

Reepithelialization of the Human Cornea Is Regulated by Endogenous Opioids

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PURPOSE. To determine the influence of endogenous opioid modulation on reepithelialization of the human cornea.

METHODS. Eight-millimeter-diameter epithelial defects were created with a trephine and mechanical scraping in the center of human corneas. Resurfacing was studied in organ culture. The size of the defect, the number of specimens with complete reepithelialization, and rate of closure were evaluated using topical fluorescein and morphometric analysis. The influence of opioid receptor blockade was studied using the potent and long-acting opioid antagonist, naltrexone (NTX; 10^{-6} M), and the effects of excess (10^{-6} M) opioid growth factor (OGF), [Met⁵]enkephalin, also were determined. The modulatory activity of NTX and OGF on DNA synthesis was evaluated by monitoring the labeling index (LI) using radioactive thymidine. The presence and location of OGF and its receptor (OGFr) were ascertained by immunocytochemistry 1 hour and 24 hours after abrasion.

RESULTS. NTX accelerated the wound-healing process, with 21% to 89% less defect than controls observed from 24 to 96 hours. At 72 hours, 62% of the subjects in the NTX group had complete closure of the corneal defects, in contrast to only 19% of the control specimens. All epithelial abrasions were resurfaced in the NTX group between 96 and 120 hours, whereas all controls were not closed until 168 hours. The rate of healing in the NTX group was 1.06 mm²/h compared to a rate of 0.68 mm²/h in the control group. OGF delayed corneal wound healing, with 24% to 260% more defect recorded than in control specimens at day 7. The healing rate of the OGF group was 0.42 mm²/h compared to 0.82 mm²/h for control subjects. The corneal epithelium adjacent to the wound had an LI that was 152% greater than control specimens, whereas OGF decreased the LI of this region by 75%. OGF and OGFr were detected in the epithelium bordering the damaged region at 1 hour, and both peptide and receptor were noted in the regenerating epithelium at 24 hours.

CONCLUSIONS. These results indicate that an endogenous opioid is present and functions as a tonically active, receptor-mediated, negative growth factor during reepithelialization of the abraded human cornea. (*Invest Ophthalmol Vis Sci.* 2000;41:73-81)

Normal hydration and transparency of the cornea is, in part, dependent on the ocular surface epithelium, which serves as an important physiologic barrier between the external and intraocular environments.¹⁻³ Injury to this epithelium necessitates prompt resurfacing to reestablish visual function, prevent infection, and restore corneal integrity. Repair of disrupted epithelium has received considerable attention.¹⁻⁷ Healing consists of a multistage process that includes cell migration, proliferation and differentiation, and reassembly of adhesion structures.^{2-4,7} A primary objective, clinically, is to decrease the timetable of wound closure and to prevent problems such as recurrent erosion.

The role of growth factors and their receptors in the regulation of key processes of wound healing, including repair of corneal abrasions, has been discussed.^{2,3,6,8} A neuropeptide documented to be a growth factor involved in cellular renewal and homeostasis of ocular surface epithelium⁹ and a modulator of corneal epithelial outgrowth in tissue culture¹⁰ is the native opioid peptide, [Met⁵]enkephalin, also termed opioid growth factor (OGF). This autocrine-produced¹¹ opioid peptide interacts with a receptor [OGFr, formerly termed zeta (ζ)] to inhibit DNA synthesis^{9,10} and regulates cell migration and tissue organization.¹⁰ Chronic blockade of opioid action using the potent and long-acting opioid antagonist, naltrexone (NTX), increases cell proliferation and cell migration and promotes architectural integrity in explant cultures of corneal epithelium.¹⁰ Both OGF and its receptor have been detected in the basal and suprabasal epithelial cells of the cornea in many classes of the phylum Chordata.^{9,10,12,13} Recently, in vitro and in vivo studies have shown that OGF is a constitutively expressed inhibitory growth factor and functions in the process of wound healing of the rabbit¹³ and rat¹⁴ corneal epithelium. This opioid peptide plays a direct role in the repair of ocular surface epithelium and does so in a receptor-mediated fashion.

The present study addressed the premise that OGF is involved in restoring the human corneal epithelium after in-

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jury. Using an organ culture model of corneal wound healing, the central region was abraded and the reepithelializing corneas subjected to (i) continuous opioid receptor blockade by NTX or (ii) increased receptor stimulation by an excess of the opioid agonist OGF. These experiments determined if opioids function in human corneal wound healing and act directly at the cellular/tissue level. Moreover, the influence of endogenous opioids on the repair process relative to DNA synthesis also was ascertained. Finally, immunocytochemical studies were performed to determine whether OGF and OGF α are found in the reepithelializing cornea.

