Prevention of Exuberant Granulation Tissue and Neovascularization in the Rat Cornea by Naltrexone

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Objective: To determine whether topical application of naltrexone prevents exuberant granulation tissue formation with neovascularization in diabetic rat corneas.

Methods: Diabetes was induced with streptozotocin. A 5-mm corneal abrasion at 9 or 11 weeks was treated topically for 7 days (4 times daily) with naltrexone or a sterile vehicle.

Results: Within 2 to 5 days after reepithelialization, diabetic rats given the sterile vehicle had a 41% incidence of corneal lesions represented by exuberant granulation tissue with corneal neovascularization extending from the limbus. These lesions exhibited edema, cellular and vascular inflammation, and disruption of stromal lamella by fibrovascular tissue and calcium mineralization, but infection was not detected. No corneal lesions were recorded in the diabetic group treated with naltrexone or the control group given the sterile vehicle. Diabetic rats with corneal lesions given the sterile vehicle reepithelialized more slowly than diabetic rats given the sterile vehicle without such lesions, but no difference in blood glucose levels were noted.

Conclusions: Using a minimally invasive model in diabetic rats, topical naltrexone normalizes corneal wound healing and prevents neovascularization.

Clinical Relevance: Direct application of naltrexone may serve as an important strategy for facilitating corneal healing and inhibiting corneal neovascularization.


CORNEAL NEOVASCULARIZATION has been estimated to affect 1.4 million Americans annually. Corneal neovascularization is associated with a wide variety of conditions, including contact lens wear, surgical and nonsurgical trauma, alkali and other chemical burns, and infection. Corneal neovascularization may be helpful in combating infections, assisting in corneal healing, and arresting autoimmune corneal melting. Nevertheless, such vascularization can lead to corneal scarring, edema, lipid deposition, and inflammation that may significantly compromise corneal transparency and result in severe visual impairment, including blindness. The etiology of corneal neovascularization is unclear, though the association of stromal edema proximal to the limbus has been proposed as necessary to allow blood vessels into the usually compact stroma. Treatment modalities include surgery, topical corticosteroids, angiostatic steroids, nonsteroidal anti-inflammatory agents, and natural inhibitors of angiogenesis. However, treatments of ocular neovascularization may have limitations, such as recurrence, adverse effects, and patients being ineligible for therapy. Thus, models of ocular neovascularization, as well as treatment regimens for corneal lesions with neovascularization, are needed.

In previous investigations, we have reported that topical treatment with the opioid antagonist naltrexone facilitates corneal reepithelialization in healthy and diabetic corneas. Subsequent preliminary histological examination has revealed that a subset of reepithelialized poorly controlled diabetic rat corneas exhibit exuberant granulation tissue formation with stromal neovascularization. These observations have led to the present study, which examines the hypothesis that topical application of the opioid antagonist naltrexone prevents granulation tissue formation and accompanying neovascularization in a unique model of delayed wound healing in rats with type 1 diabetes.
ANIMALS AND INDUCTION OF DIABETES

We obtained male Sprague-Dawley rats (weight, approximately 175 g) from Charles River Laboratories (Wilmington, Massachusetts) and housed them under standard laboratory conditions. All investigations conformed to the regulations of the Association for Research in Vision and Ophthalmology and the Institutional Animal Care and Use Committee of the College of Medicine at Pennsylvania State University.

Type 1 diabetes was induced according to Havel et al and Ahren et al. An intraperitoneal injection of 40 mg of streptozotocin (Sigma-Aldrich Corp, St Louis, Missouri) per kilogram of body weight in 0.5 mol/L of ice-cold citrate buffer (pH 4.5) was administered. A second dose of streptozotocin (40 mg/kg) was injected 24 hours later. This regimen produced insulin-deficient diabetes in 100% of the animals within 48 to 72 hours; this group consisted of 65 rats. Animals not receiving streptozotocin that were injected with citrate buffer served as the control group; this group consisted of 26 rats.

