Spontaneous Corneal Neovascularization in Nude Mice
Local Imbalance Between Angiogenic and Anti-angiogenic Factors

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Purpose. The present study considered the hypothesis that spontaneous neovascularization of the cornea of nude mice results from a local imbalance between angiogenic and anti-angiogenic factors.

Methods. The presence of angiogenic and anti-angiogenic factors was revealed by exchanging orthotopic corneal allografts between nude BALB/c mice and normal (hirsute) euthymic BALB/c mice and observing the presence, intensity, and degree of corneal neovascularization before and after grafting.

Results. Avascular corneal grafts from normal BALB/c donors resisted neovascularization after grafting to spontaneously vascularized graft beds in nude mice. In contrast, spontaneously vascularized corneal grafts from nude mice remained vascularized over 2 mo after grafting to similar nude recipients. Although corneal grafts from nude donors stimulated neovascularization in normal BALB/c recipients, most of the vessels regressed by the 6th week post transplantation.

Conclusions. The results confirm that there is an imbalance between angiogenic and anti-angiogenic factors in the nude mouse cornea. The cornea of the nude mouse displays more angiogenic activity and less anti-angiogenic activity than that of the normal mouse. Most angiogenic activity of the nude mouse cornea appears to reside in the epithelium. Invest Ophthalmol Vis Sci. 1993;34:222-230.

The normal cornea is avascular but may be invaded by blood vessels in a variety of pathologic conditions. Avascularity of a cornea was suggested to depend on the presence of anti-angiogenic factors and on the properties of a corneal stroma that form a barrier against penetrating blood vessels. Thus, corneal neovascularization would be caused by a decreased concentration of anti-angiogenic factors, or by a loosening of the stroma during inflammatory processes. Another important cause of the in-growth of blood vessels into cornea may be a local increase in angiogenic factors that would attract and stimulate proliferation of blood vessels. Such factors may originate from inflammatory cells that invade the cornea or from the cornea itself, and their over production might contribute to corneal neovascularization.

In our laboratory, a new model of spontaneous corneal neovascularization has been described re-
cently in nude and hairless mice. We noted that these mice show corneal neovascularization that starts in the perinatal period and progresses without a consistent inflammatory infiltrate or other apparent stimuli. These vessels persist without any sign of regression.

The aim of the present study was to test the hypothesis that this spontaneous corneal neovascularization results from a local imbalance between angiogenic and anti-angiogenic factors.

To reveal the presence of such factors and localize them in different layers of the cornea, we orthotopically transplanted full-thickness corneal buttons, as well as fragments of corneal epithelium and corneal stroma, and assessed the vascularity of the grafts and the host cornea using spontaneously vascularizing BALB/c nude and normal BALB/c mice in various donor-recipient combinations.

We found that corneas of the nude mice had much more angiogenic activity than those of normal mice and that this activity was localized in the epithelium. We also found that corneal stroma in nude mice, in contrast to normal mice, lacked anti-angiogenic activity.

**MATERIALS AND METHODS**

**Mice**

Female BALB/c and BALB/c nude mice (nu/nu) 8–10 wk old were obtained from Simonsen Laboratories (Gilroy, CA) and kept at UTSMC in Dallas in a laminar air flow room with filtered air and sterile equipment, food and water. The animals were used for experiments within 5–7 days after arrival. All animals were handled in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and the ARVO Resolution on the Use of Animals in Research. All studies adhered to the tenets of the Declaration of Helsinki.

**Corneal Grafting**

**Anesthesia.** General anesthesia was achieved with an intraperitoneal injection of sodium pentobarbital (1–2 mg/mouse; Abbott Laboratories, North Chicago, IL). For topical anesthesia, proparacaine hydrochloride (Alcon Laboratories Inc., Ft. Worth, TX) was administered, and to dilate pupils, tropicamide 1% (Alcon) was used.

