The purpose of this work was to study the effectiveness of heparin plus cortisone, and of cortisone alone in control of corneal vascularization in rabbit eyes. Corneal vascularization was induced by de-epithelialization of the cornea and limbus and part of the bulbar conjunctiva with concurrent trephination and excision of a central 2-mm diameter corneal button. Inhibition of vascularization by polymer pellets impregnated with heparin (Panheprin®, Abbott Laboratories; Chicago, IL) and cortisone, or neither drug was studied by implanting the pellets into the eyes at the time of injury and following the eye clinically and histologically. Wounded corneas with empty pellets developed vascularization extending from the limbus to the central cornea within 3 wk (n = 10). In other wounded eyes, heparin/cortisone pellets prevented vascularization (n = 10) while cortisone pellets slowed, but did not totally inhibit vascularization (n = 6). In other eyes, clear autografts were transplanted into vascularized eyes; and the ability of the drug-impregnated pellets to inhibit grafts vascularization was evaluated. In eyes with heparin/cortisone pellets inserted into the donor button at the time of keratoplasty, the autografts remained clear for at least 6 wk (n = 10) but subsequently vascularized if the sutures were not removed, while cortisone pellets slowed but did not block vascularization (n = 6). If heparin/cortisone pellets were inserted into the vascularized host tissue, rather than into the donor button, vascularization of the graft occurred (n = 6). Thus, heparin (Panheprin®, Abbott Laboratories; Chicago IL) plus cortisone inhibited vascularization in rabbit cornea in the models studied: The effect of other commercially available heparins remains to be studied. Invest Ophthalmol Vis Sci 27:449-456, 1986

Epithelium of conjunctival origin which grows over denuded rabbit corneas transdifferentiates through five distinct stages until it is histologically identical to normal corneal epithelium.1,2 Despite this histological identity, a penetrating wound in a cornea covered by such regenerated ocular surface epithelium induces superficial corneal vascularization, even though a similar wound in a normal cornea does not cause blood vessel ingrowth.3

In some clinical situations, there may be a parallel course of pathophysiological events. After loss of all corneal epithelium, as in chemical injury for example, additional trauma in the form of surgery (eg, penetrating keratoplasty) or perforating injury may result in additional vascularization. This vascularization may decrease vision or even lead to immune rejection of the transplant. Inhibition of such new vessel growth might, therefore, be of considerable importance in maintaining clarity of the visual axis. The identification of a specific angiogenesis factor (TAF)4 has led to numerous studies of the substances that might inhibit angiogenesis. Recently, it has been shown that heparin in the presence of cortisone, released from a polymer pellet implanted into corneas, inhibits tumor, immune, and inflammatory angiogenesis.5 To us, it seemed probable that the same drugs might inhibit corneal vascularization related to a traumatic loss of corneal epithelium and might, therefore, be useful in clinical ophthalmology. Ideally, inhibition of vascularization should be achieved without jeopardizing epithelial healing and transdifferentiation.
Fig. 1. The experimental model of traumatic corneal vascularization in which both the stimulus for vascularization and the drugs that inhibit it are presented simultaneously. After the removal of all corneal and limbal epithelia and a ring of bulbar conjunctival epithelium, a central 2-mm partial thickness trephination is done (a), a cyclo-dialysis spatula is inserted between the corneal lamellae and a pocket created (b). A polymer pellet is inserted into the pocket (c), and the remaining 2-mm button excised (d).

Therefore, we have studied (1) whether heparin in the presence of cortisone inhibits traumatic corneal vascularization in rabbits; and (2) if so, whether normal transdifferentiation of conjunctival to corneal epithelium occurs; and (3) whether heparin in the presence of cortisone inhibits vascularization of a corneal graft in rabbit corneas with preexistent blood vessels.

Materials and Methods

All animal investigations reported in this work conform to the ARVO Resolution on the Use of Animals in Research.

Pellets

Polymer pellets of ethylene-vinyl acetate copolymer impregnated after the method of Langer and Folkman\(^6\) contained heparin (Panheprin\(^\circ\), Abbott Laboratories; North Chicago, IL; 180 \(\mu g\)), cortisone acetate (1.5 mg), or neither drug. They were provided to us from Dr. Judah Folkman’s laboratory at the Children’s Hospital Medical Center, Harvard Medical School, Boston, MA. These pellets, about 1.5 X 1 mm, were implanted into rabbit eyes as described below.

