Human Serum Reduces Mitomycin-C Cytotoxicity in Human Tenon's Fibroblasts

Jonathan G. Crowston,¹ 2 Xiao Y. Wang,¹ Peng T. Khaw,³ Hans Zoellner,⁴ and Paul R. Healey¹

PURPOSE. To determine the effect of human serum factors on mitomycin-C (MMC) cytotoxicity in cultured human subconjunctival Tenon's capsule fibroblasts.

METHODS. Fibroblast monolayers were treated with 5-minute applications of mitomycin-C (0.4 mg/mL) and incubated in culture medium with or without additional human serum. Fibroblast apoptosis was quantified by direct cell counts based on nuclear morphology, flow cytometry with annexin-V/propidium iodide, and a lactate dehydrogenase release assay. The number of viable fibroblasts and fibroblast proliferation were measured with a colorimetric MTT assay and by bromodeoxyuridine (BrdU) labeling.

RESULTS. Mitomycin-C induced significant levels of fibroblast apoptosis. The addition of human serum resulted in a 40% reduction in MMC-induced fibroblast apoptosis (range, 31.3%-55.3%; P = 0.021) as determined by nuclear morphology and a 32.4% reduction measured by annexin-V/PI. There was a corresponding dose-dependent increase in the number of viable fibroblasts. Serum did not restore proliferation in MMC-treated fibroblasts.

CONCLUSIONS. Factors present in human serum reduce MMC-induced fibroblast apoptosis. The addition of human serum resulted in a 40% reduction in MMC-induced fibroblast apoptosis (range, 31.3%-55.3%; P = 0.021) as determined by nuclear morphology and a 32.4% reduction measured by annexin-V/PI. There was a corresponding dose-dependent increase in the number of viable fibroblasts. Serum did not restore proliferation in MMC-treated fibroblasts.

Several well-characterized risk factors are associated with an increased risk of postoperative scarring after glaucoma filtration surgery.¹–⁴ The mechanisms responsible for promoting scar formation in these high-risk groups are not fully understood.

The wound-healing response after trabeculectomy is unique, in that the wound site is bathed in aqueous humor, which accesses the subconjunctival tissues in a functioning bleb. The influence of aqueous humor on the wound-healing response is determined by its constituents and may either augment or suppress scar formation.⁵ ⁶ A significant increase in aqueous humor flare readings has been reported in eyes up to 4 weeks after trabeculectomy,⁷ indicating breakdown of the blood-aqueous barrier. Constituents of serum therefore have access to the filtration bleb during the postoperative period. Serum factors may also gain access to the bleb as a consequence of intra- or postoperative subconjunctival hemorrhage or from conjunctival hyperemia due to inflammation, chronic irritation, or the long-term application of topical glaucoma medications.

Serum contains a cocktail of growth factors that are essential for the survival and propagation of many cell lines in vitro. Withdrawal of serum from culture medium arrests proliferation and induces apoptotic death in some cells, including certain fibroblast lines.⁸ Primary cultured human Tenon's capsule fibroblasts (HTFs) stop proliferating when serum is excluded from culture medium; however, do not undergo rapid apoptosis.⁹

Intraoperative mitomycin-C (MMC) and 5-fluorouracil (5-FU) are widely used to limit episcleral scar formation after glaucoma filtration surgery. This effect is mediated, at least in part, through a reduction in the number of HTFs at the wound site. The number of fibroblasts may be reduced as a consequence of cell cycle arrest or cell death by apoptosis. Single applications of MMC and 5-FU induce long-term inhibition of proliferation in cultured HTFs. In addition, clinically relevant applications of MMC also induce fibroblast apoptosis.⁹ Although the effects of serum on untreated fibroblasts are well documented, the effects of serum factors on the cytotoxic action of MMC have been less well studied. We have previously demonstrated that fetal calf serum reduced MMC-induced apoptosis of HTFs in vitro.⁵ In addition, transforming growth factor-β has been shown to reduce the antiscarring effect of MMC in a rabbit filtration model, indicating that growth factors present in serum may alter the cytotoxic effect of MMC in vivo.⁶ It is, however, possible that this inhibitory effect is species specific or a property inherent only in fetal serum.¹¹ The effect of adult human serum on MMC cytotoxicity has not been reported previously. Furthermore, identification of individual growth factors that modify the antifibrotic activity of MMC may provide improved means of titrating the cytotoxic effect of the drug.

The purpose of this study, therefore, was to determine whether adult human serum or human growth factors inhibit MMC-mediated cytotoxicity in cultured HTFs.