**Full-Thickness Orthotopic Corneal Grafts.** The procedure was modified from that described previously. Briefly, a full-thickness (1.5 mm diameter) penetrating corneal button was obtained from the donor using a 1.5 mm trephine and vannas scissors. The recipient cornea was similarly scored with a 1.5 mm trephine, and the central 1.5 mm button was removed. The donor graft was sewn in place using seven interrupted sutures with 11-0 nylon (Alcon Laboratories Inc., Ft. Worth, TX). The anterior chamber was restored by injecting 5–10 µl sodium hyaluronate (Healon; Alcon Laboratories Inc.). Sutures were removed 10 days later. Topical Polysporin ophthalmic antibiotic ointment (Burroughs Wellcome Co, Research Triangle Park, NC) was applied after surgery, and every 2 days for 10 days. The eyelids were closed for 24 hr with a single suture 7-0 silk (Ethicon Inc., Johnson & Johnson Co., Somerville, NJ). Eyelids were re-opened 24 hr later by removing the sutures.

**Epithelium-Depleted Orthotopic Corneal Grafts.** Donor cornea was depleted of epithelium by scraping with a cotton swab under a slit-lamp microscope (the efficacy of this procedure was confirmed histologically in pilot experiments). A corneal button (containing stroma and endothelium) was procured and transplanted orthotopically as described above.

**Corneal Pocket Assay.** The intracorneal pocket assay was modified after that described by Muthukkaruppan and Auerbach. A central cornea was incised with a Seamless Steri-Sharps No. 15 surgical blade (Seamless Co., Wallinford, CT) to obtain a vertical incision 0.5 mm long that penetrated approximately one-half through the cornea. A corneal pocket then was made by inserting the surgical blade into the incision and carefully extending it up to 1 mm from the limbus. Full-thickness or epithelium-depleted corneal tissue fragments (approximately 0.5 mm in diameter) or scraped corneal epithelium from a single donor then were inserted into the pocket. The pocket fastened up spontaneously and healed in 24 hr. Topical Polysporin antibiotic ointment was applied after the procedure. The surgical procedure to form a pocket only (sham operation) did not induce vascularization.

All surgical procedures were done under sterile conditions, using a Zeiss (Thornwood, NY) slit-lamp microscope.

**Experimental Design.** Cross transplantation of corneal tissue between normal BALB/c and BALB/c (nu/nu) mice that had spontaneously vascularized corneas was done in all four possible donor-recipient pair combinations.

**Clinical Observation**

Grafted mice were observed with a slit-lamp microscope twice a week throughout the 2 mo study period. Graft opacity and edema were scored as described previously. The progress of vascularization of grafts was documented by counting the vessels growing toward the corneal graft and by examining vasodilation in the limbal region. The number, tortuosity, directionality, length, and caliber of blood vessels were noted.
Statistical Evaluation

A statistical evaluation of the results was done at Academic Computing Services at UTSMC with the help of Dr. D. D. McIntire. A three-factor full factorial design was used to compare the number of vessels per graft as a response to orthotopic corneal grafts. The factors (donor, recipient, and type of the graft) each were presented at two levels: (1) BALB/c and nude for each donor and recipient; and (2) full thickness and epithelium-depleted grafts as the two levels of graft type. Ten mice were randomized to each of the eight possible treatment combinations (2 donor X 2 recipient X 2 graft).

In studying the response to cornea fragments, a 2 donor by 2 recipient by 3 graft full factorial design was the selected approach. Each donor and recipient were presented at two levels—BALB/c and nude. The three levels for graft were full-thickness, without epithelium, and epithelium only. Statistical comparisons among the means were accomplished using analysis of variance of the full factorial design with two-factor interactions. Effects were considered statistically significant when the F value was less than 0.05.

Histologic Examination

After the animals were killed with methoxyHurane (Pitman-Moore Co., Washington Crossing, NJ), mouse eyes were collected and fixed in 10% phosphate-buffered formalin. The specimens were embedded in paraffin, sectioned, and stained with hematoxylin-eosin.

Fragments of cartilage were processed similarly to examine whether spontaneous vascularization in nude mice affects other avascular tissues.

