Regulation of Corneal Repair by Particle-Mediated Gene Transfer of Opioid Growth Factor Receptor Complementary DNA

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Objective: To determine whether molecular manipulation of the opioid growth factor receptor (OGFr) alters corneal reepithelialization following central corneal abrasion in rats.

Methods: The plasmid pcDNA3.1 + OGFr, carrying the rat OGFr complementary DNA in both the sense and antisense orientations, and empty vector (EV), were delivered by gene gun to the rat cornea. After 24 hours, corneas were abraded and reepithelialization was documented by fluorescein photography. Twenty-four hours after wounding, DNA synthesis (with bromodeoxyuridine) was examined.

Results: Eyes transfected with sense constructs of OGFr had corneal defects that were 24%, 52%, and 50% larger than the EV group at 16, 24, and 28 hours, respectively. Conversely, corneas transfected with antisense constructs of OGFr had corneal defects that were 56% and 48% smaller than the EV group at 16 and 24 hours, respectively. Bromodeoxyuridine labeling in the basal and suprabasal layers of the antisense group were increased 3.3- and 3.7-fold, respectively, in DNA synthesis from corresponding EV layers; DNA synthesis was comparable in the sense and EV groups.

Conclusions: Excess OGFr delays reepithelialization, whereas attenuation of OGFr accelerates repair of the corneal surface.

Clinical Relevance: Inhibition of opioid growth factor action using gene therapy could be important in the treatment of corneal diseases such as nonhealing and recurrent erosions, diabetic keratopathy, and neurotrophic keratitis.

Delayed corneal epithelial wound healing and persistent epithelial defects are serious clinical problems that threaten the integrity of the optic globe. Our research has demonstrated that an intrinsic growth regulatory system, consisting of the opioid growth factor (OGF) and its receptor (OGFr), plays a vital role in corneal epithelial homeostasis and reepithelialization. Moreover, we have shown that corneal epithelial wound healing can be pharmacologically modulated using this system. The naturally occurring OGF (chemically termed [Met5] enkephalin) functions as a tonically active, receptor-mediated (ie, OGFr) inhibitory growth factor. The addition of exogenous OGF downregulates cell division and inhibits corneal epithelial wound healing. Conversely, blockade of OGF-OGFr interactions by exogenous naltrexone, an opioid antagonist, increases the rate of DNA synthesis and cell replication, and increases the rate of corneal reepithelialization.

In the present study, we used a gene gun to introduce sense and antisense complementary DNA (cDNA) for OGFr to establish that the effects of OGF on corneal reepithelialization are specifically dependent on the OGFr.

Methods

Plasmids

The nucleotide sequence for rat OGFr is deposited in GenBank (AF156878). Following methods in our previous report, plasmids containing pcDNA3.1 + OGFr in either the sense orientation or the antisense orientation were prepared; OGFr was under the control of the cytomegalovirus immediate early gene enhancer/promoter. An empty vector (EV) pcDNA3.1 + was transfected into some animals as a control.

In Vivo Gene Transfer

Plasmid DNA was absorbed onto 1.6-µm gold particles at concentrations of 2 µg DNA per milligram of gold particles. Two bullets of DNA-coated gold particles were delivered at 300 psi with a Helios Gene Gun (Bio-Rad, Hercules, Calif) under helium pressure, according to previous methodology. DNA was delivered at a...
constant distance of 5 cm into a centrally positioned, targeted site of a 5-mm diameter on the surface of the rat cornea. The central cornea and a region of the peripheral cornea on each side received DNA, but neither the corneoscleral limbus nor the conjunctiva received DNA.

Adult male rats (250-300 g) were obtained from Charles River Laboratories (Wilmington, Mass). All animal experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research; regulations of the National Institutes of Health; and the Institutional Animal Care and Use Committee guidelines of the Department of Comparative Medicine of Pennsylvania State University College of Medicine.

Prior to transfection by the gene gun, rats were anesthetized by intraperitoneal injections of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (5 mg/kg). Two drops of 0.5% proparacaine hydrochloride (Alcaine; Bausch & Lomb, Tampa, Fla) were applied to the cornea. Only 1 eye of each animal was used for experimentation. The eyes of all animals were inspected by light microscopy for signs of ocular disease; any rat displaying ocular disease was excluded from the study.

Following transfection, animals were returned to their cages for 24 hours prior to creation of 3-mm corneal abrasions. Given a previously determined translation time between 18 and 30 hours,3 a 24-hour latency between transfection and abrasion was used to avoid complications of a fragile epithelium (eg, sloughing).

TRANSLATION OF OGFr

Translation of sense and antisense constructs was monitored by immunohistochemistry and densitometry 24 hours after transfection. Immunohistochemical procedures followed previously published guidelines,7 using a fusion protein antibody to OGFr. The preparations were quantitated by densitometry using an Olympus BH-2 fluorescent microscope (UVFL 40 x objective; Olympus, Center Valley, Pa) equipped with a Diagnostic Instruments Spot RT-KE camera (Sterling Heights, Mich). At least 10 to 15 measurements of the peripheral corneal epithelium were taken randomly from corneas transfected with the EV, sense, and antisense constructs; sections stained only with secondary antibody were included.

