Angiostatin Inhibits and Regresses Corneal Neovascularization

Balamurali K. Ambati, MD; Antonia M. Joussen, MD; Jayakrishna Ambati, MD; Yasufumi Moromizato, MD; Chandan Guha, MBBS, PhD; Kashi Javaherian, MD; Stephen Gillies, PhD; Michael S. O’Reilly, MD; Anthony P. Adamis, MD

Objective: To determine the ability of angiostatin and the angiostatin-producing low-metastatic (LM) clone of Lewis lung carcinoma (LLC) to inhibit and regress corneal neovascularization, as compared with the non-angiostatin-producing high-metastatic (HM) clone.

Methods: Three groups of C57BL6/J mice underwent chemical and mechanical denudation of corneal and limbal epithelium. One group remained tumor free while the other 2 were implanted with LLC cells (either the HM or LM clones) subcutaneously the day before, 2 weeks after, or 4 weeks after denudation. Corneas were harvested 2 weeks after tumor implantation (at 2, 4, and 6 weeks after denudation for tumor-free mice). Neovascularization was quantified by CD31 immunostaining. In a second experiment, recombinant angiostatin was delivered continuously for 2 weeks via an osmotic pump in mice with established corneal neovascularization.

Results: The mean percentages of neovascularized corneal area in mice 2 weeks after LM-LLC implantation were 4.6%, 3.7%, and 37.0%, at 2, 4, and 6 weeks after scraping, respectively. In contrast, in the mice implanted with HM-LLC, the corresponding values were 45.4% (P =.01), 90.1% (P =.03), and 80.3% (P =.005). For tumor-free mice, the corresponding values were 62.0% (P =.003), 68.9% (P =.03), and 59.3% (P =.06). Mice implanted with angiostatin pumps had a 37.7% neovascularized corneal area 2 weeks after implantation and 4 weeks after scraping while mice implanted with sham pumps had 60.5% (P =.007).

Conclusion: Angiostatin inhibits and regresses corneal neovascularization induced by mechanical and alkali corneal injury.

Clinical Relevance: This appears to be the first evidence of biologically induced regression of corneal neovascularization, and the first direct demonstration of angiostatin-induced regression of neovascularization in any tissue.

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C ORNEAL neovascularization is a central feature in the pathogenesis of many blinding corneal disorders and a major sight-threatening complication in corneal infections, chemical injury, and following keratoplasty, in which neovascularization adversely affects corneal graft survival. Antiangiogenic molecules have been shown to inhibit corneal neovascularization. However, no biologic agent has been shown to regress established corneal neovascularization, a more clinically relevant end point. Thermal laser or photodynamic therapy induces only temporary closure of new vessels and does not address the underlying biological mechanisms of neovascularization.

Angiostatin, an endogenous cleavage fragment of plasminogen, is secreted by a form of Lewis lung carcinoma (LLC) and inhibits basic fibroblast growth factor-induced corneal neovascularization in mice. Its antiangiogenic potential has been verified in numerous tumor models. We investigated the potential of an angiostatin-producing tumor to inhibit and regress corneal neovascularization in a pathophysiologically relevant murine model. In a confirmatory experiment, we also investigated the ability of pure recombinant angiostatin delivered systemically via an osmotic pump to regress corneal neovascularization in the same model.

RESULTS

There were no significant differences in corneal neovascularization between corresponding groups of control mice and mice implanted with HM-LLC (all P values >0.1).

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DESIGN AND METHODS

All animal experiments were approved by the Massachusetts Eye and Ear Infirmary (Boston) animal care committee and conformed to the Association for Research in Vision and Ophthalmology guidelines for animal use. Male C57BL/6j mice (Jackson Labs, Bar Harbor, Me) were anesthetized by intramuscular injection of 50 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine. Animals were killed by a lethal dose of pentobarbital (150 mg/kg).

EXPERIMENTAL DESIGN

Three groups of mice underwent corneal injury. One group (referred to hereafter as control) remained tumor free and the other 2 were implanted with LLC tumor cells (either the high-metastatic [HM] or low-metastatic [LM] clones) subcutaneously the day before, 2 weeks after, or 4 weeks after injury by an investigator masked to the clone type. Corneas were harvested 2 weeks after tumor implantation (at 2, 4, and 6 weeks after injury for tumor-free mice). In a separate regression experiment, angiostatin pumps were implanted 2 weeks after corneal injury. Corneas were harvested 2 weeks after implantation and compared with corneas from control mice (implanted with pumps containing phosphate-buffered saline [PBS] alone) harvested 4 weeks after corneal injury. Therefore, the time of treatment with angiostatin was 2 weeks in all experimental groups. Neovascularization was quantified in all corneas by immunostaining, using an image analyzer by a masked investigator.

