Antimetabolite-Induced Apoptosis in Tenon’s Capsule Fibroblasts

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PURPOSE. To determine whether treatment with mitomycin-C and 5-fluorouracil induces apoptotic death in cultured subconjunctival fibroblasts.

METHODS. Cultured human subconjunctival Tenon’s capsule fibroblasts were exposed to 5-minute applications of mitomycin-C (up to 1 mg/ml) or 5-fluorouracil (up to 50 mg/ml) or phosphate-buffered saline solution (PBS). Fibroblast apoptosis was determined by cell morphology, apoptosis-specific protein expression, and DNA fragmentation by TdT-mediated dUTP nick-end labeling (TUNEL). In addition, apoptosis was quantified by direct cell counts based on morphology or lactate dehydrogenase release.

RESULTS. Morphologic changes characteristic of apoptosis included nuclear and cytoplasmic condensation and occasional nuclear fragmentation while the plasma membrane remained intact. Apoptosis-specific protein expression and DNA fragmentation was observed in fibroblasts 48 hours after mitomycin-C treatment but not in control PBS-treated fibroblasts. The amount of apoptosis induced was dose dependent and partially inhibited by the addition of fetal calf serum to growth medium immediately after treatment.

CONCLUSIONS. Mitomycin-C and high-dose 5-fluorouracil induce apoptosis in cultured Tenon’s fibroblasts. Mitomycin-C-induced apoptosis is inhibited by fetal calf serum, indicating that exogenous factors influence the susceptibility of a fibroblast population to apoptosis. The induction and regulation of fibroblast apoptosis provides a novel target for the potential regulation of scarring.

Scarring at the level of the subconjunctival Tenon’s capsule is a major cause of surgical failure after trabeculectomy. Single intraoperative applications of mitomycin-C (MTC) and 5-fluorouracil (5-FU) are widely used in patients at high risk for excessive scar formation. The exact mode of action by which these agents exert their antiscarring effects, however, remains unclear.

Recent evidence suggests that the reduction in fibroblast number that accompanies resolution of the wound healing response is mediated by apoptosis. Apoptosis is a genetically directed process whereby a cell induces a death program, effectively committing suicide. Apoptotic cells display characteristic morphology, which includes condensation of the nucleus and cytoplasm, nuclear fragmentation, and cytoplasmic blebbing with an intact cell membrane. This differs from necrotic cell death, in which disruption to the cell membrane results in cellular enlargement, swelling of organelles, and the spillage of cell contents into the extracellular fluid.

Mitomycin-C and 5-FU are widely used as chemotherapeutic agents because of their capacity to induce apoptosis in malignant cells. To date, investigation into the mechanism of MTC and 5-FU activity in the conjunctiva has focused on the aspects of fibroblast function, such as proliferation, migration, and contraction.

1. Only a limited number of studies have specifically addressed the cytotoxic effects of MTC in relation to conjunctival wound healing. Jampel found no evidence of drug-induced cell death in cultured Tenon’s fibroblasts 24 hours after treatment with MTC and 5-FU at clinically used doses.

2. Smith et al., however, demonstrated that MTC and 5-FU induce cell death in transformed 3T3 fibroblasts, but only MTC was toxic to vascular endothelial cells. In addition, Occleston et al. reported that MTC was cytotoxic to Tenon’s fibroblasts seeded in collagen lattices. Histologic examination of MTC-treated drainage blebs revealed that the tissue overlying the area of MTC treatment was virtually devoid of fibroblasts. This observation implies that, in these blebs, MTC induced fibroblast death rather than purely inhibiting fibroblast proliferation.

Unlike passive necrotic cell death, the response of a given cell to an apoptotic stimulus can be altered by the addition of exogenous agents. Fibroblast apoptosis induced by dysregulated c-myc expression was inhibited by the addition of growth factors to the growth medium, such as insulin-like growth factor and platelet-derived growth factor. This is particularly interesting in light of observations from our laboratory that the addition of exogenous transforming growth factor-β to drainage blebs in rabbits reverses the antiscarring effects induced by MTC. We hypothesize that MTC and 5-FU are effective, in part, because they induce Tenon’s fibroblast apoptosis. The aim of this study, therefore, was to determine whether clinically relevant doses of MTC and 5-FU have the potential to induce apoptosis in cultured Tenon’s fibroblasts and to investigate whether this process can be modulated by exogenous factors.