METHODS

HTF Culture

HTFs were propagated from explanted subconjunctival Tenon's capsule isolated during glaucoma filtration surgery, as described previously.¹² The tenets of the Declaration of Helsinki were observed, and institutional human experimentation committee approval was granted. Ex-

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planted tissue was attached to the bottom of a six-well plate (BD Biosciences, San Jose, CA) with a sterile coverslip and overlaid with Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Poole UK). All culture media were supplemented with L-glutamine 2 mM and penicillin 100,000 U/L (all Invitrogen-Gibco, Oxbridge, UK). For expansion of the number of fibroblasts, the media were also supplemented with fetal calf serum (FCS; 10% of final volume; Invitrogen-Gibco). Once the monolayers had reached confluence, the fibroblasts were passaged and subcultured in 175 cm² tissue culture flasks.

Mitomycin-C Treatment of Fibroblast Monolayers

HTFs were seeded into multiwell tissue culture plates (BD Biosciences) and incubated overnight to allow attachment. The fibroblast monolayers were then washed and treated with a single application of MMC (0.4 ng/mL for 5 minutes; Kyowa Hakko Kogyo Ltd.), as described previously.6 MMC was reconstituted in phosphate-buffered saline (PBS; Sigma-Aldrich). No serum or additional protein was added to the MMC at the time of treatment. Control fibroblasts were treated with a 5-minute application of serum and protein-free PBS. Immediately after treatment, the monolayers were washed three times with PBS and incubated in DMEM, with or without supplemented human serum (10% total volume).

Preparation of Human Serum

Human peripheral venous blood of healthy volunteers was collected by heparin-coated syringe, pooled, and centrifuged at 3000 rpm for 10 minutes. The serum layer was then separated and filtered with a 0.2-μm pore size filter (Nalgene Labware, Rochester, NY). Serum was heat-inactivated in a water bath at 56°C for 1 hour. To provide a source of protein for serum-free culture medium, bovine serum albumin was added at a final concentration of 0.1%.

Quantifying Fibroblast Apoptosis

Nuclear Morphology. HTFs were harvested by trypsinization at intervals after treatment with MMC, as described previously.15 Cyto- spin preparations were made from a combination of the trypsinized monolayer, supernatant, and washings, to ensure that all cells were captured. The preparations were air dried overnight and stained with a Giemsa-like stain (DiffQuik; Baxter Diagnostics AG, Düdingen, Switzerland). The percentage of cells displaying features of apoptosis was determined by direct cell counts, obtained by light microscopy as described previously.15 Three hundred fibroblasts were counted for each cytospin from randomly selected fields at ×40 magnification. The number of viable cells at each time point was estimated before trypsinization by counting the number of attached cells in the monolayer from 5-randomly selected fields (at ×200 magnification) of the phase-contrast microscopy.

MTT Assay. This colorimetric assay quantifies the number of metabolically active cells based on the cleavage of the yellow tetrazolium salt MTT (5-[3-(4,5-dimethylthiazol-2-yI)] 2,5-diphenyltetrazolium bromide) to purple formazan crystals. The assay was performed according to the manufacturer’s instructions. MMC-treated fibroblasts in 96-well plates were incubated for 4 hours after 10 μL MTT labeling reagent was added. Solubilization solution was then added (100 μL) and incubated at 37°C, 5% CO₂ overnight. Absorbance from the resultant colored solution was then measured with a microplate spectrophotometer at 570 nm.

Lactate Dehydrogenase Release Assay. Cells dying by either apoptosis or necrosis release lactate dehydrogenase (LDH) into the supernatant. An LDH release assay (Roche Diagnostics, Philadelphia, PA) was performed according to the manufacturer’s instructions. As serum contains inherent LDH activity, this assay could not be used to evaluate the effect of whole serum on MMC-induced fibroblast death, but was used to determine the effect of individual growth factors on MMC-induced cell death (Table 1). Briefly, HTFs previously seeded into 48-well culture plates (BD Biosciences, Franklin Lakes, NJ) were treated with a single 5-minute application of MMC, washed three times in PBS, and incubated in 400 μL phenol red-free DMEM with 0.1% bovine serum albumin (BSA; Sigma-Aldrich). For analysis, 100 μL of supernatant was extracted from each well and placed into separate wells of a 96-well plate. To this, 100 μL of catalyst solution (at 37°C) was then added to each well and incubated at 37°C for 15 minutes. Absorbance was measured with a microtiter plate reader with a 490- to 492-nm filter.