Animal Preparation

Animal model for traumatic corneal vascularization: The eye was proptosed and the entire corneal and limbal epithelia and a 2–3 mm ring of bulbar conjunctival epithelium were removed using heptanol as described previously.\(^1,3,7\) After the heptanol had been in contact with the ocular surface for 1 min, the eyes were washed thoroughly with saline. Then, a central 2 mm trephination down to Descemet’s membrane was done, the anterior chamber entered with a Bard Parker #11\(^\circ\) (Becton Dickinson; Lincoln Park, NJ) blade, and the remaining core of tissue was excised.\(^3,8\) The wound sealed promptly with aqueous fibrin and the chamber quickly reformed. Antibiotic ointment (erythromycin) was applied every day until the cornea was covered by epithelium. The eyes were examined and checked for vascularization twice weekly, up to 3 months.

Implantation of pellets into vascularized eyes: Pellets were implanted into the corneas at the same time that epithelial removal and the 2-mm diameter trephination were done. The eye was proptosed and the cornea, limbal, and some bulbar conjunctival epithelium removed as described above. A 2-mm trephine as used to make a central penetration down to Descemet’s membrane (Fig. 1a). A lamellar button was excised, and a cyclo-dialysis spatula was inserted between the peripheral corneal lamellae at the bottom of the trephination (Fig. 1b). Horizontal movement of the spatula split the stromal lamellar and created a pocket into which the pellet could be inserted (Fig. 1c). The pellets, which were empty or contained heparin and cortisone or cortisone alone, were positioned at various distances from the limbus. After this, a Bard Parker #11\(^\circ\) blade was used to enter the anterior chamber at the trephination site, and the remainder of the 2-mm button was excised (Fig. 1d). As before, the wound sealed promptly and the chamber quickly reformed. Erythromycin was applied daily for 14 days.

The number of eyes subjected to each maneuver is shown in Table 1. An additional two eyes from each treatment group were used for histological study of vascularization and epithelial transdifferentiation.

At the end of the observation period, animals were killed by an overdose of intravenous sodium pentobarbital. The eyes were enucleated and fixed in 10% buffered formalin. Corneal histological preparations (7-\(\mu m\)) were stained with hematoxylin and eosin (H&E) or with the periodic acid Schiff reaction (PAS).

Keratoplasty: All operations were performed under the operation microscope. No drugs other than anesthetics were used before or during the operation.

Fourteen days after the procedure for producing traumatic vascularization has been performed on one
Table 1. Degree of vascularization in traumatized eyes related to location and content of polymer pellets

<table>
<thead>
<tr>
<th>Number of eyes</th>
<th>Pellet location</th>
<th>Pellet content</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>None</td>
<td>—</td>
<td>Vascularized by 1 wk. Vessels reached trephine wound by 3 wk.</td>
</tr>
<tr>
<td>10</td>
<td>Between limbus and trephination</td>
<td>Nothing</td>
<td>Vascularized as above.</td>
</tr>
<tr>
<td>10</td>
<td>Between limbus and trephination</td>
<td>Heparin: cortisone</td>
<td>No vascularization.</td>
</tr>
<tr>
<td>6</td>
<td>Between limbus and trephination</td>
<td>Cortisone</td>
<td>Vessels at 10 days. Prominent vessel at 7 wk.</td>
</tr>
</tbody>
</table>

* All eyes were observed for a period of 3 months.

eye (see Fig. 1a), 6-mm penetrating corneal grafts were interchanged between the two eyes of each animal. In the experimental eyes with a vascularized recipient cornea, the clear autograft was secured in place using eight interrupted 10-0 nylon sutures and a continuous nylon suture. In half of the cases, interrupted sutures were used. The knots were not buried. The anterior chamber was reformed with air and antibiotic ointment given postoperatively. In the opposite healthy eye, the vascularized autograft from the experimental eye was sutured with continuous 8-0 virgin silk to restore the integrity of the eye.

Control Group

The control eyes were those in which a clear autograft was transplanted into vascularized corneas with no pellet.

Experimental Groups

The experimental eyes differed from the control eyes in that all of them had a pellet implanted. Depending on where and which pellets were placed into experimental eyes, the following subgroups were formed:

(a) Pellets in the vascularized recipient cornea, outside the central clear graft.