METHODS

A primary cell line of human Tenon’s capsule fibroblasts (HTF) was established from subconjunctival Tenon’s isolated from patients during surgery as previously described. The tenets of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted. Fibroblasts were cultured at 37°C in 5% CO2 in Dulbecco modified Eagle’s medium (Sigma, Poole, England) supplemented with l-glutamine 2 mM (Gibco-BRL, Paisley, Scotland), penicil-
FIGURE 1. Human Tenon’s capsule fibroblasts 72 hours after treatment with phosphate-buffered saline (left column) or MTC (0.4 mg/ml) (right column). Evidence for apoptotic death was determined on cytospin preparations by (A) light microscopic examination; (B) transmission electron microscopic examination; (C) propidium iodide expression (red) and apoptosis-specific protein (ASP) expression (green); and (D) TUNEL. Apoptotic fibroblasts display nuclear condensation and/or fragmentation (arrows), cytoplasmic condensation, and intact plasma membrane. Apoptotic fibroblasts with nuclear changes detected with propidium iodide also express cytoplasmic ASP. The TUNEL assay provides evidence of DNA fragmentation in fibroblasts undergoing apoptosis. TUNEL-positive nuclei (brown) are clearly distinguishable (arrows) from the nuclei of viable fibroblasts stained with Mayer’s blue.
lin 100,000 U/l (Gibco-BRL), and fetal calf serum (FCS) at a final concentration of 10% (Gibco-BRL). Fibroblasts for all experiments were used between passages 2 and 6 and in the exponential growth phase. Each experiment was performed on at least three separate occasions with at least three replicates for each group under investigation.

Morphology
Tenon's fibroblasts were seeded into 24-well plates (Becton Dickinson, Mountain View, CA) and incubated overnight. The fibroblast monolayers were then washed and treated with a single application of MTC (0.4 mg/ml; Kyowa Hakko Kogyo, Japan) or 5-FU (25 mg/ml; Solopak Laboratories, Elk Grove Village, IL) for 5 minutes. Antimetabolites were diluted in phosphate-buffered saline (PBS). Control fibroblasts were treated with a 5-minute application of PBS. After treatment, cells were washed three times with 500 µl PBS and incubated in Dulbecco modified Eagle's medium. At intervals, supernatant and fibroblasts were collected from the wells by trypsinization and attached to glass slides by cytocentrifugation. The preparations were air dried overnight and stained with a Giemsa-like stain (Diff-Quik; Baxter Diagnostics, Duedingen, Switzerland). The percentage of cells displaying features of apoptosis was ascertained by light microscopic examination. The percentage was calculated as the ratio of fibroblasts with apoptotic morphology to morphologically normal fibroblasts in at least 300 fibroblasts from randomly selected fields at ×40 magnification. The viable cell number at each time point was estimated before trypsinization by counting the number of attached cells in the monolayer from five randomly selected fields (×40) of the phase-contrast microscope.

Inhibition of Mitomycin-C-Induced Apoptosis
HTF seeded in 24-well plates (Becton Dickinson) were treated with single 5-minute applications of mitomycin-C (0.4 mg/ml) or PBS as described above. After the third wash, the monolayers were incubated in growth medium ± 10% FCS (Gibco-BRL). The percentage apoptosis at 48 hours was assessed from cytospin preparations as described above. To assess the effect of growth factors on MTC-induced apoptosis, individual growth factors were added to serum free growth medium immediately after the final wash.

Transmission Electron Microscopic Examination
The monolayers and supernatants of HTF treated 72 hours previously with either MTC or PBS were collected; fixed in 3% gluteraldehyde, 1% paraformaldehyde in 0.07 M sodium cacodylate hydrochloride; and postfixed in osmium tetroxide. Specimens were washed, dehydrated through alcohols, and infiltrated with a 1:1 mixture of propylene oxide:araldite. Sections, 50 nm thick, were cut, anchored with 1% uranyl acetate and lead citrate, and examined in using a transmission electron microscopic (Model 1010 JEOL, London, England) examination at 80 kV.

Apoptosis-Specific Protein and Propidium Iodide
Apoptosis-specific protein is a cytoplasmic protein associated with the cytoskeleton, which shares homology with c-jun. Apoptosis-specific protein is expressed in cells undergoing apoptotic but not necrotic cell death.10 Cytospin preparations of HTF treated 48 hours earlier with MTC (0.4 mg/ml) for 5 minutes were air dried and fixed with 100% acetone at room temperature for 10 minutes. Nonspecific binding was blocked with 3% bovine serum albumin (Sigma) in PBS. Antiapoptosis-specific protein antibody (c-JUN/AP-1, Ab-2; Calbiochem, La Jolla, CA) was applied at a 1/20 dilution in PBS for 30 minutes. A goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FIT-c) was used for the second layer at 1/20 for 30 minutes. After this, the fibroblasts were covered with propidium iodide/RNase (Sigma) at a 1/10 dilution in PBS for 15 minutes.

