Proliferation of fibroblasts is a serious problem in ocular trauma and surgical wound healing. Depending on the location of the injury, the growth of fibroblasts can lead to different problems. In glaucoma filtering surgery, fibroblast proliferation may contribute to scar tissue formation and premature wound closure. Fibroblastic growth in proliferative vitreoretinopathy may lead to the formation of preretinal membranes, which can contract, causing retinal detachment. In an effort to find a more effective method of inhibiting ocular fibroblast proliferation, we have investigated the effect of heparin, a sulfated polysaccharide, on the proliferation of fibroblasts obtained from the sclera of donor eyes. Heparin inhibits the incorporation of $^3$H-thymidine in a dose-dependent manner in the presence of fetal bovine serum (FBS). This inhibition is partially reversed by endothelial cell growth factor (ECGF). The heparin antagonist protamine sulfate causes a reversal of heparin inhibition and, in some instances, a significant increase in $^3$H-thymidine incorporation compared to serum controls. Heparin was equally effective in inhibiting cell proliferation in control and heparin-protamine sulfate-pre-treated medium. These results were apparently unrelated to a direct toxic effect on cells, as a Trypan Blue exclusion assay showed no significant difference in viability when heparin treated cells were compared to control cells. Direct cell counts showed that heparin was effective in inhibiting cell proliferation over a long time period, but only if it was reinstalled every 2 days. Heparin treatment shows promise as a method for controlling fibroblast proliferation in the eye. Invest Ophthalmol Vis Sci 29:1272-1276, 1988

Proliferation of fibroblasts is a serious complication of eye injury and a leading cause for the failure of ocular surgical procedures. Microscopic examination of failed filtration surgery in patients with glaucoma and in a monkey model shows proliferation of fibroblasts and deposition of collagen that eventually produces a mechanical barrier to filtration. Treatment with antimitobolitic drugs like 5-fluorouracil, which are capable of inhibiting cell proliferation, is effective in controlling scarring at the surgical site. However, the toxic effects of the drug make it a less than ideal form of treatment.

Fibroblasts have been identified in preretinal membranes formed as a result of penetrating eye injuries and in membranes formed as a consequence of rhegmatogenous retinal detachments. The therapy most often used for treating intraocular membrane formation is surgical intervention, but this strategy is not always successful even with multiple operations. Many pharmacologic agents have been suggested as useful in controlling membrane formation, including steroids, 5-fluorouracil and daunomycin. The use of antiproliferative drug therapies may not be wholly satisfactory due to ocular toxicity or the transient nature of their effect on cellular proliferation. The transient nature of the antiproliferative effects may be due, in part, to rapid clearance of drug from the eye.

We investigated the sulfated mucopolysaccharide heparin as a possible agent for inhibiting fibroblast growth and controlling ocular scarring and membrane formation. Heparin is extremely effective in inhibiting vascular smooth muscle cell proliferation. The literature suggests that it is not as uniformly effective in inhibiting the proliferation of fibroblasts. However, studies with mouse LM cells and BHK fibroblasts show growth inhibition of these cell types at relatively low heparin concentrations.

There are no data on the effect of heparin on the
growth of human fibroblasts. Therefore, we investi-
gated the effect of heparin on the proliferation of
human scleral fibroblasts by assays of thymidine in-
corporation and cell proliferation.