A 6-mm diameter trephination was done to half the depth of the vascularized cornea, the cyclodialysis spatula was inserted at the bottom of the trephination site, and the peripheral corneal lamellae were split to create space for the implantation of the pellet into the recipient cornea. Four pellets containing heparin and cortisone were inserted at 12, 3, 6, and 9 o'clock about 1.5 mm peripheral to the trephination wound. After this, the anterior chamber was entered with a Bard Parker® #11 blade and the button was excised with scissors along the trephination cut. A clear autograft, 6 mm in diameter, from the contralateral eye, was sutured into this vascularized recipient bed.

(b) Pellets in the clear donor graft, placed into vascularized recipient corneas.

The healthy donor eye was proptosed, and a horizontal cut about 3 mm in length was made into the cornea near the limbus. A cyclodialysis spatula was inserted from this cut (Fig. 2), splitting the cornea lamellae and creating space into which a pellet was implanted (Fig. 3). Then, a 6-mm diameter corneal button was used to suture the button back into place.

Fig. 2. Creation of the space for implantation of the pellet into the donor cornea. The cyclodialysis spatula is inserted into the cornea of the healthy eye, splitting the lamellae.

Fig. 3. The pellet in place in the donor cornea.
Fig. 4. Trephination of the donor cornea. A 6-mm diameter cornea button with the pellet in the middle of the button is trephined. The excision is completed along the trephination cut, and the button can be sutured into the vascularized cornea of the opposite eye.

Results

Traumatic Vascularization of Cornea

Model of traumatic vascularization: The traumatically vascularized eyes (n = 10) were covered by the regenerating epithelium of conjunctival origin 7 days after wounding. All the corneas became vascularized (Table 1, Fig. 5), although not at the same time or to the same extent. At the earliest, blood vessels appeared 5 days after wounding, and at the latest, were visible 8 days after wounding.

Three weeks after injury (2 wk after re-epithelialization), at least one vessel reached the center of the cornea in all eyes. In some cases, the vessels were very numerous. In others, the vessels were farther apart and fewer of them reached the center of the cornea.

Four weeks after wounding, the accompanying conjunctival injection had faded, and the corneal vessels were less engorged. Although less conspicuous, the vessels were still visible for the 3 months the experiment was carried out. The central scar was firm enough to sustain the pressure developed during proptosing of the eye 3 wk after wounding.

Three weeks after wounding (2 wk after re-epithelialization), histology showed a thin epithelium with many goblet cells overlying the blood vessels in the superficial stroma (n = 2). Polymorphonuclear leukocyte infiltration was of a moderate degree. Both vessels and goblet cells were still present 3 months after wounding (n = 2), showing that epithelial transdifferentiation had not occurred during that time.

Vascularized corneas with empty pellets: Ten vascularized corneas with implanted pellets containing no drug showed the same sequence of events as did the control corneas described above. All ten corneas vascularized to the same extent and at the same time as the control corneas (Table 1, Fig. 6) and showed vascularization of the stroma and goblet cells in the epithelium 3 wk after wounding and implantation (2 wk after re-epithelialization) (n = 2). Vessels and goblet cells were present at the end of the observation period of 3 months (Fig. 7).

Vascularized corneas with heparin/cortisone pellets: None of the ten vascularized corneas that had polymer pellets containing heparin and cortisone implanted showed any vascularization for the 3 months after wounding (Fig. 8). All corneas were covered by epithelium 7 days after wounding (Table 1).

Two weeks after healing, (3 wk after wounding) the epithelium was in phase 2–3 of transdifferentiation with many goblet cells. Six weeks after healing, the epithelium had transdifferentiated and had morphological characteristics typical of corneal epithelium with no goblet cells (Fig. 9). At the same time, contraction of the central scar tissue was found. Both at 3 and 7
wk after wounding, the scar was firm enough to sustain the pressure exerted when the eye was proptosed (Table 1).

Vascularized eyes with cortisone pellets: Six vascularized corneas had implantation of polymer pellets that contained cortisone only. Four of these corneas were covered by epithelium 7 days after wounding, and two healed 8 days after wounding. Blood vessels became visible 10 days after wounding. They continued to grow, and the first of them reached the edge of the trephine wound 3 wk after healing. Six weeks after re-epithelialization was completed (7 wk after injury), the epithelium still had goblet cells present, and blood vessels were prominent in the superficial stroma (Table 1).