CORNEAL ABRASIONS

The procedures for wounding and observation of epithelial repair followed those reported by Zagon et al.6,7,9 In brief, animals were anesthetized with a mixture of ketamine (70 mg/kg), xylazine (7 mg/kg), and acepromazine (10 mg/kg). Eyes were examined under a dissecting microscope (SZ-ET; Olympus, Tokyo, Japan), and a superficial 3-mm-diameter circle in the center of the cornea was outlined with a disposable dermatological skin punch (Acuderm, Ft Lauderdale, Fl). The encircled corneal epithelium was removed with a scalpel blade. Wounds were created between 7:30 and 8:30AM or 4 and 5PM; or discomfort at any time throughout the experiments.

At least 10 to 15 measurements of the peripheral corneal epithelium were taken randomly from corneas transfected with the EV, sense, and antisense constructs; sections stained only with secondary antibody were included.

DNA SYNTHESIS

At 24 hours following corneal abrasion, DNA synthesis was monitored with bromodeoxyuridine (BrDU), using the procedure of Zagon et al.8 Four rats in each group (ie, antisense, sense, EV) that were not being photographed were injected with BrDU (100 mg per gram of body weight; Sigma-Aldrich, St Louis, Mo) at 3 and 6 hours prior to euthanasia. Rats were euthanized by intraperitoneal injection of sodium pentobarbital. Sections (8 µm) that included the entire corneal surface, limbus, and conjunctiva were stained with anti-BrdU antibodies (Roche, Indianapolis, Ind) and visualized with Stable 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) prior to counterstaining with hematoxylin-eosin.

The number of BrDU-positive cells was counted in basal and suprabasal layers of the peripheral cornea. A labeling index was computed as the number of labeled cells divided by the total number of cells with nuclei multiplied by 100. At least 2 sections per rat and 5 rats per experimental group were assessed.

PHOTOGRAPHY

For photography of corneal abrasions, animals were anesthetized in a Plexiglas chamber attached to a halothane vaporizer and the residual epithelial defect was stained with topical fluorescein (Fluor-I-Strip; Ayerst Laboratories, Philadelphia, Pa). Rat eyes were viewed using an Olympus dissecting scope with a tungsten light source and a gelatin Wratten 47 filter, and photographed with a Sony charge-couple device camera (Sony, Tokyo, Japan). Photographs of rat eyes were taken immediately after abrasions were made (0 hour) and 16, 24, and 28 hours later. No animal was photographed at intervals less than 12 hours to prevent disruption of the healing process.3 The area of defect was determined using Optimas software (Optimas Corporation, Bothell, Wash) and was calculated as the percentage of residual epithelial defect.

DATA ANALYSIS

Residual corneal defects (percentage) and BrDU labeling indexes were analyzed by analysis of variance with subsequent comparisons made using Newman-Keuls tests. Densitometric scores for semiquantitative immunohistochemistry were assessed with 2-tailed t tests.

RESULTS

OGFr EXPRESSION

Criteria previously established for transfections3 were used in this study. Gold particles were observed by phase microscopy to have penetrated all layers of the corneal epithelium (basal and suprabasal), but did not enter the stroma or extend to the limbus or conjunctiva.3 There was no evidence of corneal damage in any rat from the transfection procedure as judged by corneal clarity, lack of stromal swelling, inflammation, or evident irritation or discomfort at any time throughout the experiments. Transfection efficiency exceeded 85% by counting gold particles in epithelial cells (data not shown).

Immunohistochemical staining using an antibody specifically for OGFr detected OGFr protein in transfected eyes.13 Photodensitometric studies were used to ascertain the relative amounts of OGFr immunoreactivity. Exposure time of the rat corneal epithelium transfected with sense OGFr cDNA was 46% shorter than that for the EV, whereas the antisense OGFr cDNA transfected corneas
required 75% longer exposure times (Figure 1). Thus, the increase in OGFr for the sense corneas was reflected by a shorter exposure time, and the decrease in OGFr for the antisense group was indicated by an extended exposure time.

CORNEAL WOUND HEALING

The 3-mm trephine demarcated only a small region of the cornea allowing a portion of the transfected corneal epithelium to remain intact. At the light microscopic level of resolution, the method of debridement appeared to remove all cell layers of the corneal epithelium but did not disturb the basement membrane. However, it was noted that the corneal surface was more fragile in transfected than in nontransfected eyes.

Animals in the sense group exhibited epithelial defects that were 24%, 52%, and 50% larger than those in the EV group at 16, 24, and 28 hours, respectively (Figure 2). Reepithelialization was examined at 32 and 40 hours, but the small number of animals remaining with a wound, as well as the small size of the defects, precluded meaningful comparisons. Rats in the antisense group displayed residual epithelial defects that were 56% and 48% smaller than those in the EV group at 16 and 24 hours, respectively (Figure 3). Although reepithelialization was examined at 28 and 32 hours, the small number of rats with a residual wound precluded meaningful comparisons.