MATERIALS AND METHODS

Human Corneas

Pairs of donor corneas were obtained from the South Central Florida Eye Bank or the Lions Eye Bank of Oregon. All specimens were tissues rejected for transplantation based on standard operating protocols specified by the Eye Bank Association of America, as well as the Federal Drug Administration. The eyes were obtained within 36 hours of death. Corneas from donors with infectious diseases, including hepatitis and HIV, were rejected from the study. Additionally, the donor corneal tissues were subjected to biomicroscopic examination to confirm the lack of abrasions before use. Donor history was reviewed to rule out preexisting conditions (e.g., ocular surgery, trauma, glaucoma) that would render them unsuitable for experimentation. Sex and age of the donor were noted and factored in the data analysis as necessary. All procedures were approved by the Institutional Review Board of the Human Subjects Protection Office at The Pennsylvania State University College of Medicine.

Wound Healing

The procedures for wounding and observation of repair followed those of Zagon and coworkers.^{7,13,14} In brief, using a dissecting microscope (SZ-ET; Olympus, Tokyo, Japan) and a cold light source (Highlight 2000; Olympus), an 8-mm-diameter circle located in the center of the cornea was produced with a disposable dermatology skin punch (Acu-Punch; Acuderm, Inc., Ft. Lauderdale, FL). All wounds were made between 12:00 PM and 2:00 PM. The encircled corneal epithelium was removed with a No. 15 Bard-Parker scalpel blade. To facilitate accurate measurements of the wounded areas, special efforts were made to produce abrasions with round, smooth perimeters.

Immediately after wounding, the corneas were carefully dissected from the globe leaving a 1- to 2-mm scleral rim, with conjunctiva attached. Specimens were placed epithelial-side up into 7 ml of prewarmed RPMI media (Central Facility, Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine) supplemented with 10% fetal calf serum, bicarbonate, and antibiotics in a humidified atmosphere of 7% CO₂/93% air at 37°C. In the first experiment, the eyes were randomly placed in media containing either 10⁻⁶ M NTX or an equivalent volume of sterile water (approximately 5 μ l). In another experiment the eyes were randomly placed in media containing either 10⁻⁶ M OGF or an equivalent volume of sterile water; a second aliquot of OGF or water was added to the media 8 hours after the first aliquot. A

matching eye for each donor was used in each group. Both media and compound were replaced daily.

Photography

To photograph the wounded eyes, the residual epithelial defect was stained with topical fluorescein. An Olympus dissecting microscope with a tungsten light source and a gelatin Wratten No. 47 filter was used to capture images at \times 1.5 magnification with a CCD camera (Sony, Parkridge, NJ). Images were analyzed with Optimas software (Optimas Corporation, Bothell, WA). Photographs were taken at 24, 48, 72, 96, 120, 144, and 168 hours after surgery. Not all human eyes were photographed at each time point to prevent disruption of the reepithelialized surface.

Data Analysis for Wound Healing

All studies were conducted in a masked fashion, and the same individual performed the surgery and the morphometric analysis. The areas of defect were determined using Optimas software and were calculated as the percentage of residual epithelial defect. Comparisons were made for each time point by analysis of variance (ANOVA) and Newman-Keuls tests because of the design of the experiments (i.e., the use of different groups of specimens at differing time points to avoid reinjury by the fluorescein procedure).

Linear regression analysis was used to assess overall healing rates, and the slopes of the lines compared. An hourly rate of healing also was calculated between 0 and 24 hours. The number of corneas that were completely reepithelialized at a given time were compared using χ^2 tests.

Light and Electron Microscopy of Wound Healing

To confirm the injury and determine the magnitude of defect, corneas were collected immediately and 1 hour after surgery. For light microscopy, the tissues were placed in 10% neutral buffered formalin for 24 hours, processed and embedded in paraffin, and stained with hematoxylin and eosin. For electron microscopy, the tissues were fixed in 2% glutaraldehyde, 2.5% paraformaldehyde, 3% sucrose, and 0.025% CaCl₂ in 0.1 M sodium cacodylate buffer at 4°C for 18 hours, postfixed in 1% OsO₄ for 2 hours, and embedded in Epon 812. Thin sections of the abraded region were stained with 2% uranyl acetate and 0.4% lead citrate and viewed with a Philips 400 electron microscope (Philips Electronics Instruments, Mahwah, NJ).