Blood glucose levels were monitored from the tail vein using a True Track Smart System Glucometer (Home Diagnostics Inc, Ft Lauderdale, Florida). The encircled corneal epithelium was removed under a dissecting microscope with a tungsten light source and a gelatin Wratten No. 47 filter and photographed with a charge-coupled device camera at 1.5× magnification. Photographs were obtained immediately after epithelial debridement (0 hours) and 16, 24, 32, and 40 hours later. No animal was photographed at intervals shorter than 12 hours to prevent disruption of the healing process. The area of defect was measured using Optimas software (Meyer Instruments Inc, Houston, Texas) and was calculated as the percentage of the original epithelial defect.

HISTOLOGY

Animals were euthanized in a Plexiglas chamber attached to an isoflurane vaporizer, and the residual epithelial defect was stained with topical fluorescein. Rat eyes were viewed using a dissecting microscope with a tungsten light source and a gelatin Wratten No. 47 filter and photographed with a charge-coupled device camera at 1.5× magnification. Photographs were taken immediately after epithelial debridement (0 hours) and 16, 24, 32, and 40 hours later. No animal was photographed at intervals shorter than 12 hours to prevent disruption of the healing process. The area of defect was measured using Optimas software (Meyer Instruments Inc, Houston, Texas) and was calculated as the percentage of the original epithelial defect.
pentobarbital; eyes were enucleated and placed in formalin for 24 hours and prepared for paraffin embedding. Vertical sections (8 µm) that included the corneal surface, limbus, and conjunctiva were processed using hematoxylin-eosin, Masson trichrome, Brown-Hopps, or von Kossa staining protocols.

STATISTICAL ANALYSIS

Body weights and glucose measurements were analyzed using the 2-tailed *t* test. The area of residual defect was analyzed each time using 1-way analysis of variance, with subsequent analysis by the Newman-Keuls test. The incidence of corneal lesions was analyzed using the χ² test.

RESULTS

INDUCTION OF DIABETES

All rats weighed a mean (SE) of 174 (2) g at the time of streptozotocin injections (Figure 1A). Control rats gained approximately 301 g during the 8 weeks. Rats in the diabetic group were comparable in body weight with control animals until 2 weeks after injection of streptozotocin. At this time, the diabetic group had a 15% reduction (*P* < .001) in body weight relative to the control animals. Diabetic rats weighed significantly less (approximately 25%-35%) than control rats beginning week 4 and throughout the study.

Mean (SE) baseline glucose readings were 139 (8) mg/dL for all rats, and these values were consistent in the control group throughout the study (Figure 1B). Rats receiving streptozotocin became hyperglycemic within 5 days and had glucose levels greater than 450 mg/dL throughout the duration of experimentation.

SLITLAMP OBSERVATIONS OF THE CORNEA

Within 2 to 5 days after reepithelialization, 41% of the 29 diabetic animals receiving the sterile vehicle exhibited corneas with exuberant granulation tissue and blood vessels extending from the limbus to the corneal lesion (Figure 2). No additional diabetic animals receiving the sterile vehicle exhibited corneal lesions after this period. However, those animals with corneal lesions often had changes in severity of this complication (Table). None of the animals in the control group receiving the sterile vehicle or in the diabetic group receiving either 10⁻⁴ or 10⁻⁵ M of naltrexone presented with corneal lesions during the study. The incidence of corneal lesions in the diabetic rats receiving the sterile vehicle differed significantly (*P* < .001) from those recorded for the control group receiving the sterile vehicle and diabetic group receiving naltrexone.

HISTOLOGY

Examination of the corneas of rats in all groups revealed a complete epithelial layer by 2 to 3 days after debridement. However, within 4 to 7 days after mechanical abrasion, animals in the diabetic group receiving the sterile vehicle were identified with corneal lesions, segmented leukocytes in clefts in some areas of the stroma, and numerous spindle cell nuclei and melanin pigment. Edema in the stroma was observed in these animals. Rats in the diabetic group receiving the sterile vehicle that had corneal lesions exhibited capillaries containing red blood cells in the stroma, particularly at the margins of the cornea. Moreover, in some of these specimens, the epithelial layer appeared to be detached from the corneal surface.