MODEL OF CORNEAL NEOVASCULARIZATION

Topical proparacaine and 2 µL of 0.15M sodium hydroxide were applied to the right cornea of each mouse. The corneal and limbal epithelia were removed using a Toeke corneal knife (Arista Surgical Supply, New York, NY) in a rotary motion parallel to the limbus. Erythromycin ophthalmic ointment was instilled immediately following epithelial denudation.

MODEL OF LLC IMPLANTATION

Low-metastatic LLC tumors were passaged in vivo3 and HM-LLC cells were passaged in vitro.5 Recipient mice had their backs shaved, and the recipient site was cleansed with povidone-iodine and ethanol. The subcutaneous dorsa of the mice in the proximal midline were injected with 0.1 mL containing 10^6 cells of the respective tumor cell lines.

ANGIOSTATIN PUMP IMPLANTATION

Murine angiostatin was produced as a fusion protein with the murine immunoglobulin 2a Fc fragment (mFc-m-angiostatin) in a murine myeloma cell line as described

PERCENTAGE AREA OF CORNEAL NEOVASCULARIZATION

Inhibition Groups

Mean percentages of neovascularized corneal area in mice 2 weeks after tumor scraping and implantation are shown in Figure 1. The mean percentages of neovascularized corneal area in mice 2 weeks after denudation in control mice, mice implanted with LM-LLC, and mice implanted with HM-LLC were 62.0%, 4.6%, and 45.4%, respectively. Thus, mice implanted with LM-LLC had 89.9% less neovascularized corneal area than those implanted with HM-LLC (P = .01) and 92.6% less neovascularized corneal area than control mice (P = .003).

Regression Groups

Mean percentages of neovascularized corneal area in mice 2 weeks after tumor implantation and 4 or 6 weeks after corneal scraping are shown in Figure 2. The mean percentages of neovascularized corneal area in mice 4 weeks after denudation in control mice, mice implanted with LM-LLC, and mice implanted with HM-LLC were 68.9%, 3.7%, and 90.1%, respectively. Thus, mice implanted with LM-LLC had 95.9% less neovascularized corneal area than those implanted with HM-LLC (P = .03) and 94.6% less neovascularized corneal area than control mice (P = .03).

The mean percentages of neovascularized corneal area in mice 6 weeks after denudation in control mice, mice implanted with LM-LLC, and mice implanted with HM-LLC were 59.3%, 37.0%, and 80.3%, respectively. Thus, mice implanted with LM-LLC had 53.9% less neovascularized corneal area than those implanted with HM-LLC (P = .005) and 37.6% less neovascularized corneal area than control mice (P = .06). Representative photographs are shown in Figure 3.
Previously, the protein was diluted in PBS, filtered through a Millipore filter (Millipore, Bedford, Mass), and stored at −20°C until used. The purity of the mFc-m-angiostatin ranged between 90% and 95% (data not shown). The murine recombinant angiostatin was continuously delivered with a mini-osmotic pump. Mini-osmotic pumps with a pump rate of 1.0 µL per hour were implanted into the intraperitoneal cavity 2 weeks after limbal injury (n=6 animals). The total delivered dose of angiostatin was 10 mg/kg per day. After 8 days, the pumps were exchanged and animals were killed 2 weeks after the first pump was implanted. Control animals received pumps delivering equal amounts of PBS (n=7 animals).

LABELING OF CORNEAL NEOVASCULARIZATION

Immunohistochemical staining for vascular endothelial cells was performed on corneal flat mounts. Fresh corneas were dissected, rinsed in PBS for 30 minutes, and fixed in 100% acetone for 20 minutes. After washing in PBS, nonspecific binding was blocked with 0.1M PBS, 2% albumin for 1 hour at room temperature. Incubation with fluorescein isothiocyanate conjugated–coupled monoclonal antimouse CD31 antibody at a concentration of 1:500 in 0.1 M of PBS, 2% albumin at 4°C overnight was followed by subsequent washes in PBS at room temperature. Corneas were mounted with Gelmount (Biomeda Inc; San Francisco, Calif), an antifading agent, and visualized with a fluorescent microscope.