DNA Synthesis

To ascertain whether DNA synthesis is a target of opioid modulation with respect to the healing of the corneal epithelium, at least 3 corneas each exposed to NTX or vehicle were investigated for DNA synthesis at 72 hours after wounding, whereas 3 corneas each exposed to OGF or vehicle were examined at 96 hours. At these selected times, more than 50% of the defect was repaired for each drug treatment. One hour before fixation, 1 μ Ci/ml of [³H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) was added to the cultures at 1000 hours. The corneas were briefly rinsed with sterile saline, fixed in 10% neutral buffered formalin for 48 hours, embedded in paraffin, and processed for autoradiography. Seven-micrometer sections that included the entire corneal surface and limbus were collected, coated with Kodak NTB-2 emulsion, stored in light-tight boxes at 4°C for 30 days, and developed

with Kodak D-19. Sections were counterstained with hematoxylin and eosin.

Labeling Indices

The number of cells with two or more grains (background <1 grain/cell) in the basal and suprabasal epithelial layers of the superior and inferior cornea and limbus were counted from two nonserial sections per eye. Only cells in the deepest aspect of the basal epithelium, situated proximal to the basement membrane, were considered basal cells. The suprabasal layer included all cells superficial to the basal layer. Care was taken that the section evaluated passed through the axial portion of the cornea such that the superior and inferior poles were present. Labeling indices (LI) were computed as the number of labeled basal or suprabasal cells divided by the total number of labeled basal or suprabasal cells with nuclei $\times 100$.

All nucleated cells over the entire extent of the corneal epithelium were counted; the cornea was assessed by the use of a grid measuring 0.16 mm in length and observed at $\times 400$ magnification using an ocular reticule. The cornea was identified by the presence of a subjacent stroma. The central cornea consisted of 15 0.16-mm grid lengths in the middle of the cornea. The peripheral cornea was counted as 37 grids extending in each direction from the central cornea to the limbo-corneal junction. The limbus consisted of 4 grids in length on each side, beginning at the limbal-corneal junction and extending toward the bulbar conjunctiva.

Data Analysis for LI

Data are presented as the mean LI (percent) of the central cornea, peripheral cornea, and limbus. No differences were noted in the LI of the superior and inferior poles in any region of the ocular surface epithelium, and therefore data were combined. All data within a region across time were subjected to ANOVA, with subsequent comparisons made using the Newman-Keuls test.

Immunocytochemistry

In another series of experiments, immunocytochemical studies were performed to ascertain the distribution of OGF and OGF α in the human corneal epithelium. The immunocytochemical procedures described in Zagon et al.¹² were used. In brief, corneal tissue was obtained immediately (within 1 hour) and 24 hours after wounding, rinsed in 0.1 M Sorenson's phosphate buffer (SPB) (pH 7.4), frozen in isopentane chilled on dry ice, and embedded in OCT medium. Cryostat sections (15 μ m) were collected on gelatin-coated slides and stored at -20°C for no longer than 14 days until processing. Tissues were fixed and permeabilized in ice-cold 100% ethanol and acetone for 10 minutes each, rinsed with SPB, blocked with 3% normal goat serum (NGS) in 50 mM SPB, pH 7.4. Sections were incubated in a humidified chamber at 4°C for 16 to 18 hours with either anti-OGF IgG (1:150) or anti-OGF α IgG (1:150) diluted with 1% NGS and 0.1% Triton X-100 in SPB. Secondary antibody used to visualize the reaction was rhodamine-conjugated goat anti-rabbit IgG (1:100 dilution). Sections were mounted in 60% glycerol-40% SPB and observed using an Olympus BH-2 microscope equipped with fluorescent, brightfield, and phase optics. Specifics regarding the production and characteristics of each polyclonal antibody are provided elsewhere.¹² Some sections served as controls and were incubated with secondary antibody

