



Research report

Re-epithelialization of the rat cornea is accelerated by blockade of opioid receptors

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Abstract

A native opioid peptide, [Met⁵]-enkephalin, termed opioid growth factor (OGF), serves as a constitutively expressed and autocrine produced inhibitory molecule related to developing, neoplastic, renewing, and healing tissues. The present study was designed to examine the effects of interfering with opioid–receptor interaction during re-epithelialization of the cornea in the rat using both systemic injections and topical applications of the potent opioid antagonist naltrexone (NTX). A 4 mm diameter epithelial defect was made in the center of the rat cornea. NTX injected twice daily or applied as eyedrops four times daily significantly accelerated re-epithelialization compared to controls. Beginning as early as 8 h after wounding, both the systemic and topical NTX treatment groups had defects that were approximately 10% to 67% smaller than control abrasions at the time points examined. Similarly, the rate of healing for the NTX groups was 4.7- and 2.8-fold greater than controls for systemic and topical paradigms, respectively. The incidence of complete re-epithelialization in animals given systemic administration of NTX was markedly accelerated in comparison to control rats; however, differences in incidence of repair between NTX and control groups receiving topical application were not observed. These results show that native opioid peptides function in wound healing, and exert a tonically inhibitory influence at the receptor level on repair of corneal epithelial injuries. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The epithelium of the cornea modulates fluid transport for normal stromal hydration and corneal transparency, and serves as an anatomical and physiological barrier against ocular infection [10,14,18,38]. Injury to this epithelium requires prompt resurfacing in order to re-establish visual function. A three-stage process of epithelial healing has been described and includes (i) epithelial cell migration, (ii) cell proliferation and differentiation, and (iii) reassembly of adhesion structures [1,2,4,8,10,11,13,17,23,38]. Re-epithelialization of the ocular surface as a result of injury by trauma or surgery has been studied extensively [1–31,37,38]. Clinically, one of the initial goals in the treatment of corneal defects is to accelerate the rate of wound closure by stimulating epithelial cell migration.

In the past few years, the role of growth factors in the healing of the cornea has emerged as an area of potential benefit [5,7,9,12,13,15,16,28–30,37,38]. Our comprehension of the biological relevance of these factors in corneal biology in health and disease remains incomplete. One neuropeptide, [Met⁵]-enkephalin (also termed opioid growth factor, OGF), has been documented to be a growth factor in the cellular renewal of the homeostatic ocular surface epithelium [35], and a modulator of corneal epithelial outgrowth in tissue culture [34]. This native opioid peptide is autocrine produced [34] and interacts with the zeta (ζ) opioid receptor to inhibit DNA synthesis [34,35] and retard cellular migration and tissue organization [34]. Peptide and receptor have been detected in the basal and suprabasal epithelial cells of the cornea in many classes of the phylum Chordata [33–35].

In light of these findings, the present study was designed to test the hypothesis that OGF is involved with the restitution of the corneal epithelium following injury. Using an in vivo model of corneal wound healing in the rat, a

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central region of the cornea was manually denuded of epithelium and the ocular surface subjected to a continuous opioid receptor blockade by the potent and long-acting opioid antagonist naltrexone (NTX). Drugs were administered by systemic or topical routes. These experiments assayed the size of the defect, rate of repair, and incidence of complete re-epithelialization in order to examine whether opioids function in corneal wound healing.

2. Materials and methods

2.1. Animals

Adult (250–300 g) male Sprague–Dawley rats (Charles River Labs, Wilmington, MA) were utilized in this study. Animals were housed in an environment of $21 \pm 0.5^\circ\text{C}$ with a relative humidity of $50 \pm 10\%$. The room had a complete exchange of air 15–18 times/h and a 12-h light–dark cycle with no twilight. Water and Purina 5010 Rodent Chow were continuously available.

All investigations conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, the regulations of the National Institutes of Health, and the guidelines of the Department of Comparative Medicine of The Pennsylvania State University College of Medicine.