TdT-Mediated dUTP Nick-end Labeling
Immunohistochemical evidence for DNA strand breaks was obtained using TdT-mediated dUTP nick-end labeling (TUNEL) (In-situ Cell Death Detection Kit-POD; Boehringer Mannheim, Philadelphia, PA). Fluorescein-labeled nucleotides are polymer-
ized by deoxyribosenucleotide triphosphate (TdT) in a template-independent manner to the 3'-OH ends of single- or double-DNA strand breaks. Incorporated fluorescein is detected by antifluorescein antibody–Fab fragments, which are conjugated to horseradish peroxidase. The assay was performed in accordance with the manufacturer’s guidelines. Briefly, cytospin preparations of MTC-treated and untreated control HTF were fixed in 4% paraformaldehyde for 30 minutes at room temperature. Intrinsic peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes. The fibroblasts were permeabilized with 0.1% Triton-X (Rohm & Haas, Philadelphia, PA) in 0.1% sodium citrate on ice for 2 minutes. The fibroblasts were incubated with the TUNEL reaction mixture consisting of TdT and modified nucleotides for 60 minutes at 37°C. TdT was omitted from the reaction mixture in the negative controls. The slides were washed for 15 minutes in PBS, and antifluorescein antibody conjugated with POD was added for 30 minutes at 37°C. Finally, 50 μl diaminobenzidine (Sigma) substrate solution was added for 10 minutes at room temperature. A 10-second immersion in Mayer’s hemalum (Merck, Lutterworth, UK) provided background staining. Cytospin preparations of interleukin-2-deprived peripheral blood monocytes were used as a positive control.

**Lactate Dehydrogenase Release**

A lactate dehydrogenase (LDH) release assay was used to quantify fibroblast apoptosis to verify the results obtained by assessing fibroblast morphology. Dead cells in culture release LDH into the supernatant. The amount of LDH activity can be quantified by the addition of a colorless tetrazolium salt that is reduced to colored formazan.

The amount of formazan produced can be quantified with an enzyme-linked immunosorbent assay plate reader. LDH was measured in the supernatant of MTC- and 5-FU-treated HTF using a cytotoxicity detection kit (Boehringer Mannheim) according to the manufacturer’s guidelines. Briefly, HTF previously seeded into 48-well plates (Becton Dickinson) were treated with a single 5-minute application of MTC or 5-FU as described above, washed three times in PBS, and incubated in 400 μl phenol red-free Dulbecco modified Eagle’s medium with 1% bovine serum albumin (Sigma). After a 48-hour incubation, 100 μl supernatant was extracted from each well and placed into separate wells of a new 96-well plate. Catalyst solution (100 μl at 37°C) was then added to each well and incubated for 15 minutes. Absorbance was measured with a microtiter plate reader with a 490- to 492-nm filter. A standard curve was obtained by plating HTF into 48-well plates at various seeding densities from 2500 to 40,000 per 24-well plates. The HTF were lysed with 2% Triton-X in Dulbecco modified Eagle’s medium, and 100 μl was added to 100 μl catalyst solution for 15 to 30 minutes and read on the microtiter plate reader. To assess the relationship between the two quantification assays, we assessed the effect of MTC dose on a population of HTF and analyzed the amount of apoptosis induced at 72 hours using both techniques.

**RESULTS**

**Morphology**

Morphologic evidence of apoptosis characterized by nuclear and cytoplasmic condensation and occasional nuclear fragmentation was seen after treatment with MTC and 5-FU (Fig. 1A). Figure 2 shows the percentage of apoptosis induced after
treatment and the corresponding viable cell number at the same time points in one experiment that was representative of three experiments. A decrease in viable cell number correlates with an increase in the percentage of apoptosis. MTC induces apoptosis in a linear dose-dependent fashion. 5-FU was less toxic than MTC, but apoptosis was consistently observed after treatment with 50 mg/ml (Fig. 3).

Fibroblasts treated as above and subsequently incubated in growth medium with FCS (10%) subsequently underwent less apoptosis than fibroblasts incubated in a growth medium with no FCS (P < 0.05, Student's t-test). FCS inhibited MTC-induced apoptosis at concentrations below 20% (Fig. 4). A further increase in serum concentration had no inhibitory effect on MTC-induced apoptosis. These findings suggest that the susceptibility of a fibroblast population to MTC-induced apoptosis is modulated by the addition of exogenous agents.

Electron Microscopic Examination

Electron microscopic examination of HTF, treated with either a single 5-minute application of MTC (0.4 mg/ml) or PBS and subsequently incubated in serum-free growth medium, revealed morphologic changes that were characteristic of apoptosis. The plasma membrane was intact, and there was marked condensation of the nuclear chromatin (Fig. 1B). The cytoplasm appeared electron dense in some fibroblasts; in others, there were accumulations of intracytoplasmic membrane-bound vesicles containing organelles that appeared to bud from the cell membrane and become externalized, leaving a cytoplasm devoid of organelles (not shown). These changes were not observed in the PBS-treated control population.