Keratoplasty

Controls: Seven days after keratoplasty, blood vessels started to invade the clear graft in 6 of 6 transplanted eyes. By the day 21 post keratoplasty, they covered the whole graft (Table 2, Fig. 10). However, if the sutures were removed 10 days after keratoplasty (n = 4 eyes), the vessels that had crossed the graft-host junction ceased to grow and started to fade.

Experimental: For the heparin-cortisone pellet in vascularized recipient cornea surrounding clear graft, the vessels crossed the host-graft junction on the day 7 postoperatively and covered the whole graft by 3 wk after the operation, at about the same rate as those eyes in which no pellet had been placed (Table 2).

For Heparin-cortisone pellets in clear donor graft placed into vascularized recipient bed, all ten grafts remained avascular and clear for 6 wk. At that time, the sutures were removed from five eyes, and these
grafts remained avascular (Fig. 11). In the other five eyes from this group, the sutures were left for an additional week during which time the vessels crossed the host-graft junction. The sutures were then removed from these eyes (at 7 wk postoperatively), and the vessels did not grow any further (Table 2).

The eyes (n = 6) with cortisone pellets in the clear donor graft placed in vascularized recipient bed showed some inhibition of the rate of vascularization, with the vessels entering the graft about 12 days after keratoplasty and continuing to grow during the following 4½ wk (Table 2).

Upon histologic examination, all autografted corneas showed healing with scar formation and minimal retrocorneal fibrosis. There was a minimal leukocytic infiltration of the stroma, which otherwise looked normal. The epithelium was slightly attenuated over the pellet, but at the base of the prominence created by the implanted pellet, it was thicker than normal.

Table 2. Outcome of grafting into vascularized corneas

<table>
<thead>
<tr>
<th>Number of eyes</th>
<th>Pellet location</th>
<th>Pellet content</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6—sutures not removed</td>
<td>None</td>
<td>Nothing</td>
<td>Vessels entered cornea at 7 days, total vascularization at 21 days.</td>
</tr>
<tr>
<td>4—sutures removed at 10 days</td>
<td>None</td>
<td>Nothing</td>
<td>As above for 10 days, then vessels stopped growing, and faded at 2 wk.</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6*</td>
<td>In host cornea</td>
<td>Heparin: cortisone</td>
<td>Vessels at 7 days.</td>
</tr>
<tr>
<td>5*</td>
<td>In graft</td>
<td>Heparin: cortisone</td>
<td>No vascularization of graft.</td>
</tr>
<tr>
<td>5—sutures removed at 7 wk</td>
<td>In graft</td>
<td>Heparin: cortisone</td>
<td>Vessels crossed into graft at 7 wk, then stopped.</td>
</tr>
<tr>
<td>6*</td>
<td>In graft</td>
<td>Cortisone</td>
<td>Vessels entered graft at 12 days.</td>
</tr>
</tbody>
</table>

* Grafts had sutures removed at 6 wk except as indicated. All eyes were observed for 2 months.
Discussion

Neovascularization, or angiogenesis, is a component of many tissue responses: chronic inflammation, immune reaction, and reaction to neoplasia. It also plays a role in wound healing. The corneal vascularization induced in the traumatically vascularized corneas in this study undoubtedly is a tissue response associated with the healing process. The exact stimulus for vascularization in the traumatically vascularized eyes is unclear. Resurfacing of the cornea from conjunctival epithelium can, by itself, induce up to 2 mm of blood vessel ingrowth into the cornea.\(^9\) Corneal edema, although important for vascularization,\(^9\) does not per se cause it.\(^10,11\) Excision of Descemet's membrane, which is resistant to blood vessel ingrowth, seems not to be able to induce vascularization to such an extent by itself. Polymorphonuclear leukocytes enhance angiogenesis in those eyes, since removal led to a cessation of the angiogenesis. Although the action might have been related to its anti-inflammatory capabilities, the cortisone effect was probably a direct inhibition of the angiogenesis. Although the action might have