2.2. Wound healing

Rats were anesthetized by i.m. injections of ketamine (10 mg/kg) and xylazine (5 mg/kg). Using an Olympus SZ-ET dissecting microscope (Tokyo, Japan) and a High-light 2000 cold light source (Olympus), a 4 mm diameter circle located in the center of the right cornea was produced with a disposable dermatology skin punch (Acu-Punch, Acuderm, Ft. Lauderdale, FL). All wounds were made between 1000 and 1200 h. Only one eye in each rat was wounded according to policies for humane animal treatment. The encircled corneal epithelium was removed with a #15 Bard-Parker scalpel blade. To facilitate accurate measurements of the wound areas, special efforts were made to produce abrasions with round and smooth perimeters. Following surgery, 50 μl of Polytrim (Allergan Pharmaceuticals, Irvine, CA) was applied to the injured eye; in preliminary studies, application of Polytrim had no effect on wound healing.

In the first experiment, animals received i.p. injections of either 30 mg/kg NTX or an equivalent volume of sterile water (CO). After debridement, all animals were injected twice daily at 0800 and 1700 h, with initial drug administration occurring immediately after surgery. The dosage of NTX chosen was based on earlier experiments indicating a duration of 24 h for opioid receptor blockade [32].

In a second series of experiments, rats with abraded corneas received NTX (10^{-6} M) or an equivalent volume (50 μl) of sterile water in the form of eyedrops at 0800, 1200, 1700, and 2100 h each day. Compounds were given as a single drop to the central cornea of the wounded eye, with the lower eyelid held away from the eye to avoid overflow. The initial application of NTX or sterile water was administered immediately following surgery, and all other doses were given to awake, unanesthetized animals. The regimen of NTX administration was chosen to compensate for its potentially rapid removal from the ocular surface by nasolacrimal drainage.

2.3. Photography

In order to photograph the wounded eyes, animals were anesthetized in a chamber attached to a halothane vaporizer and the residual epithelial defect was stained utilizing topical fluorescein (Fluor-I-Strip, Ayerst Laboratories, Philadelphia, PA) [31]. Using an Olympus dissecting microscope with a tungsten light source and a gelatin Wratten #47 filter, images were captured at $1.5 \times$ magnification with a Sony CCD camera. Images were analyzed with Optimas software (Optimas, Bothell, WA). Photographs of the eyes of rats receiving intraperitoneal injections were taken at 0, 8, 16, 24, 28, 32, 36, 42, and 48 h, whereas animals receiving topical application were photographed at 0, 8, 12, 20, 28, 32, 36, and 48 h following injury. Animals were randomized for photography. Fluorescein was not applied to each abraded cornea at every time point because this procedure disrupted re-epithelialization (unpublished observations). All injured animals had an interval of at least 12 h between examination with fluorescein, except at the 8 h time point. At least eight animals/group were used for each time point.

2.4. Data analysis

All studies were conducted in a blind fashion, and the same individual performed the surgery and the morphometric analysis. The areas of defect were determined using Optimas, and were calculated as the percent of residual epithelial defect. Comparisons were made for each time point by analysis of variance and Newman–Keuls tests because of the design of the experiments (i.e., the use of different groups of animals at differing time points to avoid reinjury by the fluorescein procedure). The rate of healing was calculated only between 0 and 8 h for the intraperitoneal and topical applications because the rate of wound closure was not a linear process; therefore, linear regression analysis was not appropriate to assess overall healing rates as discussed elsewhere [2,3]. The number of corneas that were completely re-epithelialized at a given time were compared using chi-square tests.

2.5. Light microscopy

In order to confirm the injury and determine the magnitude of defect, eyes were collected immediately and 1 h after surgery. The tissues were placed in 10% neutral buffered formalin for 24 h, processed and embedded in paraffin, and stained with hematoxylin and eosin.

3. Results

The normal central corneal epithelium was composed of a single layer of basal cells, and approximately four layers of suprabasal cells (Fig. 1A). The procedures for wounding the cornea were found to remove all layers of the epithelium (Fig. 1B). Wound healing occurred in a manner consistent with the description by Dua and Forrester [4], with a leading edge containing the presence of convex fronts. The initial area of debridement ranged from 13.0 to 13.9 mm², and corresponded to corneal injuries of approximately 4.0 mm diameter. No differences in the size of the initial lesion could be observed between CO and NTX groups.

All of the rats tolerated the procedures well. Wounded eyes were grossly free of infection or exudate throughout the duration of the experiments. Animals were examined in the post-operative period and were excluded if the eyes were infected or appeared abnormal.