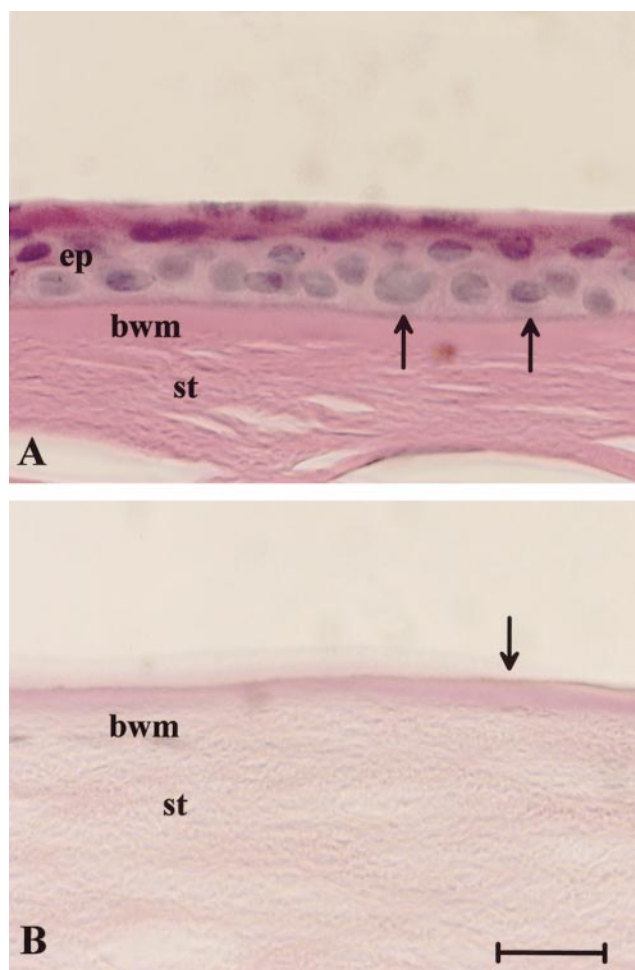


FIGURE 1. Histologic preparations of the ocular surface of the human cornea placed in media for 1 hour. The corneal epithelium (ep) (A) consisted of approximately five cell layers. Abrasion of this epithelium (B) removed the cell layers, but the basement membrane (arrow) was observed. ep, epithelium; bwm, Bowman's membrane; st, stroma. Bar, (A, B) 25 μ m.

only or with primary antibodies preabsorbed with either an excess of OGF or the 17-kDa subunit of OGF α .

RESULTS

Organ Culture of the Human Corneal Epithelium

In organ culture, the human corneal epithelium was composed of a single layer of basal cells, and approximately four to five layers of nucleated suprabasal cells (Fig. 1A). The limbus contained a layer of basal epithelial cells along with a suprabasal region that averaged two to three nucleated cell layers in thickness. Organ cultures of the human cornea were maintained for no longer than 7 days. Throughout this period, the epithelium displayed (i) a uniformity of cell layers, (ii) a tight packing of cells organized in five to six layers, (iii) DNA synthesis (see below), (iv) normal repair processes (e.g., tapering of migrating epithelium), (v) cellular differentiation, with well-defined wing cells and superficially located squamous cells, (vi) negligible intercellular edema and sloughed cells, and (vii) an absence of corneal ulceration. The basement membrane and

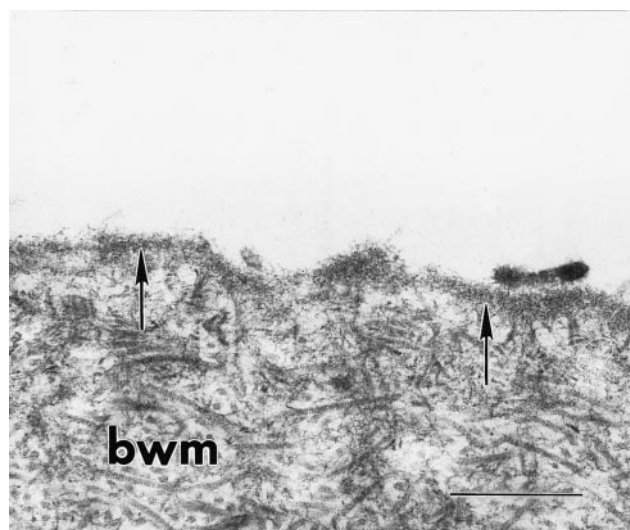


FIGURE 2. Ultrastructural preparation of the ocular surface of the human cornea placed in media for 1 hour. Abrasion of this epithelium removed the cell layers, but the basement membrane (*arrows*) remained intact. bwm, Bowman's membrane. Bar, 300 nm.

Bowman's membrane were intact, and undulations of these membranes were not recorded. The stroma had no edema, and a keratocyte density and morphology of normal appearance. The endothelium was characterized by a lack of intercellular spaces and intracellular vacuoles.

Wounding of the Human Corneal Epithelium

Abrasion of the demarcated region with a scalpel blade completely removed all the cell layers constituting the corneal epithelium, but preserved the basement membrane (Figs. 1B, 2).

