The Effects of D-penicillamine and Daunorubicin on Conjunctival Fibroblast Proliferation and Collagen Synthesis

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Two drugs, D-penicillamine and daunorubicin, were tested for their effect on proliferation and collagen synthesis of cultured conjunctival fibroblasts. This cell type is likely responsible for scar formation and ultimate filter surgery failure in glaucoma patients. Both drugs were antiproliferative; however, D-penicillamine required 2000 times the concentration of daunorubicin to achieve a similar degree of inhibition. D-penicillamine had a more consistent effect on intracellular collagen synthesis than daunorubicin at the doses tested. In contrast, at concentrations of daunorubicin where all proliferation ceased, intracellular production and extracellular transport of collagen were maintained. Invest Ophthalmol Vis Sci 29:112-118, 1988

Filtering surgery for medically unresponsive glaucoma fails in approximately 10% of patients. Fibroblast proliferation occurs following surgery and can lead to scarring that closes the scleral filtering site. The probable source of fibroblasts is from Tenon's capsule, the fibrous inner portion of the conjunctiva.

An antiproliferative agent, 5-fluorouracil, retards cellular proliferation when injected subconjunctivally or intravitreally. 5-fluorouracil has been shown to prolong filter patency in primates and seems to reduce the risk of filter failure in glaucoma patients if repeated subconjunctival injections are given. 5-fluorouracil has corneal epithelial toxicity, however, and its present regimen of daily injections for 2 weeks is potentially dangerous and impractical.

In searching for other agents to prevent or retard the scarring that occurs after filtering surgery it was decided to experiment with a collagen crosslinking inhibitor, D-penicillamine, which acts differently from an antimitotic agent, and to study a second antiproliferative agent which was potentially more potent than 5-fluorouracil. If both agents exerted different effects at the cellular level on proliferation and collagen synthesis, then perhaps the two could be used in conjunction to prevent scarring at the surgical sites. It is anticipated that the combined effect would enhance the effect of any singular agent.

D-penicillamine has been shown to inhibit collagen fibril formation by binding copper anions which are needed as catalysts for lysyl oxidase. The drug has also been shown to inhibit T-cell rosette formation and the production of IgM antibodies. It was postulated that D-penicillamine's prevention of mature collagen formation and its anti-inflammatory properties might be useful in inhibiting wound healing following thermal sclerostomy and thereby prolonging filter function.

Another drug, daunorubicin hydrochloride, possesses both antiproliferative and cytotoxic activity. Although its mode of action is unknown, it is thought to affect the breakage-reunion reaction of DNA with topoisomerase II. We therefore evaluated the effect of D-penicillamine and daunorubicin on cultured fibroblasts from Tenon's capsule. For each agent, we measured its effect on cell proliferation and on collagen synthesis.

Materials and Methods

Subjects

All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research. A 5 mm × 4 mm section of conjunctiva was excised from two anesthetized cynomolgus monkeys (Macaca fascicularis) and placed into 60 mm tissue culture plates (Corning, Corning, NY) containing Ham's F12 medium (Gibco, Grand Island, NY). Consent was obtained from three human patients to
obtain a 4 mm² section of tenon’s capsule at the time of cataract extraction. All sections were obtained under sterile conditions and immediately transported in medium to the laboratory for culture.

This paper will discuss results obtained for cell doubling times using both monkey and human cells. The proliferation assay, collagen synthesis assay, and cellular morphology study are presented only for human cells.

Cell Culture

Tissue was minced, placed under a coverslip and incubated at 37°C in a humidified incubator. After several days, fibroblasts explanted from the tissue attached to the plate. At that time, tissue sections were removed and cultures were allowed to grow for 7 days. Cells were then exposed to 0.25% trypsin (Sigma, St. Louis, MO) and transferred to 25 cm² tissue culture flasks (Corning). When confluency was achieved, the cells were transferred to 75 cm² flasks and these flasks were used for all subsequent passages.

Cultures were grown in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 units penicillin/ml and 100 µg streptomycin/ml (all from Gibco), and were not used beyond passage 7.