Bromodeoxyuridine Assay for Fibroblast Proliferation. Fibroblast proliferation was measured with a 5-bromo-2-deoxyuridine (BrdU) assay that quantitates BrdU uptake into newly synthesized DNA of replicating cells. HTFs (1 × 10⁶) were plated into separate wells of a 24-well microtiter plate and incubated with BrdU on days 2, 3, and 7 after treatment. BrdU labeling was detected by immunohistochemistry with a BrdU labeling and detection kit (Kit II; Roche Diagnostics). Five hundred to 1000 cells were randomly counted in each well. The BrdU labeling index was calculated as the percentage of BrdU-positive cells.

Flow Cytometry. Flow cytometry was performed (FACStar plus; BD Biosciences) with a 100-nW 488 argon laser light source. Light was filtered with an FL-1 filter at 520 ± 20 nm or FL-2 (580 ± 20 nm). Acquisition and analysis was performed on computer (Lysis II software; BD Biosciences). Attached fibroblasts were detached with trypsin and collected together with fibroblasts in the supernatant and washings, centrifuged at 1000 rpm for 5-minutes, and resuspended in 100 μL PBS. At least 5000 cells were analyzed from each specimen. Triplicate specimens were prepared per treatment group. Viable cells were gated according to forward- and side-scatter profiles. Markers were set on histograms plotting fluorescence intensity against cell counts (WinMDI software ver. 1.3.3; Windows 3.1 Multiple Document Interface Flow Cytometry application; developed by Joe Trotter and provided in the public domain by the Scripps Research Institute, La Jolla, CA; available at facs.scripps.edu/software.html).

Annexin-V Propidium Iodide. Annexin-V staining and propidium iodide uptake were determined in nonpermeabilized cells (Apoptosis Detection Kit; R&D Systems) according to the manufacturer’s instructions. HTFs were treated with MMC 0.4 mg/mL or PBS for 5 minutes. For analysis, fibroblasts were harvested by trypsinization, and 1 × 10⁶ cells were resuspended in 100 μL of binding buffer containing 5 μL annexin-V FITC and 5 μL propidium iodide and kept in the dark at room temperature for 15 minutes. Binding buffer (400 μL) was then added, and samples were analyzed immediately by flow cytometry. Scatter profiles for fluorescence intensities in FL-1 (annexin-V) and FL-2 (propidium iodide) were plotted. The distribution of viable, necrotic and apoptotic fibroblasts is shown (Fig. 1). Viable cells exclude propidium iodide and are annexin-V negative. Apoptotic cells exclude propidium iodide and label with annexin-V. Necrotic cells are positive for both. Quadrants were set to differentiate positive and negative staining. The percentage of cells in each quadrant was determined on computer (Lysis-II software; BD Biosciences). Fibroblasts labeled with 5 μL of an irrelevant (anti-CD45-RB-FITC) isotype control antibody were used as the negative control. Apoptotic lymphocytes from an IL-2-dependent T-cell line deprived of IL-2 for 24 hours served as the positive control for apoptotic cells (data not shown).

Table 1. Growth Factors Used in Initial Screening

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Concentration (ng/mL)</th>
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</thead>
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<tr>
<td>Platelet-derived growth factor-AB (PDGF)</td>
<td>3.0</td>
</tr>
<tr>
<td>Insulin-like growth factor-1 (IGF-1)</td>
<td>3.0</td>
</tr>
<tr>
<td>Transforming growth factor-β1</td>
<td>3.0</td>
</tr>
<tr>
<td>Transforming growth factor-κ2</td>
<td>2.5</td>
</tr>
<tr>
<td>Interleukin-2 (IL-2)</td>
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</tr>
<tr>
<td>Interleukin-4 (IL-4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Tumor necrosis factor-α (TNF-α)</td>
<td>2.5</td>
</tr>
<tr>
<td>Interferon-γ (IFN-γ)</td>
<td>3.0</td>
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</tbody>
</table>

Reduction of Mitomycin-C Cytotoxicity in Tenon’s Fibroblasts
Statistical Analysis

Data were analyzed on computer (JMP ver. 5.1; SAS, Cary, NC). Comparison of means was performed by Student’s t-test. Multiple comparisons were performed by ANOVA, with appropriate post hoc corrections when necessary.

RESULTS

Mitomycin-C-Induced Fibroblast Apoptosis

Nuclear Morphology. Evaluation of cytospin preparations generated 72-hours after exposure to MMC revealed nuclear changes characteristic of apoptosis (Fig. 2A). These included condensation of nuclear chromatin with occasional nuclear fragmentation and are similar to those described in a previous report that demonstrated apoptosis as the prime mechanism of fibroblast death after exposure to MMC, according to several different apoptosis assays.9 Human serum significantly reduced the percentage of cells with apoptotic morphology (P = 0.021, Student’s t-test) with a mean reduction of 40.6% (range, 31.3%–55.3%; Fig. 2B).