3.1. Intraperitoneal studies

The effects of NTX given by systemic injection on corneal wound healing were examined in the first experiment (Figs. 2 and 3). Animals with an opioid receptor blockade using NTX had defects that were significantly reduced in size from CO animals at the first point examined—8 h, and at 16, 24, 28, and 32 h. Thus, animals with epithelial debridement and receiving systemic application of NTX had wounds that were decreased by 15%, 25%, 46%, 33%, and 61% at 8, 16, 24, 28, and 32 h, respectively, from rats injected with vehicle.

The rate of healing of the corneal epithelium calculated over the first 8 h of the experiment showed that the rate for the CO group was 0.06 ± 0.02 mm²/h in contrast to a statistically reliable ($p < 0.0002$) increase of 0.28 ± 0.04 mm²/h for animals in the NTX group.

An acceleration in the incidence of wound healing was observed in the NTX group compared to the CO animals at 28, 32, 36, and 42 h. Inspection of corneas at 28 h following injury revealed 6% of the animals in the NTX group were re-epithelialized in comparison to none of the rats given vehicle; the difference between NTX and CO groups was significant at $p < 0.05$. At 32, 36, and 42 h the number of healed corneas in the NTX group was 17%, 40%, and 86%, respectively, in contrast to 7%, 14%, and 53%, respectively, of the CO animals; differences between the NTX and CO groups were significant at $p < 0.05$. All corneal wounds were healed by the 48 h time point.

3.2. Topical application

The effects of NTX given by topical application on corneal wound healing were examined in the second experiment (Fig. 4). Animals receiving NTX had defects that were significantly reduced from CO animals at the first point examined—8 h, and at 12, 28, 32, and 36 h. Thus, animals with epithelial debridement and receiving topical application of NTX had wounds that were reduced significantly (either at $p < 0.05$ or $p < 0.01$) from CO rats by 10%, 11%, 38%, 55%, and 67% at 8, 12, 28, 32, and 36 h, respectively. At 20 h, the NTX treated rats had epithelial defects that were substantially smaller than in CO animals, but this change of 20% was not statistically significant ($p < 0.06$).

The rate of healing of the corneal epithelium calculated over the first 8 h of the experiment revealed that the rate for the CO group was 0.15 ± 0.01 mm²/h in contrast to a significantly ($p < 0.0001$) increased rate of 0.42 ± 0.03 mm²/h for NTX-exposed subjects.

The incidence of complete re-epithelialization at 32 and 36 h was 31% and 53%, respectively, for the NTX group

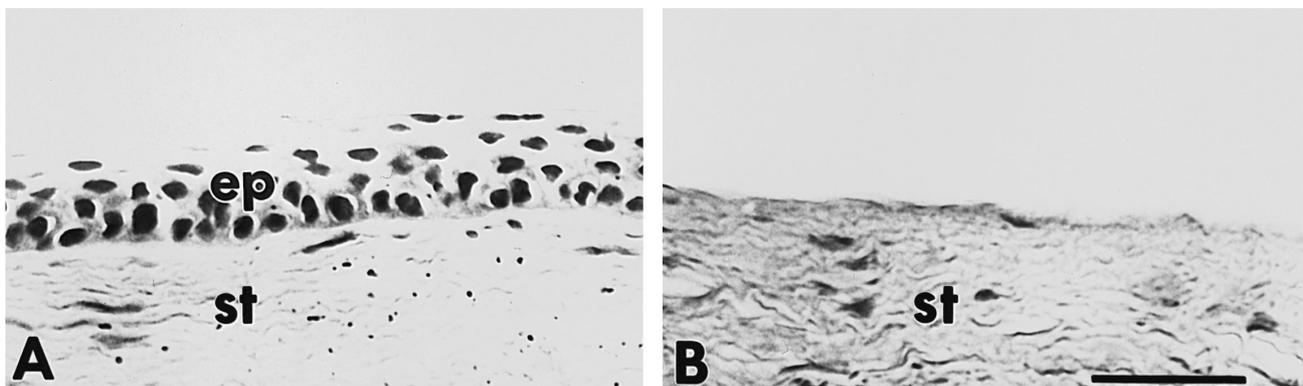


Fig. 1. Photomicrographs of histological sections of the rat central cornea before (A) and after (B) abrasion, and stained with hematoxylin and eosin. Note the removal of the epithelium (ep) (see A) following debridement (B); st = stroma. Bar = 40 μ m.