RESULTS

Morphology of Nude Mouse Cornea

Corneas of BALB/c (nu/nu) mice showed extensive vascularization by blood vessels that penetrated from the limbus into the stroma. These vessels extended closely to the center of the cornea, leaving only a tiny central part nonvascularized. Some of them formed loops and returned to the limbus. A majority of the vessels had a rather straight course and showed few, if any bifurcations. On histologic preparations, blood vessels were located in the superficial stroma in the vicinity of the epithelial layer (Fig. 1).

Orthotopic Full-Thickness and Epithelium-Depleted Corneal Grafts

Normal BALB/c to BALB/c Donor-Recipient Combination. The neovascularization elicited by syngeneic full-thickness grafts was not noted until day 5 (Fig. 2) and persisted up to 10 days, reflecting a nonspecific response to corneal injury. The blood vessels originated from the limbus and reached the graft, but did not penetrate it. These vessels were of medium caliber and ran a straight course without loop formation. They started to regress on the 10th day after transplantation, when sutures were removed. Most of the blood vessels around the suture placement sites regressed within 4 days after suture removal, but occa-
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Fig. 2. BALB/c orthotopic corneal graft on a syngeneic BALB/c recipient. Photography taken 3 days post-transplantation. Note avascularity of the graft.

sionally some of the small vessels persisted throughout the study period. Generally, in the control syngeneic group, there were no blood vessels in the graft observed by the 14th day post-transplantation, except those few related to suture placement.

Initial, nonspecific vascular response to epithelium-depleted orthotopic corneal grafts was less intense than the response to full-thickness grafts. No vessels that penetrated the graft were seen after 14 days.

Nude BALB/c to Nude BALB/c Donor-Recipient Combination. Vascularized full-thickness grafts not only retained their vascularity, but gained new blood vessels from the host limbus. These new vessels were of medium caliber and were straight and without bifurcations. Generally, nude to nude corneal grafts stayed very well vascularized over the entire 2 mo observation period. The vascular response to epithelium-depleted grafts was lower than in full-thickness grafts, but more pronounced than in normal syngeneic BALB/c recipients.

Nude BALB/c to Normal BALB/c Donor-Recipient Combination. Vascularized full-thickness grafts stimulated the growth of host vessels from the limbus that resulted in additional graft vascularization (Fig. 3). However, most of the donor and recipient vessels started to regress around the 6th wk after transplantation; by 2 mo, no blood vessels could be seen in the grafts, and only "ghost" vessels were noted on histologic preparations.

Epithelium-depleted grafts induced less vascular response than full-thickness grafts, with similar regression of blood vessels occurring by 6 wk.

Normal BALB/c to Nude BALB/c Donor-Recipient Combination. Corneal full-thickness grafts transplanted onto vascularized recipient cornea remained avascular over the entire observation period. Initial host vascular response to such grafts was similar to that observed in syngeneic normal controls, with several blood vessels running toward the graft. However, a striking feature was that these vessels never penetrated the graft. They usually were able to reach the graft border, but then changed their direction and encircled the graft. Epithelium-depleted grafts did not induce significant vascular response.

The results of the full-thickness corneal graft experiments are summarized in Table 1.

Corneal Pocket Grafts

Normal BALB/c to BALB/c. Full-thickness and epithelium-depleted corneal fragments (0.5 mm diameter) and isolated corneal epithelium failed to induce any vascular response in the recipient corneas.

Nude BALB/c to Nude BALB/c. All types of grafts induced a strong vascular response in the already vascularized recipient corneas, but the intensity of this response was highest for full-thickness fragment grafts. The number of blood vessels always increased, but no changes in the vascularization pattern were observed.
FIGURE 3. Corneal graft from nude BALB/c donor grafted to a normal BALB/c recipient. Photograph taken 27 days post-transplantation. Note intense neovascularization of entire corneal graft.