In this study, inflammation does not appear to be the prime stimulus. Previous studies have shown that the inflammatory cell infiltrate was the same in eyes which vascularized following the central trephination as in those which did not. In those studies, the obvious difference was that the eyes that vascularized were covered by epithelium of conjunctival origin whereas those that did not were covered by epithelium of corneal origin.\(^14\) Whether a combination of these factors or some other stimulus causes vascularization in this model is uncertain. It is certain, though, that the initial event responsible for vascularization in these corneas differs from those in which heparin and cortisone were originally tested: tumors, implantation of silica particles, and implantation of lymph node tissue.\(^5\)

In the eyes with keratoplasty, the sutures used to secure the graft also play a role in stimulating the angiogenesis in those eyes, since removal led to a cessation of the vascularization of the graft. In fact, the stimulus for extensive autograft vascularization in this model probably comes from the unburied sutures as well as the epithelial type. However, sutures in the absence of prior epithelial removal and trephination do not cause vascularization of the autograft.

In this study we were able to inhibit traumatic angiogenesis by implanting heparin:cortisone pellets into corneas at the time the stimulus for vascularization was presented. In addition, heparin in the presence of cortisone inhibited the growth of blood vessels from a vascularized recipient into the graft. Cortisone alone failed to inhibit vascularization completely: Grafts containing a polymer pellet with cortisone alone became vascularized, although vascularization was slower in the presence of cortisone as compared to controls. This effect of cortisone was probably a direct inhibition of the angiogenesis. Although the action might have

Heparin alone would not be likely to inhibit graft vascularization since it has been shown that it increases the migration of capillary endothelial cells in vitro\(^15\) and enhances tumor angiogenesis.\(^16\) Moreover, the literature suggests that the ratio of heparin to cortisone is important and that an excess of heparin may, in fact, stimulate vessel growth.\(^5\) The effect of excessive heparin was excluded from our experiments by not giving any intravenous or intramuscular heparin for prevention of aqueous clotting in the rabbits. The heparin used in these studies was Panheprin\(^R\) (Abbott Laboratories; North Chicago, IL). Recently, however, it has been reported that anti-angiogenic effects in the tumor models of corneal vascularization can be obtained with other heparins used in combination with cortisone (Lund, Dennis, Children's Hospital Medical Center, Boston, MA, personal communication) suggesting that the effect is due to heparin, not to a subunit of heparin or contamination of the Panheprin\(^R\).

The synergism of heparin and cortisone in the inhibition of vascularization may relate to one drug facilitating the uptake of the other into capillary endothelial cells or to activation by cortisone of an inhibitor of angiogenesis within the large heparin molecule.

When used in pellets, the heparin:cortisone combination had some limitations in inhibiting vascularization. First, the effect lasted only about 6 wk in the presence of a continued stimulus for vascularization,
the sutures. The reason for this rather short action is obvious: Heparin is released from the pellet for 14 days only, and cortisone for more than 30 days. Nevertheless, it appeared that vascularization was inhibited after the drug combination was no longer present, since it takes 4 wk for the renewal of vascularization to occur.

Another limitation is that the pellets that contained heparin and cortisone inhibit angiogenesis only when placed in the graft, and not when inserted into the vascularized recipient cornea. The explanation for this is not obvious. It may be that the surrounding vessels carry the drug away, or that the concentration of drug is less because much of the medication diffuses into the recipient cornea with little reaching the graft, or that there is no effect when the heparin:cortisone concentration is highest behind the progressing capillary sprouts.

Nevertheless, heparin:cortisone pellets may have some application in clinical ophthalmology. In the case of chemical injury, for example, the following sequence of events may occur: (1) total loss of the corneal and limbal epithelium followed by (2) their replacement by epithelium of conjunctival origin with (3) concurrent vascularization of the cornea in response to the injury, interfering with normal transdifferentiation of the epithelium, with (4) the epithelium retaining its conjunctival characteristics. Subsequent keratoplasty would mimic the animal model reported here, leading to additional vascularization into the graft. The use of the heparin:cortisone pellets in the transplant button might, at least for the short term, counteract this tendency for vessels to grow into the graft. Candidates for the application of heparin:cortisone pellets would be those patients who have a poor prognosis with any type of corneal grafting. The pellets could be put at the periphery of a penetrating graft, or in the bed of a lamellar graft. While angiogenesis might be inhibited for a rather short period, in some cases this may be long enough to outlast the stimulus for neovascularization.

**Key words:** angiogenesis, vascularization, keratoplasty, heparin, cortisone

**References**