Histological examination of sections of corneas collected 3 weeks after debridement from diabetic rats receiving the sterile vehicle were characterized by cellular and vascular indications of inflammation and edema (Figure 3). Focal necrosis and loss of surface epithelium with segmented leukocytes and macrophages were visible within the subjacent stroma. The inflammatory cells and capillary proliferation disrupted the normally transparent lamellar pattern of collagen within the stroma (Figure 3F). There was focal degeneration and mineralization of the subepithelial basement membrane that was confirmed positive for calcium with the von Kossa stain (data not shown). Examination of sections stained with the Brown-Hopps tissue gram stain revealed an absence...
of bacteria associated with the corneal lesions. Animals in the control group receiving the sterile vehicle, as well as rats in the diabetic group receiving naltrexone at concentrations of $10^{-4}$ and $10^{-5}$ M, did not exhibit any histological abnormalities.

**CORNEAL REEPITHELIALIZATION**

The 5-mm trephine demarcated the entire corneal region of the rat eye but did not encroach on the limbus or conjunctiva (Figure 4A). Wound healing occurred in a manner consistent with previous studies in healthy rats, rabbits, and humans as well as in diabetic rats.9,13,14 The initial area of the abrasion ranged from 19.3 mm$^2$ to 25.6 mm$^2$ and corresponded to corneal injuries of 4.9 to 5.7 mm in diameter. No differences in the size of the initial abrasions were noted between groups.

Diabetic animals (with or without corneal lesions) receiving the sterile vehicle had corneal epithelial defect measurements that indicated a significant retardation from the control group receiving the sterile vehicle at 32 ($P < .05$) and 40 ($P < .001$) hours and from the diabetic groups given $10^{-4}$ M or $10^{-5}$ M naltrexone treatment at 16 ($P < .05$), 24 ($P < .001$), 32 ($P < .01$), and 40 ($P < .001$) hours (data not shown). The diabetic animals receiving the sterile vehicle that had corneal lesions had greater epithelial defects than diabetic rats receiving the sterile vehicle without corneal lesions at 16 and 40 hours (Figure 4). Diabetic animals receiving naltrexone at concentrations of either $10^{-4}$ Mo r1 $10^{-5}$ M did not differ from each other in corneal reepithelialization at any time (data were collapsed for comparison). However, the diabetic
animals receiving naltrexone had smaller defects than control animals receiving the sterile vehicle at 24 hours but were comparable at 16, 32, and 40 hours.

**BLOOD GLUCOSE LEVELS**

In the diabetic group receiving the sterile vehicle, blood glucose levels in animals exhibiting corneal lesions did not differ from glucose levels in rats without corneal lesions at 1, 4, and 8 weeks. Specifically, mean (SE) blood glucose levels were 564 (18) mg/dL for 12 diabetic rats receiving the sterile vehicle that exhibited corneal lesions and 524 (19) mg/dL for 17 diabetic rats receiving the sterile vehicle that had no corneal lesions.

Our study reveals that more than 40% of rats with poorly controlled diabetes and corneal abrasions exhibit exuberant granulation tissue formation with neovascularization as determined by slitlamp microscopy and confirmed with histopathology. This abnormality following reepithelialization of the cornea, with indications at the histological level, was recorded as early as 2 days after debridement. These complications involved inflammation, edema, mineralization, and neovascularization but did not appear to include an infectious process.

Our data show that these complications following corneal epithelial repair can be completely prevented by topical treatment with either $10^{-10}$ or $10^{-11}$ M of naltrexone. Interestingly, diabetic animals with corneal lesions were found to be correlated with significantly slower rates of reepithelialization than those detected in diabetic rats without corneal lesions. These results would suggest that the appearance of granulation tissue with neovascularization may be related to an increased susceptibility for damage in these diabetic corneas brought on by delays in repair of injury. In this regard, it is well known that there is an irregular thickening and multilamination of the epithelial basement membrane in diabetic humans and animals. It may be conjectured that the subset of diabetic animals displaying more exaggerated delays in reepithelialization than others have a problem with the integrity of the basement membrane. However, we did find that the blood glucose levels in both groups were similar; so it can be concluded that the magnitude of hyperglycemia was not an issue in this regard. Presumably, the exuberant granulation tissue disrupts the repaired corneal epithelium as the excessive granulation tissue protrudes above the level of the surrounding epithelium. Given the edema in the stroma and the appearance of new blood vessels from the limbus, our observations support the hypothesis of Cogan, that stromal edema occurring near the limbus may be necessary to allow blood vessels into the corneal stroma. Thus, for the first time, we have defined a model for a pathologic response to complications in reepithelialization that involves exuberant granulation tissue formation and neovascularization. Moreover, we have demonstrated a treatment modality using naltrexone that aborts this process.