PERCENTAGE AREA OF CORNEAL NEOVASCULARIZATION: ANGIOSTATIN PUMP VS CONTROL PUMP

Mean percentages of neovascularized corneal area in mice 2 weeks after pump implantation and 4 weeks after corneal scraping are shown in Figure 4. Mice with angiostatin pumps inserted had 37.7% less neovascularized corneal area than mice with sham pumps (P=0.007).

TUMOR BURDEN

No animal that had been implanted with LM-LLC had visceral metastases, whereas all animals implanted with HM-LLC were noted to have liver and/or lung metastases. The mean±SD weights of LM-LLC mice at 2, 4, and 6 weeks after implantation were 16.5±0.5 g, 19.3±0.3 g, and 21.8±0.6 g, respectively. The mean±SD weights of HM-LLC mice at 2, 4, and 6 weeks after implantation were 16.5±0.5 g, 19.3±0.3 g, and 22.0±0.4 g, respectively (all P values >0.1).

COMMENT

These data demonstrate that the LM clone of LLC, an angiostatin-producing tumor, can inhibit and regress corneal neovascularization induced by corneal injury secondary to mechanical and alkali trauma. In contrast, HM-LLC, a spontaneous variant of the LM-LLC tumor that is unable to generate angiostatin, was unable to inhibit angiogenesis or regress neovascularization. These data were confirmed in experiments showing the regression of corneal neovascularization induced by recombinant angiostatin delivered systemically via an osmotic pump. To our knowledge, this is the first report of biologically induced regression of corneal neovascularization as well as the first direct demonstration in any tissue of regression of blood vessels likely mediated by angiostatin.

TABLE 1: PERCENTAGE AREA OF CORNEAL NEOVASCULARIZATION

<table>
<thead>
<tr>
<th>Time</th>
<th>LM-LLC</th>
<th>HM-LLC</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.4</td>
<td>0.8</td>
<td>0.005</td>
</tr>
<tr>
<td>Week 6</td>
<td>0.6</td>
<td>1.0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Figure 2. Mean fraction of corneal neovascularization (±SEM) 2 weeks after tumor implantation and 4 weeks after corneal scraping in control mice (solid) (n=3), mice implanted with low-metastatic Lewis lung carcinoma (LM-LLC) (n=3), and mice implanted with high-metastatic LLC (HM-LLC) (n=3), and mice implanted with HM-LLC (n=4).
The results indicate near complete inhibition of corneal neovascularization by LM-LLC (in accordance with previous data) and near-complete regression of corneal neovascularization when LM-LLC was implanted 2 weeks after corneal injury. The regressive effect was substantially less when LM-LLC was implanted 4 weeks after injury. This may indicate that the more mature vessels are less amenable to the ability of angiostatin to induce regression or they may require further exposure to angiostatin to regress. This was not addressed in our study, as mice generally died of tumor burden from LM-LLC between 18 and 24 days, and supplies of recombinant angiostatin pumps did not permit longer-term or varied dose-effect experiments. The mechanisms of the regression of the new vessels are not fully characterized. Down-regulation of vascular endothelial growth factor can induce the regression of capillaries not covered by pericytes in the corpus luteum of ovaries, while increased levels of angiopoietin-2 destabilize mature capillaries and induce their regression.9 It would be valuable to determine to what extent angiostatin is capable of the 2 effects.

Angiostatin inhibits the development of basic fibroblast growth factor– and vascular endothelial growth factor–induced corneal neovascularization in mice.3 Other studies have shown that thalidomide, curcumin, integrin antagonists, cyclooxygenase inhibitors, prolactin, octreotide, herbal extracts, matrix metalloproteinase inhibitors, angiostatic steroids, thrombospordin-2, kringles 1-3, and beta-cyclodextrin tetradecasulfate all exert inhibitory effects on the development of corneal neovascularization induced by various methods.10-23 Our study demonstrates the ability of angiostatin to inhibit neovascularization in a clinically relevant model of mechanical and chemical corneal trauma. The method of neovascularization induction affects pharmacological efficacy, eg, integrin antagonists inhibit basic fibroblast growth factor–induced corneal neovascularization but not that caused by chemical injury.10 Moreover, this study demonstrates for the first time, to our knowledge, the biologically induced regression of corneal neovascularization. The only other interventions demonstrated to affect established corneal neovascularization have been laser photocoagulation, fine-needle diathermy, and photodynamic therapy.24-28 However, these methods do not induce biological vessel regression, as was observed with angiostatin. Angiostatin’s ability to inhibit blood vessels on the basis of tumor regression and endothelial apoptosis has been demonstrated in culture9,29; our study provides a direct in vivo correlate.