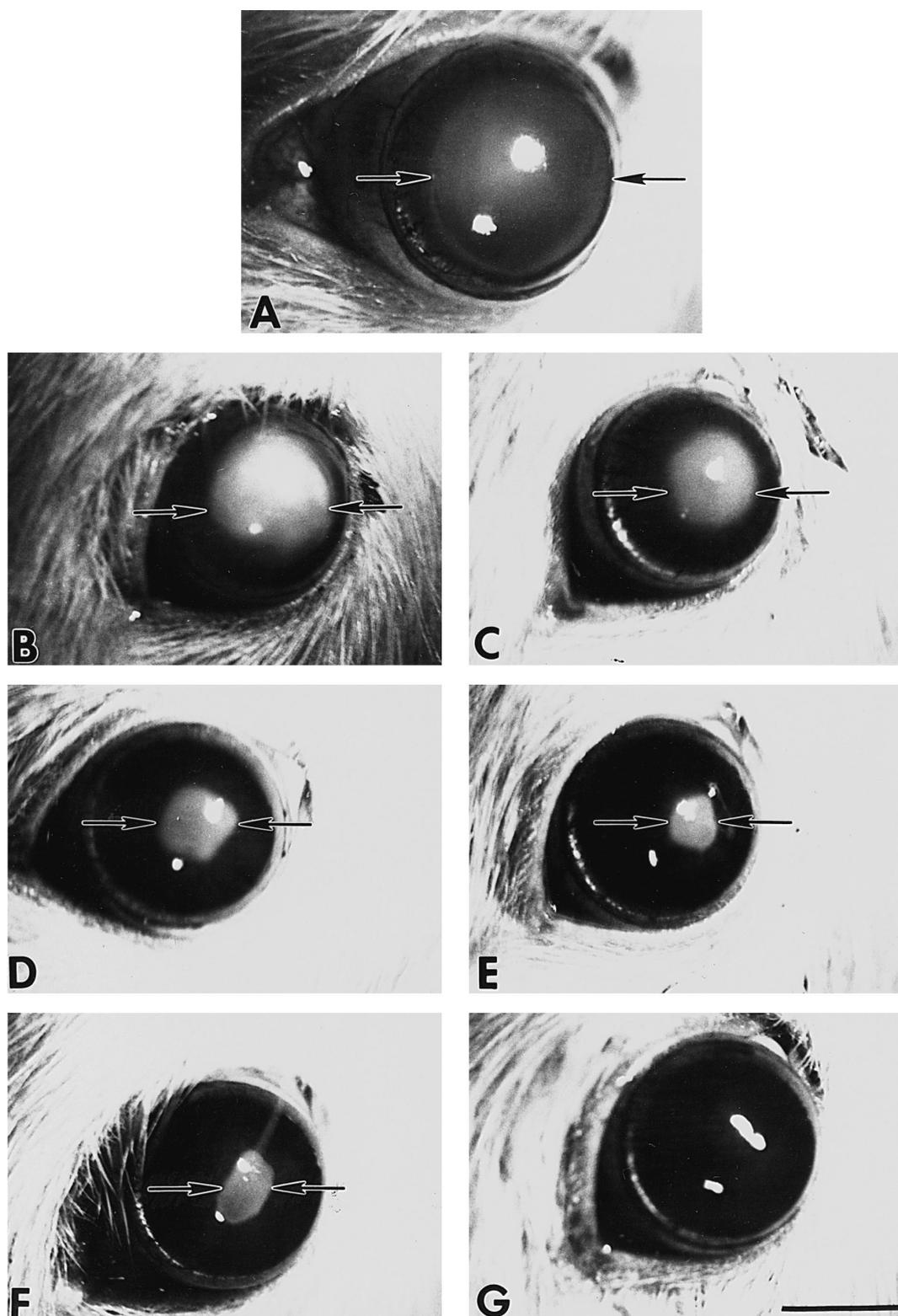


Fig. 2. Photographs of the rat eye stained with fluorescein immediately (A) or at 16 h (B, C), 24 h (D, E), or 32 h (F, G) following the formation of a 4 mm corneal wound that denuded the central region of epithelium. Animals received twice daily injections of 30 mg/kg NTX (C, E, G) or an equivalent volume of vehicle (B, D, F). Arrows indicate the boundaries of the fluorescein positivity observed in the cornea. Note that wounds heal somewhat circularly, but convex fronts of re-epithelialization may not produce completely symmetrical closures. Bar = 10 mm.