Materials and Methods

Cell Culture

All procedures were performed in a tissue culture
room, using sterile instruments and vessels. Scleral
fibroblast (SF) cultures were prepared by dissection
of sclera from human donor eyes. The eyes were ob-
tained from the Lion’s Eye Bank of Northeastern
New York. The pieces of sclera were washed several
times with Pharmadine solution (Sherwood Pharma-
cutical Company, Mahwah, NJ), and the internal
and external surfaces were scraped with a scalpel
blade. The pieces of sclera were finely minced using a
pair of curved scissors, and incubated at 37°C with
500 units/ml of collagenase type II (CooperBiomed-
cal, Malvern, PA) in a 2.5% bovine serum albumin
(BSA) solution in Dulbecco’s Modified Eagle’s Me-
dium (DMEM). Incubation was for 30 min, and the
tubes containing enzyme solution and tissue pieces
were mixed periodically with a vortex mixer. By the
end of the incubation period the tissue had begun to
dissociate, and was then washed once with DMEM
supplemented with 20% fetal bovine serum (FBS)
(Hyclone Laboratories, Inc., Logan, UT), 0.1 mM
nonessential amino acids (Gibco Labs, Grand Island,
NY) and 50 μg/ml gentamicin sulfate (Whittaker
M.A. Bioproducts, Walkersville, MD). After washing,
the partially dissociated tissue pieces were placed in
tissue culture dishes with just enough medium to
cover the bottom of the dish. The dishes were left in
an incubator at 5% CO₂ and 37°C for several days,
and, when visual inspection revealed that the tissue
pieces were attached to the bottom of the dish, more
medium was added. When confluent, the cells were
passaged, using an enzyme solution containing 0.05%
trypsin, 0.02% ethylene glycol bis-(beta-aminoethyl
ether) N,N,N’,N’-tetraacetic acid (EGTA), 1% polyvi-
nylpyrrolidone, and 18 mM 4(2-hydroxyethyl)-1-pi-
perazineethanesulfonic acid (HEPES) buffer in 0.9%
nasium or calcium (cmf-PBS), once with 10% trichlo-
roacetic acid, and 0.55 ml of 1% sodium lauryl sulfate
in 0.05 M NaOH was added. After incubation at
37°C for 15 min, a 0.5 ml aliquot was taken, added to
10 ml of scintillation fluid and counted. The test sub-
stances examined for their effects on SF proliferation
were: heparin (Sigma, St. Louis, MO), endothelial cell
growth factor (ECGF) (Meloy Labs, Springfield, VA)
and protamine sulfate (Sigma).

For cell counts, the cells were seeded at 1.0 × 10⁶
cells/dish into 35 mm dishes in DMEM supple-
mented with 20% FBS. The cells were allowed to at-
tach overnight and medium was added with or with-
out heparin. After the various incubation times, the
cells were washed twice with cmf-PBS and removed
by treatment with 0.25 ml of PET. Cell numbers were
determined by hemocytometer counts. Three dishes
were counted for each time point. The results were
expressed as the average of three dishes with the stan-
dard deviations for each time point.

Cell Viability Assay

The viability of cells was determined by a Trypan
Blue exclusion assay. Scleral fibroblasts were seeded
into 60 mm dishes at 6.0 × 10⁴ cells/dish in DMEM
supplemented with 10% FBS. After allowing them to
attach overnight, the cells were incubated for 24 h in
DMEM with 0.5% serum. The cells were then incu-
bated for 24 h with various concentrations of heparin
in DMEM with 10% FBS, or in DMEM with 10%
FBS alone. Since nonviable cells may either detach
from the surface of the culture dish or remain at-
tached, we attempted to account for both situations.
We assessed cell detachment by performing a cell
count and comparing the cell number to the cell
number in control dishes. We determined the viabil-
ity of the cells that remained attached by dye exclu-
sion. Cells were removed from the dishes by treat-
ment with PET. Trypan Blue at a concentration of
0.5% was mixed in an equal volume with the cell
suspension in DMEM and 10% FBS. The percentage
of cells that excluded the dye was determined. A
minimum of 100 cells were counted for each determi-
nation. Both cell counts and the Trypan Blue exclu-
sion assay were performed on four dishes for each of
the concentrations of heparin tested.
Statistical Analyses

Data is presented as the mean and one standard error of the mean (SEM) or standard deviation (SD), as indicated. All experiments were performed at least twice, with different cell lines, and the results from representative experiments were reported. Significance was determined by student t-test at the 95% level of confidence.

Results

Thymidine incorporation into SF was inhibited by the presence of heparin at all the concentrations of FBS tested, and this inhibition was reversed by the precipitation of heparin with the heparin antidote protamine sulfate (Fig. 1). In some cases the ability of the growth medium to stimulate proliferation of cells was actually enhanced when treatment with heparin was followed by heparin precipitation with protamine sulfate.