The antiangiogenic effect observed in animals implanted with LM-LLC, as opposed to HM-LLC, has been...
localized to angiostatin. O’Reilly et al\(^7\) purified the serum and urine of LM-LLC and HM-LLC mice with reverse-phase chromatography and gel electrophoresis and assayed different fractions for inhibition of endothelial proliferation; inhibitory activity corresponded to angiostatin. Dong et al\(^8\) performed fast protein liquid chromatography and Western blot analysis and found that the antiangiogenic activity of serum from LM-LLC mice localized to angiostatin and also found that a monoclonal antibody against angiostatin blocked the antiangiogenic effect (measured by an assay of endothelial cell proliferation) of LM-LLC serum in culture. O’Reilly et al\(^8\) found that blocking the production of angiostatin by LM-LLC cells in culture using antibodies to gelatinase A (required for angiostatin production) eliminated the antiangiogenic effect of these cells, and that only fractions generated by gelatinase A that contained angiostatin had antiangiogenic effects (measured by ability to inhibit endothelial cell proliferation).

The level of regression in tumor-implanted mice was far greater than that induced by the angiostatin pump. This may be owing to several factors. The concentration of angiostatin produced by the tumor may be much higher than that delivered by the pump, although it is not possible to quantitate serum or urine levels of angiostatin because of lack of commercial assays. The concentration of angiostatin in the tumor model is also variable and likely steadily rises as the tumor grows, and this variability may enhance the regressive effect.

In our study, the tumor burden was not significantly different between the LM-LLC and the HM-LLC groups. The HM-LLC clone arose as a spontaneous variant of the LM-LLC clone in mice and it did not produce angiostatin in vitro or in vivo. Therefore, the antiangiogenic effects observed herein can very likely be attributed to angiostatin. The implantable pump study results are consistent with this conclusion.

Digital quantification of corneal neovascularization, described previously\(^7,8\) using flat-mounted corneas, can potentially be confounded by the 3-dimensional nature of specimens and the density of blood vessels. The dehydration of corneas prior to analysis and the use of a microscope with a good depth of field minimize the effect of 3-dimensionality. This is especially true in the very thin mouse cornea. The density of blood vessels in corneas with a high degree of neovascularization is potentially a problem because individual vessels sometimes cannot be discerned, leading to an overestimation of vascularization as areas of clear cornea in between are not resolvable. This is countered by the effect of underestimation of overlapping vessels. Previous investigators\(^7,8\) have found the magnitude of these errors to be small and not to affect the consistency among samples.

In summary, angiostatin inhibits and regresses the corneal neovascularization induced by mechanical and alkali corneal injury. This finding could advance the management of blinding disorders, such as Stevens-Johnson syndrome, cicatricial pemphigoid, corneal allograft rejection, and corneal injury from infection, trauma, or alkali. The potential of topical or sustained-release forms of angiostatin with distribution constrained to the eye should be a next line of investigation. Topical forms of angiostatin have already been used in the rat and rabbit.\(^31,32\) Gene transfer methods generating angiostatin have been developed to halt angiogenesis in mouse tumor models.\(^33,34\) and naked plasmids have been shown to be taken up by corneal epithelium.\(^35\) Future research should also determine the effect of recombinant angiostatin in this model and the molecular interactions among angiostatin, inflammatory phenomena, and neovascularization. This model can also be extended to neovascularization in other ocular tissues, including the iris, retina, and choroid.

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Corresponding author and reprints: Anthony P. Adams, MD, Department of Ophthalmology, Massachusetts Eye & Ear Infirmary, 243 Charles St, Boston, MA 02114 (e-mail: tony_adams@meei.harvard.edu).

REFERENCES

24. Fig. 1. The optic fundus shows marked excavation with absent central vessels in the left (A) and right (B) eyes of a 17-year-old who underwent renal transplantation.