Drugs

Lyophilized daunorubicin hydrochloride (Ives Laboratories, New York, NY) was used at concentrations of 25, 12.5, 5 and 2.5 µg/ml while D-penicillamine (Merck Sharp and Dohme, West Point, PA) was used at concentrations of 50, 25, 10 and 5 mg/ml. Both drugs were diluted in Ham's F12 medium immediately before use in all experiments. Both drugs' solutions were sterilized and their pH was adjusted to a level compatible with the drug culture media, pH = 7.0, before they were added to culture plates.

Proliferation Assay

Cells for assay were washed three times in Dulbecco’s Ca++/Mg++-free phosphate buffered saline (PBS), trypsinized, counted, and aliquoted to culture plates. Proliferation assays similar to those used to test other antineoplastic drugs in vitro⁹,¹⁴ were carried out in triplicate in flat bottom microtiter wells (Linbro, McLean, VA) with 8.4 X 10⁵ fibroblasts/well in 100 µl medium. The pH of the medium equilibrated in the incubator was 7.2. Cells were allowed to attach for 22 hr at 37°C at which time the drugs were added. One hundred µ of each of the four concentrations of either D-penicillamine or daunorubicin was added to each well resulting in a final volume of 200 µl well. Negative control wells received 100 µl of complete medium without drug. Cultures were incubated an additional 28 hr in 5% CO₂ at 37°C at which time 1 µCi/well of ³H-thymidine (New England Nuclear, Boston, MA), (specific activity 6.7 mCi/mmol) and 100 µg/ml of ascorbic acid (Sigma) were added.¹⁵ Cultures were then incubated 18 hr, harvested and counted in a liquid scintillation counter (Tracor, Chicago, IL; Unilux III). Counting efficiency of tritiated thymidine for this machine was calculated to be 47%. The entire cellular proliferation assay was repeated three times.

Collagen Synthesis Assays

Intracellular and extracellular collagen synthesis was assayed by measuring incorporation of ¹³C proline (New England Nuclear) as previously described.¹⁶,¹⁷ Twenty-four-well flat bottom plates (Linbro) containing 2.0 X 10⁴ fibroblasts/well and 0.5 ml of each of the four concentrations of either D-penicillamine or daunorubicin were cultured in triplicate in a final volume of 1.0 ml. Control wells contained cells and 0.5 ml of complete medium without drug. Each well was assayed three times: once for unused proline (supernatant), once for intracellular proline (cell lysate), and once for extracellular matrix proline (SDS extract).¹⁸

Cells were allowed to adhere 18 hr prior to experimentation. At that time, growth medium was replaced with fresh medium containing 100 µg/ml ascorbic acid.¹⁵ Immediately after adding the drugs, 2 µCi/well of ¹³C proline (specific activity 250 mCi/mmol) was added. Cultures were incubated an additional 48 hr, at which time 0.5 ml cell-free supernatants were counted in Budget Solve solution in a liquid scintillation counter to determine the amount of unused proline. Each well was then washed three times with 1 ml of Ca⁺⁺/Mg⁺⁺-free PBS and cells were removed by 1 hr incubation on a rotary shaker at 37°C in 0.5 ml of 1.0 mM ethyleneglycol bis(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA, Sigma), pH = 6.5, and pipetted into separate counting vials. Each vial was counted for intracellular proline as above. The time necessary for all cell detachment from each well was determined by examination under a Nikon (Yokohama, Japan) inverted phase microscope.

Extracellular matrix that remained in the wells was extracted after rinsing each well with PBS by adding distilled water containing 0.5 ml of 0.2% sodium deoxycholate (SDS, Biorad, Richmond CA), and incubating the plates on a rotary shaker at 37°C for 30 min. The extracellular matrix in each well was then harvested and counted as described above. The entire collagen synthesis assay was repeated twice.
Fig. 1. Fibroblast culture proliferation times extrapolated from the slopes of the lines of Coulter-counted cells obtained by cell attachment assay. Data on each curve represents three determinations on each of two subjects.