Nude BALB/c to Normal BALB/c. Only full-thickness and isolated corneal epithelium grafts elicited the vascular response in the recipient corneas. This response was weak, and blood vessels barely reached the grafts.

Normal BALB/c to Nude BALB/c. Transplanted tissues were not vascularized by host vessels. The vessels that normally are present in the nude mouse cornea seemed to be repelled from the grafts of normal BALB/c corneal fragments (Fig. 4) but not of corneal epithelium alone. The results of the pocket graft experiments are summarized in Table 2.

TABLE 1. Vascular Response to Orthotopic Corneal Grafts, Noted 2 wk After Transplantation

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Full-Thickness Graft</th>
<th>Epithelium-Depleted Graft</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>BALB/c</td>
<td>5 (3–6)</td>
<td>4 (2–5)</td>
</tr>
<tr>
<td>Nude</td>
<td>Nude</td>
<td>23 (20–33)</td>
<td>17 (11–19)</td>
</tr>
<tr>
<td>Nude</td>
<td>BALB/c</td>
<td>18 (14–28)</td>
<td>11 (8–12)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Nude</td>
<td>6 (3–8)</td>
<td>6 (4–7)</td>
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</table>

* Results are means of newly formed blood vessels per graft with ranges in parentheses. Ten mice per group.

The statistical evaluation showed the two significant interactions: donor × recipient ($P = 0.0037$) and donor × graft ($P = 0.0001$). In both cases, the nude donors provided a greater number of vessels per graft. The full-thickness nude corneal grafts exhibited a greater mean of vessels than the epithelium-depleted grafts.

DISCUSSION

The results indicate there is an imbalance between angiogenic and anti-angiogenic factors in the spontaneously vascularized corneas of the nude mice. The cornea of the nude mouse displays more angiogenic activity than that of a normal mouse. Full-thickness corneal grafts from nude donors elicited a massive, persistent in-growth of blood vessels from the limbus in normal recipients. In contrast, similar grafts from normal mice failed to induce any significant, persistent angiogenic responses. The angiogenic activity was localized in the epithelium, because isolated nude corneal epithelium of a total volume not exceeding 0.2 mm$^3$ induced a marked angiogenic response after implantation into a corneal pocket in normal mice. This response was, however, not as massive as a full-thickness graft. This difference might be explained by the much smaller volume of the epithelial implant, compared to the full-sized corneal grafts. We found that even epi-
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FIGURE 4. Normal BALB/c full-thickness corneal fragment 14 days after transplantation into a corneal pocket in BALB/c nude mouse. Note the relative avascularity of the graft (arrowhead). Blood vessels in the superficial stroma of the nude mouse cornea are faintly visible. There is a single large caliber blood vessel (arrow) near the graft that seems to have been repelled from the graft.

Epithelium-depleted nude corneal grafts were weakly angiogenic. We speculate that this was due to an accumulation of a putative angiogenic factor that diffused into the stroma from the corneal epithelium. The concentration of such a factor probably was low and was not sustained because of the absence of corneal epithelium. Further support for the localization of putative angiogenic factors in the epithelium is suggested by the histologic observation that most of the blood vessels in the nude corneas were near epithelium.

In contrast to nude corneal grafts, isolated epithelial fragments from normal mice elicited a weak and transient vascular response in the normal host, as did normal full-thickness corneal grafts. Epithelium-depleted grafts failed to induce any neovascularization.

We also found that the corneal stroma in nude mice, in contrast to normal mice, showed diminished anti-angiogenic activity.

Epithelium-depleted corneal grafts from normal donors seemed resistant to vascular invasion when transplanted into nude mice. Although some host blood vessels initially were directed toward such grafts, they never penetrated them, and instead bypassed the grafts. This encircling movement seemed to

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Full-Thickness</th>
<th>w/o Epithelium</th>
<th>Epithelium Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>BALB/c</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nude</td>
<td>Nude</td>
<td>9 (5–10)</td>
<td>5 (4–6)</td>
<td>3 (1–4)</td>
</tr>
<tr>
<td>Nude</td>
<td>BALB/c</td>
<td>4 (2–5)</td>
<td>2 (1–4)</td>
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<tr>
<td>BALB/c</td>
<td>Nude</td>
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<td>0</td>
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</table>

* Results are means of newly formed blood vessels per graft (ranges in parentheses) noted 2 wk after transplantation. Five mice per group.