Reepithelialization of the Human Corneal Epithelium

Wound healing occurred in a manner consistent with previous descriptions,¹⁵ with a leading edge containing the presence of convex fronts (Fig. 3). Corneas remained transparent throughout the healing process. The initial area of abrasion ranged from 55.9 to 62.5 mm² and corresponded to corneal injuries of 8.4 to 8.9 mm in diameter. No differences in the size of the initial lesion could be observed between control and experimental groups.

Exposure to NTX. The role of the endogenous opioid system relative to growth in corneal wound healing was examined in the first experiment (Figs. 3, 4, 5). Corneas placed in culture with NTX, to render a complete opioid receptor blockade, had a 21% smaller defect than measured in control specimens at 24 hours, the earliest time point examined. Measurements at 48, 72, and 96 hours revealed that the residual abraded areas were 38%, 51%, and 89%, respectively, less than in control subjects. At 120 hours and 144 hours, when all the NTX-exposed corneas were reepithelialized, there was a 13% and 7%, respectively, defect present in the control group.

With regard to the incidence of wound closure, at 48 hours, 12% of the corneas in the NTX group were healed compared to 0% in the control group; this difference was statistically significant at $P = 0.04$. At 72 hours the NTX group

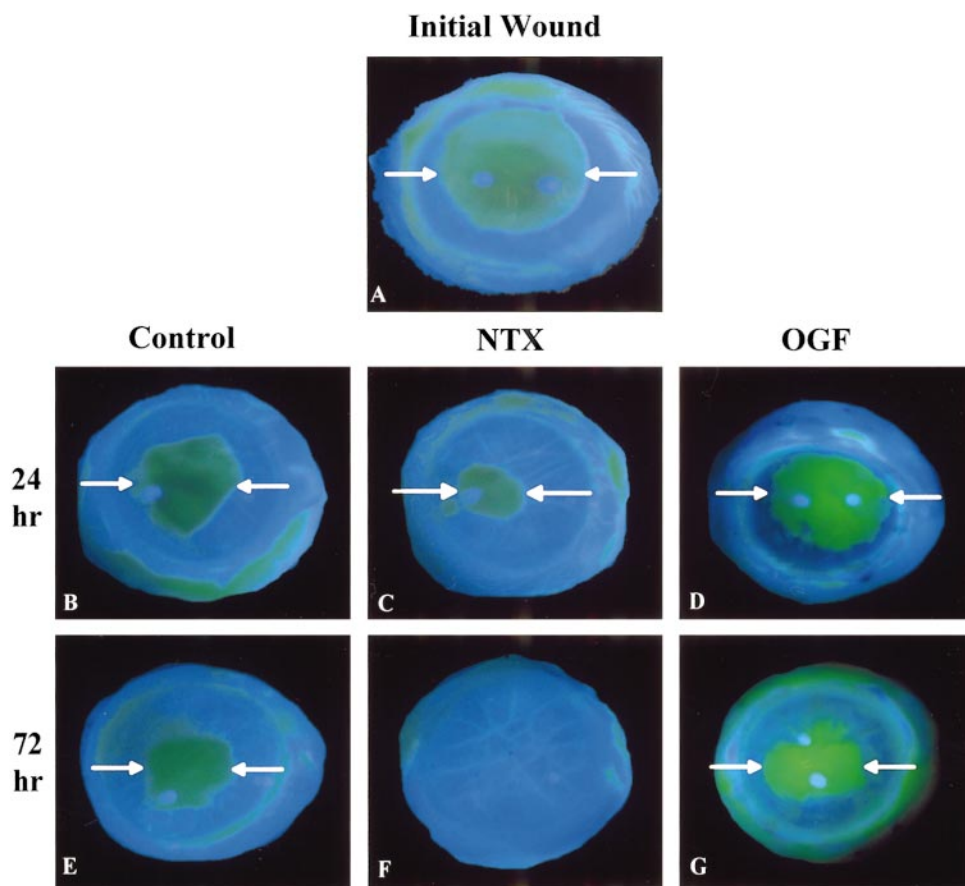


FIGURE 3. Photographs of the human eye stained with fluorescein immediately (A), 24 hours (B through D), or 72 hours (E through G) after formation of an 8-mm corneal wound that denuded the central region of the epithelium. Corneas were placed in culture containing either sterile water (control) (B, E), 10^{-6} M NTX (C, F), or 10^{-6} M OGF (D, G). *Arrows* indicate boundaries of fluorescein positivity observed in the cornea. Note that wounds healed somewhat circularly, but convex fronts of reepithelialization may not produce completely symmetrical closures. Magnification, $\times 3$.