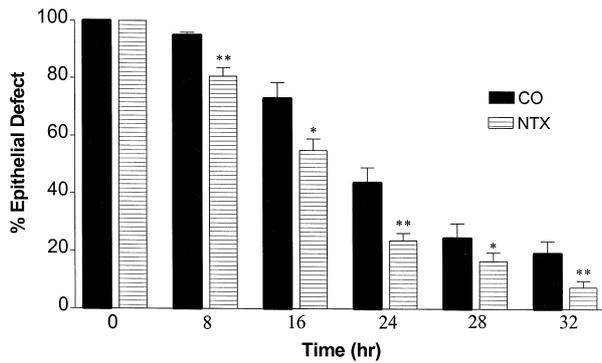


Fig. 3. Histogram of residual epithelial defect in rats following formation of a 4 mm central corneal wound. Two times daily the animals were given injections of either 30 mg/kg M NTX or an equivalent volume of sterile water. Photographs of fluorescein stained corneas were projected, the images captured with a Sony CCD camera, and areas analyzed by Optimas software. Residual epithelial defects are presented as percentage of the original wound. Data are expressed as means \pm S.E.M. Significantly different from controls at $p < 0.05$ (*) or $p < 0.01$ (**).

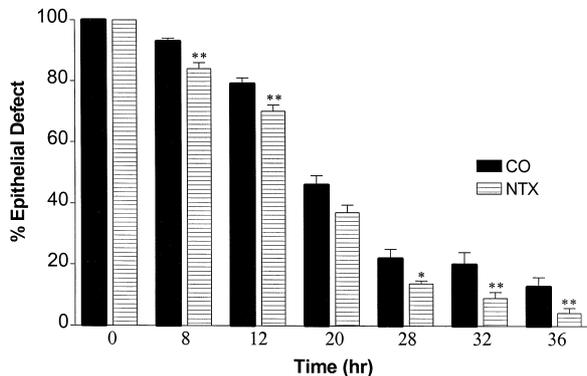


Fig. 4. Histogram of residual epithelial defect in rats following formation of a 4 mm central corneal wound. Four times daily the animals were given eyedrops of either 10^{-6} M NTX or an equivalent volume of sterile water. Photographs of fluorescein stained corneas were projected, the images captured with a Sony CCD camera, and areas analyzed by Optimas software. Residual epithelial defects are presented as percentage of the original wound. Data are expressed as means \pm S.E.M. Significantly different from controls at $p < 0.05$ (*) or $p < 0.01$ (**); at 20 h, the difference between the CO and NTX group was $p < 0.06$.

and 41% and 50%, respectively, for animals with abraded corneas receiving vehicle (CO group); these differences were not statistically significant. All corneal wounds were healed by the 48 h time point.

4. Discussion

A number of pieces of evidence have been published suggesting that OGF is an important modulator of DNA synthesis, tissue organization, and cell migration of the corneal epithelium, and that both OGF and its receptor, ζ , are associated with ocular surface epithelial cells [33–36]. Using full thickness explants of the rabbit peripheral cornea

from which Descemet's membrane and endothelium were removed, persistent blockade of opioid–receptor interaction for 7 days increased the extent of outgrowths and the number and labeling index (DNA synthesis) of epithelial cells relative to control levels [34]. Outgrowths exposed to OGF were subnormal in extent and labeling index, and displayed alterations in architectural pattern; these effects were receptor-dependent [34].

Homeostatic renewal of ocular surface epithelium in the rat also is known to be governed by OGF [35]. Prolonged disruption of opioid peptide–opioid receptor interfacing using NTX resulted in an elevation ranging from 30% to 72% in DNA synthesis of basal epithelial cells in the peripheral cornea, limbus, and conjunctiva, with these effects noted under both in vivo and in vitro conditions. NTX in this experimental paradigm had no effect on epithelial cells of the central cornea which do not undergo DNA synthesis. In contrast to opioid receptor blockade, excessive exposure to OGF depressed DNA synthesis of basal epithelial cells in the peripheral cornea, limbus, and conjunctiva by 25% to 50% in a receptor mediated manner, but had no effect on basal cells of the central cornea.