Table 1. Effect of ECGF on heparin-mediated thymidine incorporation

<table>
<thead>
<tr>
<th>Heparin (mg/ml)</th>
<th>Heparin alone</th>
<th>Heparin plus 120 µg/ml ECGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>100.0 ± 0.0</td>
<td>102.0 ± 10.7</td>
</tr>
<tr>
<td>0.02</td>
<td>57.7 ± 5.3</td>
<td>115.4 ± 11.8</td>
</tr>
<tr>
<td>0.05</td>
<td>54.1 ± 4.0</td>
<td>120.7 ± 9.7</td>
</tr>
<tr>
<td>0.10</td>
<td>69.3 ± 4.3</td>
<td>121.0 ± 3.2</td>
</tr>
<tr>
<td>0.20</td>
<td>54.3 ± 3.1</td>
<td>103.3 ± 4.5</td>
</tr>
<tr>
<td>0.40</td>
<td>50.2 ± 3.7</td>
<td>54.9 ± 10.3</td>
</tr>
<tr>
<td>0.80</td>
<td>41.0 ± 1.3</td>
<td>85.0 ± 4.3</td>
</tr>
<tr>
<td>1.00</td>
<td>42.1 ± 4.2</td>
<td>80.3 ± 2.5</td>
</tr>
<tr>
<td>2.00</td>
<td>31.4 ± 1.9</td>
<td>76.2 ± 7.0</td>
</tr>
<tr>
<td>4.00</td>
<td>20.0 ± 1.3</td>
<td>49.9 ± 4.6</td>
</tr>
<tr>
<td>8.00</td>
<td>12.2 ± 1.4</td>
<td>38.5 ± 2.2</td>
</tr>
</tbody>
</table>

This table shows a dose response for heparin in inhibiting thymidine incorporation into scleral fibroblasts. Significant inhibition of incorporation was shown with the lowest dose used (0.02 mg/ml). At all concentrations studied, except 0.4 mg/ml, the inhibitory effect was blunted by the addition of endothelial cell growth factor (ECGF). Used alone, ECGF does not affect thymidine incorporation into scleral fibroblasts.

Treatment of cells grown in 5% FBS showed a dose-response relationship at heparin concentrations of 0.0016 to 8.0 mg/ml. For concentrations of heparin from 0.05 to 8.0 mg/ml, thymidine incorporation was significantly lower when compared to control values (Fig. 2).

It appears from our assay for direct toxic effects that heparin is not acutely toxic to scleral fibroblasts. We tested for cell viability with doses of heparin of 1.0, 2.0, 4.0 and 8.0 mg/ml, and the cell numbers and percentage of viable cells were not significantly different from control values (data not shown). Thus, the effect of heparin in controlling cell number is due mainly to an inhibition of cellular proliferation. This view is further supported by the observation that a single dose of heparin (1 mg/ml) is not able to inhibit cell proliferation of scleral fibroblasts over a sustained time period (Fig. 4). Repeated instillations of the heparin are required for it to be fully effective (Fig. 5).

We speculated that the potentiation of thymidine incorporation in serum containing medium to which both heparin and protamine sulfate were added was due to the removal of an inhibitor present in the serum. Another possible explanation is that residual heparin or protamine sulfate, which may remain after the precipitation reaction, may have a stimulatory effect on scleral fibroblast proliferation. However, low doses of heparin (Fig. 2, table 1) and protamine sulfate alone (we tested concentrations of 0.00125, 0.0025, 0.0125, 0.5 and 1.5 mg/ml, data not shown) did not stimulate thymidine incorporation.
If the action of heparin was owing to the binding and internalization of an inhibitor present in serum, we reasoned that we might remove the inhibitor by heparin treatment followed by protamine sulfate precipitation. To test this hypothesis, we first treated medium with heparin, precipitated the heparin with protamine sulfate, decanted the supernatant, and treated cells with heparin and the supernatant. If heparin was acting through an inhibitor that we removed, retreatment should be less effective in inhibiting thymidine incorporation. However, heparin was equally effective in inhibiting thymidine incorporation in cells in growth medium whether or not it was pretreated with heparin-protamine sulfate (Fig. 3).

In order to measure the effects of heparin on sustained cell proliferation, 1 mg/ml of heparin was instilled at the time of seeding and cells were allowed to grow without changing the medium for 6 days. The cell numbers were assessed by hemocytometer counts every 24 h. Once the control culture entered log phase, the heparin-treated cultures had less cells compared to controls until day 4. By days 4 through 6 the cell numbers for the heparin-treated cultures were not significantly different from the control cell numbers (Fig. 4). When heparin was administered 96 h after seeding, a slight depression in cell number was noted, but the number of cells in heparin-treated and control cultures was not significantly different for the 7 days they were counted (data not shown).