**Cell Doubling Time**

To determine doubling times, a cell attachment assay was performed in triplicate in 24-well flat bottom plates with $2.0 \times 10^4$ fibroblasts and 0.5 ml of each of the four concentrations of either D-penicillamine or daunorubicin in a final volume of 1.0 ml as described above. The sera used for each cell line were the same. Experimental plates were counted after 70 hr (48 hr after time 0). An additional set of control wells on a separate plate was harvested at 22 hr (time 0). Cells were first washed with 1 ml PBS and trypsinized using 300 $\mu$l of 0.1% trypsin containing 0.05% EDTA (Sigma). After 10 min, 700 $\mu$l of modified Eagle's media with 10% FBS (Gibco) was added to each well. The contents of each well were added to 9 ml of Isoton counting fluid (American Scientific, McGraw Park, IL) and counted twice with a model ZF Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). The counting efficiency for this machine was calculated to be 90%. The entire cell attachment assay was repeated twice for each species.

**Cell Morphology**

To determine the effect of each drug on morphology, 24-well flat bottom plates containing $2.0 \times 10^4$ fibroblasts and 0.5 ml of each of the four concentrations of either D-penicillamine or daunorubicin were plated in a final volume of 1.0 ml, as before. Forty-eight hours after adding the drugs all wells were washed, stained and photographed. To do this, all fluid was aspirated from each well and cells were fixed with 1.0 ml of 10% neutral buffered formalin for 24 hr. When the fixative was aspirated, 1.0 ml of absolute methanol was added to each well for 3 min. Once that was aspirated, 150 $\mu$l Wright-Giemsa Stain (Anderson Labs Inc., Fort Worth, TX) was added, immediately followed by 235 $\mu$l distilled water. After 3 min, each well was then washed three times with distilled water, and the plates were allowed to air dry for 48 hr. Photographs were taken with a Nikon M-35S inverted phase microscope.

**Statistical Analysis**

Comparisons of proliferation, and intracellular and extracellular collagen synthesis at different dosage levels of daunorubicin and D-penicillamine were made using the two-tailed student t-test for independent groups.

**Results**

**Cell Proliferation**

The proliferation rate for each cell culture as determined by the number of Coulter-counted cells is depicted by the slope of the lines in Figure 1. Extrapolating from the lines, human cell cultures doubled in approximately 54 hr, or three times faster than monkey cell cultures, which doubled in approximately 169.5 hr. Each point on each line represents three determinations of each of two subjects.

Both drugs caused a decrease in proliferation as measured by thymidine uptake (Fig. 2). Proliferation
significantly decreased with D-penicillamine at 5 mg/ml ($P < 0.01$) and at 10 mg/ml ($P < 0.001$). A significant difference between 5 mg/ml and 10 mg/ml ($P < 0.01$) was also seen. D-penicillamine’s activity was therefore dose-dependent. A similar analysis showed that the antiproliferative activity of daunorubicin was significantly decreased at 2.5 µg/ml ($P < 0.001$). A significant difference between 2.5 µg/ml and 5 µg/ml ($P < 0.05$) was also seen. Therefore, both drugs’ activities were dose-dependent. Daunorubicin’s activity plateaued at 5 µg/ml and D-penicillamine’s action plateaued at 10 mg/ml. A significant decline in proliferation was achieved with as little as 2.5 µg/ml daunorubicin while 5 mg/ml D-penicillamine (2000X) was required to achieve similar inhibition.

Collagen Synthesis

$^{14}$C-proline uptake in the presence of each drug was assessed on a per cell basis to determine the amount of collagen synthesized. Proline incorporation into cellular collagen decreased significantly with 10 mg/ml of D-penicillamine ($P < 0.005$) and with as little as 5 mg/ml ($P < 0.025$). D-penicillamine caused statistically significant decreases in extracellular collagen produced per cell (Fig. 4) at 10 mg/ml ($P < 0.025$), at 25 mg/ml ($P < 0.05$), and at 50 mg/ml ($P < 0.05$). There was a significant difference in extracellular collagen synthesis between the 5 mg/ml and the 10 mg/ml concentrations ($P < 0.01$).

Daunorubicin affected intracellular incorporation of labelled proline only at the highest concentration, 25 µg/ml, (Fig. 3, $P < 0.25$). Extracellular collagen production per cell (Fig. 4) was unaffected by daunorubicin even at the highest concentration ($P > 0.20$).

Cell Morphology

Normal human fibroblasts in a proliferating phase are seen in Figure 5. At 5 mg/ml, D-penicillamine had a significant effect on cultured cells (Fig. 6). These same effects were seen at much lower concentrations (2.5 µg/ml) of daunorubicin (Fig. 7) and consisted of cell-cell contact interruption, and retraction and rounding of cell processes. These effects suggest interference with cell adhesion.