The statistical evaluation showed a significant interaction of recipient X graft (*P = 0.0103*). Vascularity of grafts from nude donors (full-thickness and w/o epithelium) increased significantly in nude recipients compared to BALB/c recipients.
be caused by the presence of a repelling substance that prevented vessel penetration. Similar morphologic events occur when blood vessels induced by a tumor or lymphocytes interact with anti-angiogenic substances from cartilage.12,13

When nude epithelium-depleted corneal grafts containing pre-existing blood vessels were transplanted to nude mice, they were penetrated by additional new blood vessels and remained vascularized over a 2 mo observation period. However, when they were transplanted into normal corneas, their stromal vessels regressed within 2 mo, probably because of an insufficient angiogenic stimulus, which would be needed to sustain the presence of vessels, or because of the presence of anti-angiogenic factors in the normal host cornea.

Taken together, our results seem to suggest that in the nude mouse cornea there is more of an imbalance in angiogenic factors (most probably epithelium derived) than in corneas of normal mice. At the same time, the corneal stroma in the nude mice seems to lack anti-angiogenic factors that are present in the corneas of normal mice.

It recently was proposed that spontaneous vascularization of the cornea in the nude and hairless mutant mouse strains results from chronic inflammation in response to persistent bacterial infections of the conjunctival cul-de-sac.14 Although we often have observed bedding and other foreign debris in the conjunctival cul-de-sac of nude and hairless mice, we have not found convincing evidence to support the aforementioned hypothesis. Histologic examination (light microscopy) of corneas collected from nude and hairless mice on days 4, 6, 8, 13, 14, 18, 21, 23, 24, 28, 30, 44, 49, 52, and 56 post-partum failed to establish a temporal relationship between the presence of polymorphonuclear neutrophils (PMNs) and corneal blood vessels (unpublished findings). Some corneas contained numerous PMNs, yet were free of corneal vessels, whereas other corneas (especially those of adult mice) contained vessels throughout the entire cornea, yet were free of PMNs. Although a significant number of corneas did contain extensive PMN inflammation and blood vessels, we are reluctant to conclude that the presence of corneal vascularization in nude and hairless mice is a direct result of chronic inflammation, because the association between vessels and PMNs is very inconsistent. Moreover, the inherent angiogenic activity of nude corneal grafts transplanted to hirsute BALB/c recipients and the anti-angiogenic activity of BALB/c corneas grafted to nude recipients further argues against the proposition that corneal vascularization in nude and hairless mutant mouse strains simply is the product of chronic inflammation. Nonetheless, this issue warrants further scrutiny and could be resolved by a carefully controlled prospective study.

The vascularization in nude mice seemed to affect only corneas, because histologic observations of other avascular tissues, such as cartilage, revealed normal structure with no blood vessels. Another possibility for spontaneous neovascularization could be an excessive susceptibility of nude mouse vascular endothelium to angiogenic stimuli. The in vivo reactivity of nude vascular endothelium to angiogenic stimuli from tumor and lymphoid cells was tested in vivo using a modified lym-
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Spontaneous corneal neovascularization in nude mice probably is related to an excess of angiogenic factors and a deficiency of anti-angiogenic factors. The balance between angiogenic factors (mainly from corneal epithelium) and anti-angiogenic factors from corneal stroma thus may be crucial for maintaining the avascular state of a normal cornea.

This model of spontaneous corneal neovascularization in nude mice may help gain a better understanding of the mechanisms that control the ingrowth, persistence, and regression of blood vessels into the cornea.

Key Words

angiogenic factors, anti-angiogenic factors, cornea, neovascularization, nude mice.

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References