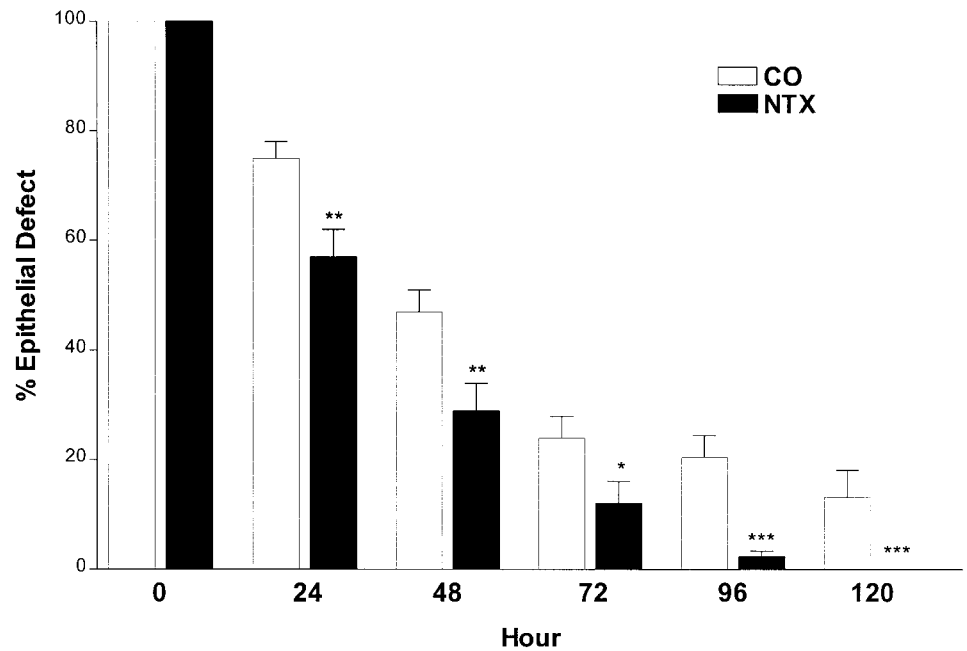


FIGURE 4. Histogram of residual epithelial defect in the human cornea after formation of an 8-mm corneal wound. Human eyes were placed in culture media containing either sterile water (CO) or 10^{-6} M NTX. Photographs of fluorescein-stained corneas were captured with a Sony CCD camera, and areas were analyzed by Optimas software. Residual epithelial defects are presented as percentage of the original wound. Data are expressed as means \pm SEM. Significantly different from controls at * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$.

had 62% of the corneas repaired in contrast to 19% of the controls ($P = 0.0018$) and, at 96 hours, 81% of the NTX-exposed corneas were reepithelialized and only 32% of the controls were healed ($P = 0.0006$). All corneas in the control group were healed by 168 hours.

The rate of corneal wound repair was assessed by dividing the total area that was reepithelialized by the number of hours elapsed. Between 0 and 24 hours, the NTX group had a rate of wound healing of $1.06 \text{ mm}^2/\text{h}$ relative to a rate of $0.68 \text{ mm}^2/\text{h}$ in the control group; this difference was statistically significant at $P < 0.05$. The slope of the healing rate for the NTX treated corneas was -0.67 ± 0.13 , and the slope for the control group was -0.63 ± 0.09 ; these slopes differed significantly at $P < 0.05$. Linear regression indicated an $r = 0.90$ or better for both slopes.

Exposure to OGF. In the second experiment, the effects of an excess of the growth factor, OGF, on corneal reepitheli-

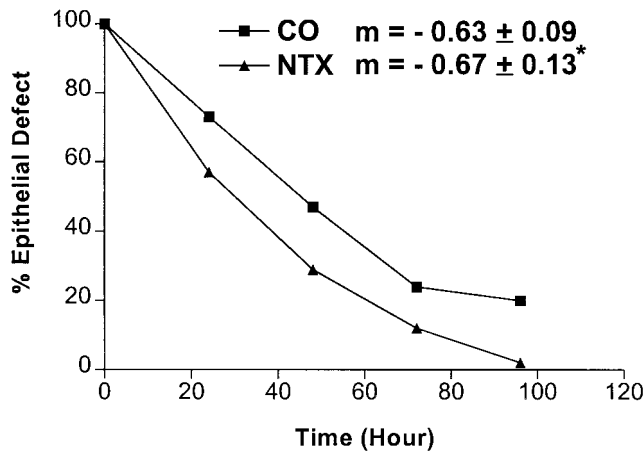


FIGURE 5. Overall healing rates of NTX-treated and control (CO) corneas analyzed by linear regression. Values for the slopes (m) of the lines are presented in the figure; significantly different from control at * $P < 0.05$.