In another experiment, the cultures were refed every 2 days with DMEM containing 20% FBS or the same medium with 1 mg/ml of heparin. Every 48 h the medium was aspirated and replaced with the complete medium, with or without heparin. By the third day after administration of heparin the number of control cells was greater than the heparin-treated cells, the difference in cell number increased with every successive day, and by the seventh day the cells with the heparin had accomplished 2.6 doublings, while the control cells had accomplished 4.9 population doublings (Fig. 5).

Discussion

Most of the studies concerning the effect of heparin on cell growth have involved the effect of heparin on the growth of smooth muscle cells (SMC). With this cell type the phenomenon of inhibition is well characterized, but the mechanism is not well understood.

![Fig. 3](image_url) Effect of pretreatment of medium with heparin and precipitation with protamine sulfate. The graph compares thymidine incorporation into cells incubated in medium with 2% FBS (complete medium) (A); cells incubated in complete medium and 1 mg/ml heparin (B); cells incubated in complete medium in which heparin was added and precipitated with protamine sulfate (C); and cells incubated in medium "C" and retreated with heparin (D). Bars indicate 1 SEM.

![Fig. 4](image_url) Effect of a single heparin treatment on the growth of scleral fibroblasts. Cells were seeded at 1.0 x 10⁴ cells/dish in DMEM containing 20% FBS in 35 mm tissue culture dishes. After allowing the cells to attach overnight, they were counted and treated with medium containing 20% FBS with or without heparin. The cells were counted every day for 6 days. Bars indicate 1 SD.

![Fig. 5](image_url) Effect of multiple treatments on the growth of scleral fibroblasts. Cells were seeded at 1.0 x 10⁴ cells/dish in DMEM containing 20% FBS in 35 mm tissue culture dishes. After allowing the cells to attach overnight they were counted and treated with medium and 20% FBS with or without heparin. Every other day, the cultures were fed with growth medium with or without heparin. The cells were counted every day for 7 days. Bars indicate 1 SD.
Growth-arrested SMC are nearly 100 times more sensitive to heparin-mediated growth inhibition compared to exponentially growing SMC. This is consistent with the observation that heparin halts the proliferation of cells in the Go-S transition or at a very early S-phase event, and with the observation that exponentially growing SMC bind eight-fold less heparin per cell than growth-arrested SMC.

The binding data and the kinetics of the heparin antiproliferative effect suggest that the antiproliferative effect is owing to a direct effect of heparin on the SMC. Recent data suggests that growth factors may have a role in modulating the heparin effect; the addition of epidermal growth factor (EGF), at concentrations of 5–250 ng/ml, to serum-containing medium decreased the growth-inhibitory action of heparin by 60–77%. With heparin pretreatment, the binding capacity of EGF is decreased by 50%. We noticed a significant blunting by ECGF of the heparin inhibition of serum-stimulated cells at all of the concentrations of heparin tested, with a slight but significant increase in thymidine incorporation at the lower concentrations of heparin tested. Heparin is known to bind avidly to various growth factors, including ECGF. This may affect the binding of heparin to cells or act in some other manner to reduce heparin’s effectiveness in preventing cell proliferation. This fact may be important in considering the intraocular use of heparin, as ECGF may be related to other anionic, neurally derived growth factors such as retinal-derived growth factor (RDGF). RDGF may be present at the posterior pole of the eye.

The necessity of replenishing heparin in SF cultures in order for it to be effective in preventing long-term proliferation may be a specific trait of fibroblasts compared to smooth muscle cells, or it may be that dividing fibroblasts are refractory to heparin’s action and, therefore, only a small number of cells in the cycling population are susceptible to heparin’s action at any one time. In SMC cultures, treated with heparin while growing in medium with 20% serum, the growth rate is unaffected during the first 24–48 h, after which a steady decline in growth rate begins. Our results suggest a more immediate effect, but one that cannot be sustained, and may be related to factors in the serum inactivating the heparin, or to an inactivation of the heparin after binding to fibroblasts. The transient nature of the heparin effect substantiates the fact that the dose of heparin used is nontoxic; after a single treatment with heparin, the cell population was able to rebound after an initial suppression in cell numbers.

The data suggest that heparin treatment is an effective method of inhibiting SF proliferation, and as such may be an ideal method of controlling ocular scarring.

Key words: heparin, fibroblast proliferation, inhibition

References