Annexin-V–Propidium Iodide Labeling. Viable cells exclude propidium iodide and do not bind annexin-V. Apoptotic and necrotic cells externalize phosphatidylserine on the plasma membrane and are labeled with annexin-V. Apoptotic cells with intact membranes exclude propidium iodide but label with annexin-V (bottom right). Necrotic cells lose membrane integrity and therefore take up propidium iodide and remain annexin-V positive (top right).

Effect of Human Serum on Viable HTFs after MMC

A dose-dependent increase in the number of viable fibroblasts was observed in MMC-treated fibroblasts when human serum was added to the culture medium immediately after treatment (Fig. 4). A similar number of viable fibroblasts were present in the MMC-treated wells containing 25% human serum and the untreated serum-deprived fibroblast (P = 0.39, Student’s t-test).

FIGURE 1. Forward- and side-scatter profile associated with annexin-V labeling and plasma membrane permeability in viable, apoptotic, and necrotic fibroblasts. Viable cells exclude propidium iodide and do not label with annexin-V (bottom left). Apoptotic cells with intact plasma membranes exclude propidium iodide but label with annexin-V (bottom right). Necrotic cells lose membrane integrity and therefore take up propidium iodide and remain annexin-V positive (top right).

FIGURE 2. (A) Light microscopy of MMC-treated human Tenon’s fibroblasts. Apoptotic cells (*) display condensation of nuclear chromatin, a characteristic feature of apoptosis. (B) The percentage of fibroblasts with apoptotic nuclear morphology 72 hours after a single application of mitomycin-C (0.4 mg/mL) or phosphate buffered saline (control). Fibroblasts were maintained in culture medium supplemented with 10% human serum or in serum-free medium supplemented with 0.1 mg/mL of BSA. The data shows the mean ± SD from a representative of three similar experiments (n = 4 per treatment group).
Effect of Serum on Fibroblast Proliferation

Control fibroblasts maintained in serum had high levels of proliferation, which was highest at 48 hours (30.2% ± 1.6%; Table 2) and decreased with time as the monolayer reached confluence and fibroblasts became contact inhibited. No proliferation was observed in MMC-treated fibroblasts that were maintained in serum-free medium. A small number of BrdU-positive cells were observed at 48 hours in MMC-treated fibroblasts cultured in serum-containing medium (5.0% ± 1.2%, Fig. 5). No proliferation was evident at 72 or 168 hours.

Effect of Individual Growth Factors on Fibroblast Apoptosis

Our purpose was to identify individual growth factors that reproduce the effect of human serum. Initial screening was performed with growth factor concentrations between 1 and 5 ng/mL. Initial pilot experiments suggested that PDGF-AB and IGF-1 might inhibit apoptosis, whereas TGF-β2 augmented

<table>
<thead>
<tr>
<th>Timepoint (h)</th>
<th>Control-SF</th>
<th>Control-HS</th>
<th>MMC-SF</th>
<th>MMC-HS</th>
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<tbody>
<tr>
<td>48</td>
<td>0.94 ± 0.56</td>
<td>30.2 ± 1.6</td>
<td>0</td>
<td>5.0 ± 1.2</td>
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<tr>
<td>72</td>
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<td>20.3 ± 5.4</td>
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<tr>
<td>168</td>
<td>1.04 ± 0.32</td>
<td>0.9 ± 0.8</td>
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Percentage of positive BrdU labeled HTFs. (mean ± SEM; >500 randomly selected cells counted per well; n = 4).
MMC-induced apoptosis. The other growth factors tested had no effect on fibroblast apoptosis at the dose tested (data not shown). Subsequent experiments were performed to evaluate the effect of TGF-β2, PDGF-AB, and IGF-1 over a wider range of concentrations with the LDH release assay. No single growth factor afforded significant inhibition of MMC-induced apoptosis at the concentrations tested (Fig. 6).

**DISCUSSION**

Intraoperative application of MMC has a profound inhibitory effect on postoperative scar formation after trabeculectomy. There is currently no consensus regarding the optimum concentration or treatment time for MMC application during trabeculectomy, perhaps because of the wide variation in the scarring response between different patient risk groups, as well as a potential variation in the response to antifibrosis treatment. The contents of aqueous humor may account for some of this variation. These data demonstrated that factors present in human serum significantly increased fibroblast survival after exposure to MMC. This effect was a direct result of serum-mediated inhibition of fibroblast apoptosis, as human serum had little effect on reversing MMC inhibition of fibroblast proliferation. Serum factors gain access to the filtration bleb as a consequence of intraoperative hemorrhage and/or breakdown of the blood aqueous barrier during and immediately after trabeculectomy and may therefore modulate MMC cytotoxicity in vivo.