Finally, observations from immunocytochemical studies have revealed that both OGF and the ζ receptor are associated with basal and suprabasal epithelial cells of the ocular surface of the rat [35]. Both peptide and receptor also have been reported in the corneal epithelium of a wide variety of vertebrates, including humans [33,34]. Recently, in situ hybridization investigations have demonstrated that the message for preproenkephalin, which encodes OGF, is present in basal and suprabasal epithelial cells of the peripheral and central cornea, limbus, and conjunctiva [36]. These findings have permitted the conclusion that both peptide and gene expression occur in epithelial cells of the mammalian cornea, and that autocrine production of OGF is a common feature of the corneal epithelium.

The present study investigated the hypothesis that OGF is involved with the repair of the cornea after abrasion. The results show that continuous disruption of opioid peptide–opioid receptor interaction either through systemic or topical drug administration following abrasion of the corneal surface significantly accelerates re-epithelialization. Thus, measures of the size of the defect, rate of healing, and the incidence of animals with re-epithelialized corneas support the concept that opioids are inhibitory growth factors. Although systemically administered NTX markedly influenced the incidence of re-epithelialization, differences were not detected between rats receiving topical application of NTX or vehicle. Further studies using more time points and the defining of optimal conditions (e.g., number of applications/day) need to be explored to elucidate any differences in the impact of systemically vs. topically applied NTX.

The fact that interruption of opioids from their receptors permits an increase in the temporal course of wound healing would suggest that opioids are constitutively ex-

pressed growth factors. These results are consonant with data in previous reports showing that opioids are important in normal processes of homeostatic cellular renewal of the epithelium composing the ocular surface [35], as well as the outgrowth of corneal epithelial explants [34], and reveal for the first time that native opioid peptides also play an active role in the repair of the damaged cornea.

The experiments conducted herein utilized an opioid antagonist, NTX, to probe the function of endogenous opioids in wound healing. The question remains as to which opioid(s) is(are) involved with re-epithelialization. Based on previous reports, the most likely candidate would be OGF—[Met⁵]-enkephalin [33–36]. Earlier work has demonstrated that OGF is an inhibitory growth factor regulating the biology of ocular surface epithelium [33–36]; this molecule meets the requirements suggested by the results of the present study. Further investigations are needed to inquire as to whether OGF and/or other opioid peptides serve in the modulation of corneal epithelial repair. Moreover, it will be valuable to learn about the repercussions as to OGF expression in response to injury of the corneal epithelium.

The process of wound healing of the corneal epithelium consists of a number of events: cell migration, cell replication and differentiation, and the reassembly of adhesion structures [1–3,10,11,13,17,23,28]. The resurfacing of a wound is an initial and critical component of re-epithelialization. Cells proximal to the injured area become flattened, elaborate filopodial processes, and migrate as a sheet over the wounded surface, with cell division not required. The present study has utilized a well documented method of corneal injury, and the data are consonant with the results of controls from other reports on the healing process. Integration of the findings in this study with our knowledge about re-epithelialization of the cornea would suggest that opioids exert a distinct effect on early events, particularly cell migration. The importance of opioids in subsequent processes of wound healing (e.g., cell proliferation) requires clarification. However, earlier work [34,35] has shown that opioids are regulatory elements involved with DNA synthesis, and one could postulate that these peptides have other mechanistic features that impact on the entire course of wound healing of the corneal surface. Finally, the experiments presented herein reveal that opioids must be an active component of the repair process; otherwise, interruption of peptide–receptor interfacing would be predicted to have no influence on re-epithelialization.

Blockade of growth-related endogenous opioid peptides from opioid receptors by opioid antagonists such as NTX could prove to be of therapeutic advantage. For example, ulcers and erosions of the corneal epithelium are a major cause of ocular morbidity and visual loss [14,18,38]. Delayed corneal epithelial healing may be associated with or follow microbial infections, alkali burns, penetrating keratoplasties, radiation keratoconjunctivitis, toxic keratopathies, dry eyes, and the recurrent corneal erosion

syndrome. Prolonged ulceration or erosion of the cornea may result in thinning or melting of the corneal stroma. Given that conventional therapeutic alternatives for long-standing corneal ulcers or erosions are not uniformly successful, the present data showing that NTX can accelerate at least the initial stages of the wound healing process are intriguing. Conversely, although speculative at this juncture, it may be envisioned that an overproduction of opioids that serve as negative growth factors occurs in some individuals, and excessive opioids would be expected to repress re-epithelialization.

Acknowledgements

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