Figure 1. Immunohistochemical preparations of peripheral corneal epithelium from eyes transfected with empty vector (A), sense (B), and antisense (C) opioid growth factor receptor (OGFr) complementary DNA 24 hours earlier. Tissues were stained with an antibody to OGFr. Note the increase in immunoreactivity in the specimen overexpressing OGFr (sense), and the decrease in immunofluorescence in the specimen with a reduced OGFr expression (antisense), compared with the empty vector. D, Histogram of mean ± SEM of the mean for exposure times of at least 15 readings per group for empty vector, sense, and antisense transfected corneas. Abbreviations: EP, epithelium; ST, stroma. Bar=15 µm. Asterisk indicates P<.001.

Figure 2. Photomicrographs of the rat eye stained with fluorescein immediately (0 hour) (A) and at 16, 24, and 28 hours following a 3-mm corneal wound in animals receiving empty vector (B) or the sense (C) of opioid growth factor receptor (OGFr). The arrow indicates the boundaries of the fluorescein positivity (orange, original magnification ×1.5). D, Histogram of residual epithelial defect in rat corneas after formation of a 3-mm corneal wound (empty vector or the sense of OGFr). Residual epithelial defects are presented as percentage of the original wound. Data are expressed as mean ± SEM. Asterisk indicates significantly different from the empty vector group (P<.05).
DNA SYNTHESIS

Examination of DNA synthesis in the peripheral cornea with BrdU at 24 hours following abrasion revealed that the antisense group had 3.3- and 3.7-fold increases in the basal and suprabasal layers of the peripheral corneal epithelium, respectively, compared with the EV group (Figure 4). No changes in the number of BrdU-labeled cells between the sense and EV groups were observed for basal or suprabasal cells in the peripheral cornea (Figure 4).

Figure 3. Photomicrographs of the rat eye stained with fluorescein immediately (0 hour) (A) and at 16 and 24 hours following a 3-mm corneal wound in animals receiving empty vector (B) or the antisense (C) of opioid growth factor receptor (OGFr). The arrow indicates the boundaries of the fluorescein positivity (orange, original magnification ×1.5). D, Histogram of residual epithelial defect in rat corneas after formation of a 3-mm corneal wound (empty vector or the antisense of OGFr). Residual epithelial defects are presented as percentage of the original wound. Data are expressed as mean ± SEM. Asterisk indicates significantly different from the empty vector group (P<.05).

Figure 4. The percentage of bromodeoxyuridine (BrdU)-labeled suprabasal (A) and basal (B) epithelial cells in the peripheral cornea of rats transfected with sense and antisense constructs of opioid growth factor receptor, or empty vector, and examined after 24 hours. Six and 3 hours prior to euthanasia, animals received an injection of BrdU. Data represent mean ± SEM. Asterisk indicates significantly different from empty vector (P<.001); dagger, significantly different from the antisense group (P<.001).

The present study demonstrates that the OGF-OGFr system is a determinant of the course of corneal reepithelialization. Overexpression of OGFr gene and protein using sense OGFr cDNA, which thereby facilitated OGF-OGFr interactions, had a marked effect on retarding reepithelialization. These data from molecular manipulations of OGFr support earlier pharmacological findings in which delays were noted in healing of corneal abrasions in rabbits and humans, with the addition of exogenous OGF. The results also show that a reduction in the expression of the OGFr gene and protein by using antisense OGFr cDNA accelerated corneal epithelial wound healing and increased DNA synthesis. These findings are consonant with earlier pharmacological reports that blockade of the OGF receptor by naltrexone increased the rate of corneal reepithelialization in diabetic rats, nondiabetic rats, rabbits, and donor human corneas. The present study establishes, for the first time, that the OGF-OGFr axis is an autocrine loop that serves as a crucial regulatory system for corneal epithelial wound healing. This investigation suggests that abnormalities in either the OGF peptide or its receptor could lead to disturbances in corneal reepithelialization.

Naltrexone is an approved medication for systemic use for opioid and alcohol dependence and abuse.

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icity studies for the topical use of naltrexone on the ocular surface epithelium of rats are ongoing, with initial reports indicating that dosages of $10^{-4}$ to $10^{-7}$ mol/L administered 4 times daily were not toxic, as monitored on a series of noninvasive (eg, pachymetry, specular microscopy) and invasive (eg, histology, apoptosis/necrosis) measures. Moreover, efficacy studies indicate that naltrexone at dosages of $10^{-4}$, $10^{-5}$, and $10^{-6}$ mol/L significantly improves reepithelialization of corneal abrasions. Blockade of the OGFr by naltrexone produces similar effects as those resulting from transfection with antisense cDNA for OGFr. Therefore naltrexone therapy may be capable of using the body’s own natural cell proliferation machinery to correct abnormalities of corneal reepithelialization. Moreover, if inborn errors of this growth regulatory system are confirmed, gene therapy using a more permanent method of transfection may provide a feasible method for treating such disorders.

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REFERENCES