The mechanism(s) concerning naltrexone's capacity to attenuate neovascularization in the repair of the abraded cornea of diabetic rats is unclear. One possibility is that naltrexone directly diminishes the proliferation of these blood vessels. However, it is known that 5 µg of naltrexone placed on a methylcellulose disc stimulates angiogenesis (blood vessel number and length) compared with controls given vehicles in a chick chorioallantoic membrane preparation. Moreover, naltrexone at a dosage that induced a continuous opioid receptor blockade (ie, 30 mg/kg daily) has been reported to elevate DNA synthesis in the intima and media and to increase intimal thickness and reduce luminal area in the carotid artery that was denuded with balloon catheterization in comparison with vehicle-exposed control rats. These data are consistent with findings that sustained opioid receptor blockade with naltrexone or other opioid antagonists results in enhanced DNA synthesis in tissues undergoing development, cellular renewal, or repair as well as in neoplasia. Thus, the evidence does not appear to support the hypothesis that naltrexone has a direct inhibitory effect on neovascularization. Another possible mechanism regarding naltrexone treatment and diminished neovascularization is based on the observation that the cornea of the diabetic rats receiving the sterile vehicle heals slower than that in the control group receiving the sterile vehicle.

In the current study, when the diabetic group receiving the sterile vehicle is subdivided into those with and without corneal lesions, those with corneal lesions exhibited even more retarded reepithelialization than those without corneal lesions. This finding suggests that the pace of repair of corneal defects is more highly associated with the appearance of neovascularization, with a greater risk of neovascularization in animals with the slowest rates of reepithelization. This hypothesis is consonant with the observation that diabetic animals exposed to topical naltrexone have an accelerated rate of corneal wound healing comparable with that of the control animals receiving sterile vehicles, and these diabetic rats receiving naltrexone did not display neovascularization. Thus, it could be conjectured that the mechanisms for the promotion of neovascularization in the cornea of diabetic animals may be factors and events occurring in the extended period of reepithelialization. If this is the case, then a test of this hypothesis would be an examination of the repercussions of a drug- or mechanical-induced retardation in corneal repair in diabetic rats, which would be predicted to increase the incidence of neovascularization. Finally, it is known that naltrexone treatment restores corneal sensitivity in diabetic rats. In this manner, it may optimize the homeostatic milieu and thereby facilitate healing by reestablishing the blink reflex and tear production.

The complications following corneal reepithelialization in rats we documented has not been reported in humans. One factor that may contribute to the development of the corneal lesions we observed is the severity in degree and duration of hyperglycemia (blood glucose levels in these rats were routinely $>450$ mg/dL for $>8$ weeks). Moreover, humans have a Bowman membrane that is absent in rats. If a corneal abrasion in a rat can be equated to a superficial corneal ulcer in humans, the lack of this ana-
tomic barrier (ie, the Bowman membrane) in the rat could make their corneas more susceptible to inflammatory mediators accompanying reepithelialization. Further contributing to the complications following corneal epithelial repair in these poorly controlled diabetic animals may be the presence of decreased corneal sensitivity that we have previously reported, which can be remedied by topical treatment with naltrexone. Decreased corneal sensitivity would be expected to decrease tear production and/or blink reflex, thereby subjecting the newly repaired cornea to an adverse environment.

The healthy human cornea is free from blood vessels, but neovascularization is a common clinical problem seen as a response to chronic hypoxia or various inflammatory stimuli, such as bacterial keratitis, alkali burns, and graft rejection. The severity of clinical disorders involving corneal neovascularization, which, untreated, may lead to pronounced visual impairment, emphasizes the importance of broadening our base of knowledge in this field. That the model we describe is occurring on the ocular surface makes these lesions very accessible for defining related structural, biochemical, and physiological events, particularly neovascularization. Additionally, such a model provides the unique opportunity to delineate the effect of various treatment regimens. Therefore, for the first time, we have characterized a unique model of exuberant granulation tissue with neovascularization that accompanies complications of corneal reepithelialization that can be tested for toxicity and efficacy of therapeutic modalities.

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References