Figure 5. BrdU-labeled proliferating Tenon’s fibroblasts. Fibroblasts were treated with MMC or PBS (control) for 3 minutes. Images are representatives of day 2 of culture. Arrows: BrdU-positive nuclei. Magnification, ×20.

Single 5-minute applications of MMC inhibits HTF proliferation at a concentration of 0.1 mg/mL. Treatment strategies used to inhibit scarring in clinical practice usually exceed the doses necessary to inhibit fibroblast proliferation in vitro which suggests that inhibition of fibroblast proliferation per se is not sufficient to exert clinically significant reductions in scar formation in many patients, that the wound environment in vivo reduces the efficacy of antiscarring agents, or that the pharmacokinetics of MMC in vivo are not the same as in vivo. Growth-arrested fibroblasts treated with sublethal doses of MMC and 5-fluorouracil are still capable of contributing to the scarring response by maintaining their ability to migrate as well as secrete growth factors and lay down extracellular matrix. Effective long-term inhibition of scarring, particularly in the presence of persistent profibrotic stimuli, may therefore require fibroblast clearance through the induction of fibroblast apoptosis. The price of this apoptosis is hypocellular, avascular drainage blebs that may have an increased risk of bleb leak, infection, and persistent hypotony.

The trabeculectomy bleb is bathed in aqueous humor, which contains a large number of growth factors. The constituents of aqueous humor not only influence postoperative scar formation per se, they may also affect the fibroblast response to MMC. An early observation in the course of the study was that MMC induced increased apoptosis if fibroblasts were maintained in serum-free medium after treatment. Further investigation revealed that fetal calf serum significantly inhibited MMC-induced apoptosis. Serum contains a cocktail of growth factors that can have profound effects on HTF activity. Patients in the high-risk categories for scarring after trabeculectomy frequently have disrupted blood–aqueous barriers, where the constituents of blood (serum) leak into aqueous humor. The factors in serum that inhibit apoptosis therefore may be relevant in vivo. Several growth factors, particularly insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) have been shown to inhibit apoptosis in fibroblasts. Furthermore, the addition of exogenous TGF-β to rabbit and mouse blebs reversed the antiscarring effect of MMC. We therefore attempted to identify individual factors that would reproduce the inhibitory effect of serum. Identification of single agents that alter the response to treatment has two potential applications: First, measurement of aqueous or bleb growth factor levels pre- or perioperatively may provide quantitative means for better predicting an individual’s response to surgery. Second, the addition of growth factor to the wound site, or the inhibition of a specific growth factor may permit postoperative manipulation of the scarring response. The growth factor concentration range was chosen on the basis of reported normal serum concentrations in humans (IGF23 100–500 ng/mL, PDGF24 15–20 ng/mL. TGF-β25
induced apoptosis in rat fibroblasts and Chodon et al. re-
IGF-1 (100 ng/mL) and PDGF (10 ng/mL) inhibited c-Myc-
It is possible that the effects of serum are derived from
The growth factor profiles of human or commercially avail-
able animal sera have, to our knowledge, not been evaluated. It is likely that the aqueous humor of patients with disrupted
The synergistic action of more than one growth factor, are
induced by growth factor concentrations outside the range
tested or that inhibition may be derived from an as yet untested
growth factor. The absence of an inhibitory effect by TGF-β is
contrary to that observed in vivo in rabbits and mice. This
may reflect limitations of our cell culture system, which does
not allow for possible secondary effects of TGF-β on other cell
types, including macrophages and lymphocytes, that may alter
the susceptibility of fibroblasts to MMC-induced death.27

The growth factor profiles of human or commercially avail-
1–10 ng/mL). Growth factor levels present in 10% serum there-
fore lay well within the chosen range. In addition, previous
reports have demonstrated growth factor-mediated inhibition
of apoptosis within this range. Harrington et al.19 reported that
IGF-1 (100 ng/mL) and PDGF (10 ng/mL) inhibited c-Myc-
induced apoptosis in rat fibroblasts and Chodon et al.26 re-
port that TGF-β inhibited Fas-induced apoptosis in vivo at
concentrations between 5 and 20 ng/mL. None of the growth
factors tested replicated the effect of whole serum and signif-
ically inhibited MMC-induced apoptosis at the concentrations
tested. It is possible that the effects of serum are derived from

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