechanisms underlying this response were not determined in this study, it may be that exposure to these agents reduces receptor numbers by antimetabolic effects on mRNA and subsequent protein production.\textsuperscript{20} Alternatively, this reduction in receptor numbers may be an overall cellular response to DNA damage after exposure to these agents, or it may be due to a combination of DNA damage and antimetabolic effects. Interestingly, the expression of growth factor receptors in control cultures in this study appeared to be in the order of TGF/β receptor > bFGF receptor > EGF receptor. These findings may partially or fully explain the differential potencies of the growth factors TGF/β, bFGF, and EGF on the stimulation of fibroblast functions observed by Khaw et al (manuscript in preparation), who reported a decrease in potency in the order of TGF/β > bFGF > EGF for equimolar concentrations.

Exposure to 5FU or MMC significantly reduced the production of collagen types I and III and fibronectin throughout the 48-day assay period both at the mRNA and protein levels. These results suggest that 5FU and MMC reduce the production of ECM molecules primarily by means of their antimetabolic effects on RNA and subsequent protein synthesis. The biological significance of these findings is unclear. It may be that although the levels of ECM molecules were reduced after growth arrest, compared to controls, their production may still be significant enough to support skin epithelial cell growth in vitro or to contribute to a healing response in vivo.

Therefore, growth-arrested cells appear to be capable of producing growth factors, expressing growth factor receptors, and producing ECM molecules. These findings may partly explain why growth-arrested feeder layers can support skin epithelial cell growth in vitro. This is important because coculture techniques are used to produce a great deal of clinically useful skin epithelial cell cultures, and these techniques are also potentially useful for corneal epithelial culture. Also, because both growth factors and growth factor receptors are involved in the regulation of cellular wound-healing behavior, the production of potentially biologically active growth factors and receptors may also partly explain the scarring that sometimes occurs in vivo after exposure to antiproliferatives. Finally, the production of ECM molecules may have profound effects on the healing response with respect to tissue structure and the regulation of other aspects of
cellular behavior, such as migration and gene expression.\textsuperscript{32-36}

The migratory capacity of fibroblasts exposed to antiproliferatives was reduced compared to controls. Recovery of cellular function during these experiments was similar to previous results for proliferation,\textsuperscript{7} in that the migration of fibroblasts after exposure to 5FU appeared to consist of a lag phase for 7 to 14 days before these cells exhibited a marked increase in migration. Exposure to MMC had similar but less marked effects than 5FU. Other studies have reported conflicting data on the effects of growth arrest on cellular migration. Yamamoto et al\textsuperscript{10} reported that exposure to 5FU or MMC, although causing growth arrest, did not inhibit the ability of rabbit Tenon’s capsule fibroblasts to migrate, using an agarose drop technique. Reports from Hogg et al\textsuperscript{37} suggested that growth arrest induced by exposure to sodium butyrate reduced the ability of human trabecular meshwork cells to migrate. Unlike the Yamamoto study, both the current study and that performed by Hogg et al used migration assays based on modified Boyden chambers (i.e., the ability of cells to migrate through pores in membranes). The conflicting data from the Yamamoto study may be explained either by species or migration assay differences. The current study using single, 5-minute exposures to 5FU and MMC also differed from the continuous exposure to these agents\textsuperscript{10} or continuous exposure to a different growth-arresting agent, sodium butyrate, which also induces cellular differentiation.\textsuperscript{39} Hogg et al used trabecular meshwork cells, not Tenon’s capsule fibroblasts. As such, it is difficult to compare the data from these studies.

It may be that in response to DNA damage, complicated processes such as proliferation and migration, requiring the coordination of several intra- and extracellular mechanisms, are shut down until the cell repairs the damage sustained. However, further investigation is required to elucidate the mechanisms underlying the inhibition of this process. The fact that growth-arrested cells can still exhibit long-term migration may be significant to the healing process in vivo. It may be that these cells eventually can move into and around the wound site and elicit a scarring response by means of other aspects of their healing behavior.

In conclusion, growth-arrested fibroblasts may still be able to contribute either directly or indirectly to a scarring response by their own behavior or by influencing the behavior of surrounding cells. This may help explain why certain patients fail glaucoma surgery after exposure to antiproliferatives. The determination of the actual ability of growth-arrested fibroblasts to interact with other cells may give further insight into the mechanisms underlying the healing process after exposure to antiproliferatives. This area is being actively investigated in our laboratory.

**Key Words**

antiproliferatives, fibroblasts, glaucoma, wound healing

**References**