Discussion

Both D-penicillamine and daunorubicin exerted a significant effect on human conjunctival fibroblast proliferation as shown by thymidine incorporation.
and cell count, and as inferred by cellular morphology. This was true for D-penicillamine only at much higher concentrations (2000×) than for daunorubicin. We had not expected D-penicillamine to exhibit any substantial antiproliferative action since its known major action is prevention of collagen cross-linking.

Both intracellular and extracellular collagen synthesis were suppressed with D-penicillamine, indicating that de novo collagen production decreased and consequently less collagen was available for transport out of the cell. In contrast, at concentrations of daunorubicin where all proliferation ceased, intracellular production and extracellular transport of colla-
Fig. 7. Human fibroblasts after 48 hr culture with 0.025% daunorubicin (Wright-Giemsa stain, original magnification ×1440). Surviving cells have rounded up and lost attachment sites, reflecting the cytotoxic effect of the drug.

gen were maintained. We demonstrated no significant difference from the control in the amount of extracellular labeled collagen per cell at any concentration of daunorubicin. Even when the number of cells was reduced by 80% (at 5–25 µg/ml) there was no significant difference in the amount of extracellular labeled collagen. Therefore, the cells that survived the drug continued to produce collagen, suggesting that both the collagen synthesis mechanism and the collagen transport mechanism were still intact.

The antiproliferative agent 5-fluorouracil has been used clinically to retard filtration failure and proliferative vitreoretinopathy and has been evaluated in vitro. It inhibited fibroblast proliferation by 50% at a dose of 0.2 µg/ml. Other drugs which have been evaluated in fibroblasts in vitro are cytarabine, for which the I-50 value was 0.05 µg/ml, and doxorubicin, for which the I-50 value was 0.004 µg/ml.

In the present work, we were unable to determine the I-50 value for daunorubicin because the lowest concentration tested, 2.5 µg/ml, caused marked inhibition. The I-50 value clearly would be well below that concentration, and could well be as low as the value of 0.004 µg/ml reported by Blumenkranz for the structurally related compound doxorubicin.

In deciding upon a dose of daunorubicin to use in a model of filtration surgery in a glaucomatous primate model, we were guided by the reported experience with 5-fluorouracil and by an estimate of the relative potencies of the two drugs. Since 5-fluorouracil is generally used at a dose of 5 mg twice a day, in the form of a 0.5 ml injection of 10 mg/ml, an equivalent dose of daunorubicin was estimated at 0.1 mg, in the form of a 0.5 ml injection of 0.2 mg/ml (200 µg/ml). However, our initial studies were performed with even lower concentrations, 5 µg/ml and 25 µg/ml. Concentrations of 50 µg/ml were shown to be toxic to skin fibroblasts in tissue culture. Even with these lower doses used once daily we observed a substantial loss of corneal endothelial cells by light and electron microscopy, and clinical corneal edema was evident (unpublished observations). Therefore, concentrations less than 5 µg/ml (1 × 10⁻⁸M) should be used in future experimentation to attempt to find a safe level of drug that is effective in preventing filter failure.

D-penicillamine used subconjunctivally at concentrations of 100 mg/ml per day for 5 days in glaucomatous monkeys prolonged filter patency only briefly before ultimate failure. As this concentration is the limit of this drug's solubility a novel slow delivery system may be necessary for its ultimate clinical use.

While neither drug, D-penicillamine or daunorubicin, is ideal for the control of fibroblast proliferation following filtering surgery, as D-penicillamine has solubility problems and daunorubicin has demonstrated corneal endothelial toxicity, we have shown that these agents are capable of inhibiting the fibroblastic response seen with subconjunctival scarring. By examining both these agents' cellular effects, we would hypothesize that to prevent filtering surgery failure a combination therapy might be beneficial. Early in the postoperative period an antimitotic agent
is necessary to prevent cellular proliferation, and, either simultaneously or later, a collagen inhibitor could further prevent scarring by obstructing the production or transportation of collagen from existing cells at the surgical site. Further research with the help of tissue culture systems and/or collagen synthesis assays seems warranted.

Key words: fibroblasts, D-penicillamine, daunorubicin, collagen synthesis, filtering surgery, glaucoma

References