zation were examined (Figs. 3, 6, 7). Corneas placed in culture with OGF had 24% more defect than in control specimens at 24 hours, the earliest time point examined. Measurements at 48, 72, and 96 hours revealed that the remaining abraded areas were 45%, 83%, and 90%, respectively, greater than in control subjects. At 120 and 144 hours, corneas subjected to OGF contained residual defects that were 173% and 260%, respectively, larger than in control specimens.

With regard to the incidence of wound closure, the OGF group often exhibited a delay in healing compared to control corneas. For example, at 96 hours when only 12% of the OGF

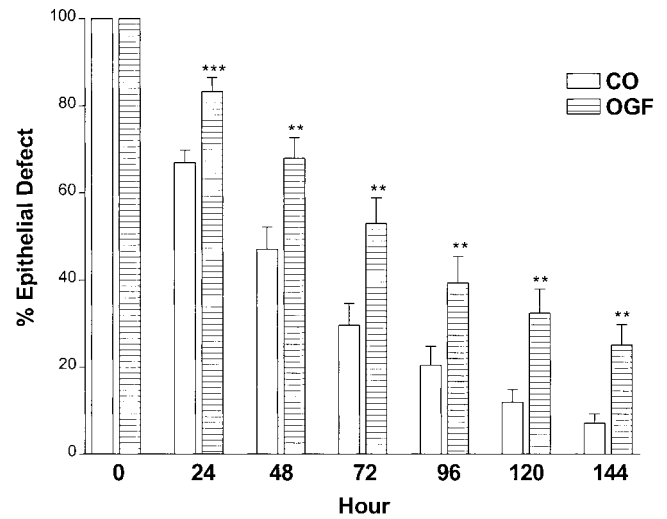


FIGURE 6. Histogram of residual epithelial defect in the human cornea after formation of an 8-mm corneal wound. Human eyes were placed in culture media containing either sterile water (CO) or 10^{-6} M OGF. Photographs of fluorescein-stained corneas were captured with a Sony CCD camera, and areas were analyzed by Optimas software. Residual epithelial defects are presented as percentage of the original wound. Data are expressed as means \pm SEM. Significantly different from controls at ** $P < 0.01$, or *** $P < 0.001$.

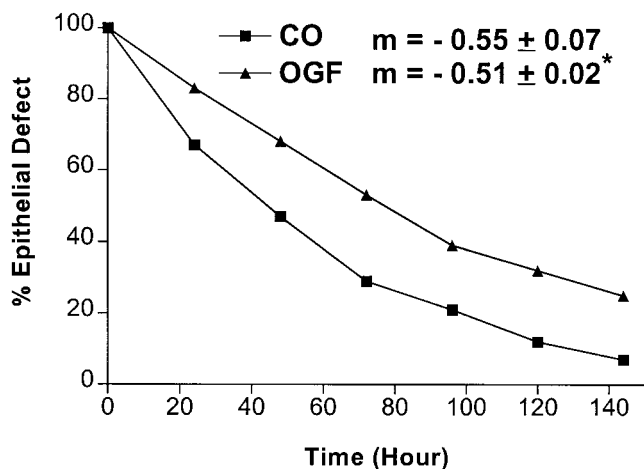


FIGURE 7. Overall healing rates of OGF-treated and control (CO) corneas analyzed by linear regression. Values for the slopes (m) of the lines are presented in the figure; significantly different from control at $*P < 0.05$.

exposed corneas were repaired, 37% of the corneas in the control group were reepithelialized. At 120 hours, the OGF group had 19% of the corneas healed but the control group had 44% repaired. Although the incidence of total reepithelialization in OGF-exposed corneas often appeared to be retarded, no statistically significant differences were recorded between the OGF and the control groups at any time point examined.