Apoptosis-Specific Protein and Propidium Iodide

Staining, positive for apoptosis-specific protein, was seen in the cytoplasm of a proportion of fibroblasts 48 hours after treatment with MTC (0.4 mg/ml). Propidium iodide labeling of the fibroblast nuclei clearly demonstrated nuclear changes in some, but not all, fibroblasts showing positive staining for apoptosis-specific protein. These changes included condensation and fragmentation of the fibroblast nucleus.

LDH Release

The standard curve for LDH released after Triton-X-mediated lysis of varying numbers of HTF in 24-well plates revealed a linear relationship between fibroblast number and LDH release (r² = 0.97). Analysis of the same population of MTC-treated fibroblasts revealed a close correlation between MTC dose and percentage of apoptosis using both techniques. LDH is released from a cell after disruption of the cell membrane. In our system, this may have resulted from primary necrosis induced by MTC or by secondary necrosis, which occurs in apoptotic cells if they are not cleared by apoptosis. Direct cell membrane damage by MTC would result in LDH release at or shortly after the time of treatment. No significant difference was found in LDH levels between MTC-treated and untreated HTF at 3 hours after treatment (data not shown). LDH release, therefore, occurs more than 3 hours after treatment. In addition, time-lapse videomicroscopic examination revealed that apoptotic HTF are not phagocytosed by neighboring viable HTF after MTC treatment. In the absence of phagocytosis, apoptotic cells undergo further degradation by secondary necrosis.

DNA Fragmentation

Evidence for DNA fragmentation was obtained using TUNEL in HTF treated 48 hours earlier with MTC. No DNA fragmentation was detected in the PBS-treated negative controls at 72 hours.

DISCUSSION

In this study we present evidence that MTC and 5-FU, at clinically relevant doses, induce apoptotic cell death in Tenon's fibroblasts in vitro. Apoptotic fibroblasts display cytoplasmic and nuclear condensation, nuclear fragmentation, and an intact plasma membrane. Fibroblasts deemed apoptotic by nuclear morphology express apoptosis-specific protein. This cytoplasmic protein is expressed by cells undergoing apoptosis but not primary necrosis. In addition, evidence of DNA fragmentation was obtained in MTC-treated fibroblasts using TUNEL.
Fibroblast apoptosis was quantified by two techniques. The ratio of apoptotic to viable fibroblasts was counted from cytospin specimens and by measuring LDH release in the supernatant. Using both techniques, we have shown that MTC is more potent than 5-FU at inducing fibroblast apoptosis. Although the exact mechanism by which these agents induce apoptosis is unclear, a possible explanation for this may lie in their different modes of action. MTC damages DNA by cross-linking bases in the same or adjacent strands of DNA. This effect is independent of the cell cycle. DNA damage is a powerful stimulus for apoptosis. 5-FU is a thymidilate synthetase inhibitor and prevents the synthesis of DNA. 5-FU induces less direct DNA damage than MTC, and its effects are cycle specific and mediated primarily on proliferating cells.

MTC-induced apoptosis was inhibited by the addition of FCS to the growth medium. This suggests that drug-induced apoptosis can be regulated. To identify factors that mediate this inhibition, we investigated a panel of growth factors constituent in FCS and also present at sites of inflammation, at sites of wound healing, or in aqueous humor. To date, we have been unable to identify a single growth factor that reproduces the significant inhibition of MTC-induced fibroblast apoptosis observed with FCS. The panel of growth factors tested included transforming growth factor-beta, insulin-like growth factor-1, platelet-derived growth factor-alpha, tumor necrosis factor-alpha, and interleukins (IL-2, IL-4, IL-7). The identification of factors that regulate the response of fibroblasts to the cytotoxic effects of antimitabolites may enable more accurate prediction of treatment outcome and may reduce the incidence of complications that can arise in devitalized blebs.

Fibroblast apoptosis mediates the reduction in fibroblast number that accompanies resolution of the normal wound healing response. We have presented evidence that MTC and 5-FU have the capacity to induce apoptosis in cultured Tenon's capsule fibroblasts. It is, therefore, possible that these agents exert their antiscarring effect not only by inhibiting fibroblast proliferation but also through the induction of fibroblast apoptosis. It is interesting to speculate whether some of these agents may be accelerating the resolution phase of the wound healing. We believe that the investigation of fibroblast apoptosis provides a novel approach for the understanding and potential regulation of the wound healing process.

References