The rate of corneal wound repair was assessed by dividing the total area that was reepithelialized by the number of hours elapsed. Between 0 and 24 hours, the OGF group had a rate of wound healing of $0.42 \text{ mm}^2/\text{h}$, compared to a rate of $0.82 \text{ mm}^2/\text{h}$ in the control group; this difference was statistically significant at $P < 0.05$. The slope of the healing rate for the OGF treated corneas was -0.51 ± 0.02 , and the slope for the control group was -0.55 ± 0.07 ; these slopes differed significantly at $P < 0.05$. Linear regression indicated an $r = 0.98$ for the OGF exposed corneas and $r = 0.90$ for the control corneas.

DNA Synthesis in the Human Corneal Epithelium

The influence of the endogenous opioid system on basal epithelial cell DNA synthesis was examined during wound healing (Fig. 8). At 72 hours, the LI of cells in the central cornea and limbus in both the NTX and control groups was comparable. However, the number of cells undergoing DNA synthesis in the peripheral cornea of NTX-exposed corneas was 152% greater than that in control corneas.

Measurements of DNA synthesis in injured corneas exposed to OGF for 96 hours showed a 75% and 82% decrease in the peripheral cornea and limbus, respectively, from control specimens (Fig. 8). The central corneal region had an LI that was similar in both the OGF and the control groups.

Presence and Distribution of OGF and OGFr

Immunocytochemical preparations of human cornea within 1 hour of wounding (Figs. 9A-9C) and 24 hours after wounding (Figs. 9D-9F) were used to examine the effects of epithelial injury on the presence and distribution of OGF and OGFr. Both peptide and receptor could be observed in the cytoplasm, but not in the nucleus, of cells located in the region adjacent to the

wound shortly after abrasion. At 24 hours postinjury, at least one layer of cells could be seen in the reepithelialized region. These cells were elongated in a plane parallel to the basement membrane. Immunoreactivity for OGF and OGFr was observed in these cells. At least subjectively, there appeared to be no differences in staining intensity between the 1- and 24-hour specimens stained with either OGF or OGFr antibodies. Preabsorbed control preparations and those stained with the secondary antibody showed little reactivity (data not shown).

DISCUSSION

A number of models have been used to examine human and animal corneal wound healing.¹⁶⁻²⁸ The organ culture model was chosen in the present study because it (a) preserves corneal architecture, (b) permits the interaction of different cell types (e.g., epithelial cells, keratocytes), (c) retains limbal cells, a source of epithelial cells, (d) maintains characteristics such as centripetal migration, (e) enables the utilization of human tissue, and (f) provides a means to examine the importance of endogenous and exogenous growth factors.¹⁶ Subsequent to McCarey and Kaufman's²⁹ report of media (MK media) in which human corneas could be stored for up to 14 days before transplantation, two major methods of culturing human corneal tissues have been published. These include culturing of explants/corneas in media by immersion¹⁷⁻²⁶ or by an air-liquid interface technique.^{16,27,28} The advantages and disad-

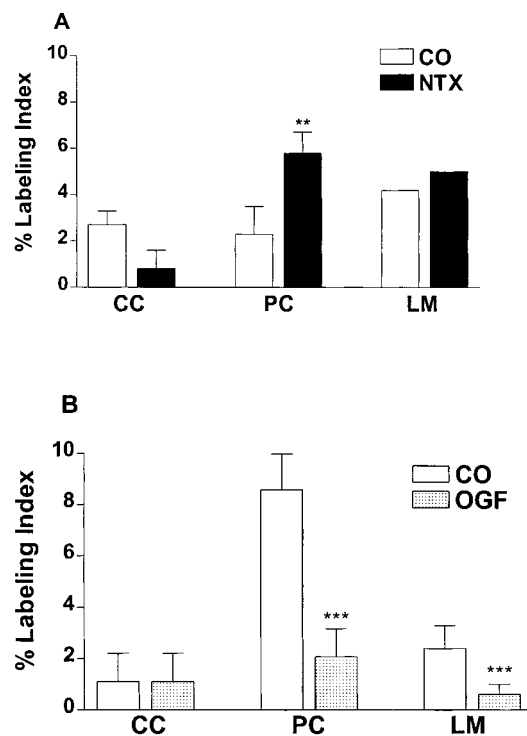


FIGURE 8. Histograms of the labeling index of human basal epithelial cells in the central cornea (CC), peripheral cornea (PC), and limbus (LM). Specimens were evaluated 72 hours (A) after placement in culture media with sterile water (CO) or naltrexone (NTX), or 96 hours (B) in culture media containing sterile water (CO) or OGF; drugs and media were replaced daily. Values represent means \pm SEM. Significantly different from controls at $**P < 0.01$ or $***P < 0.001$.

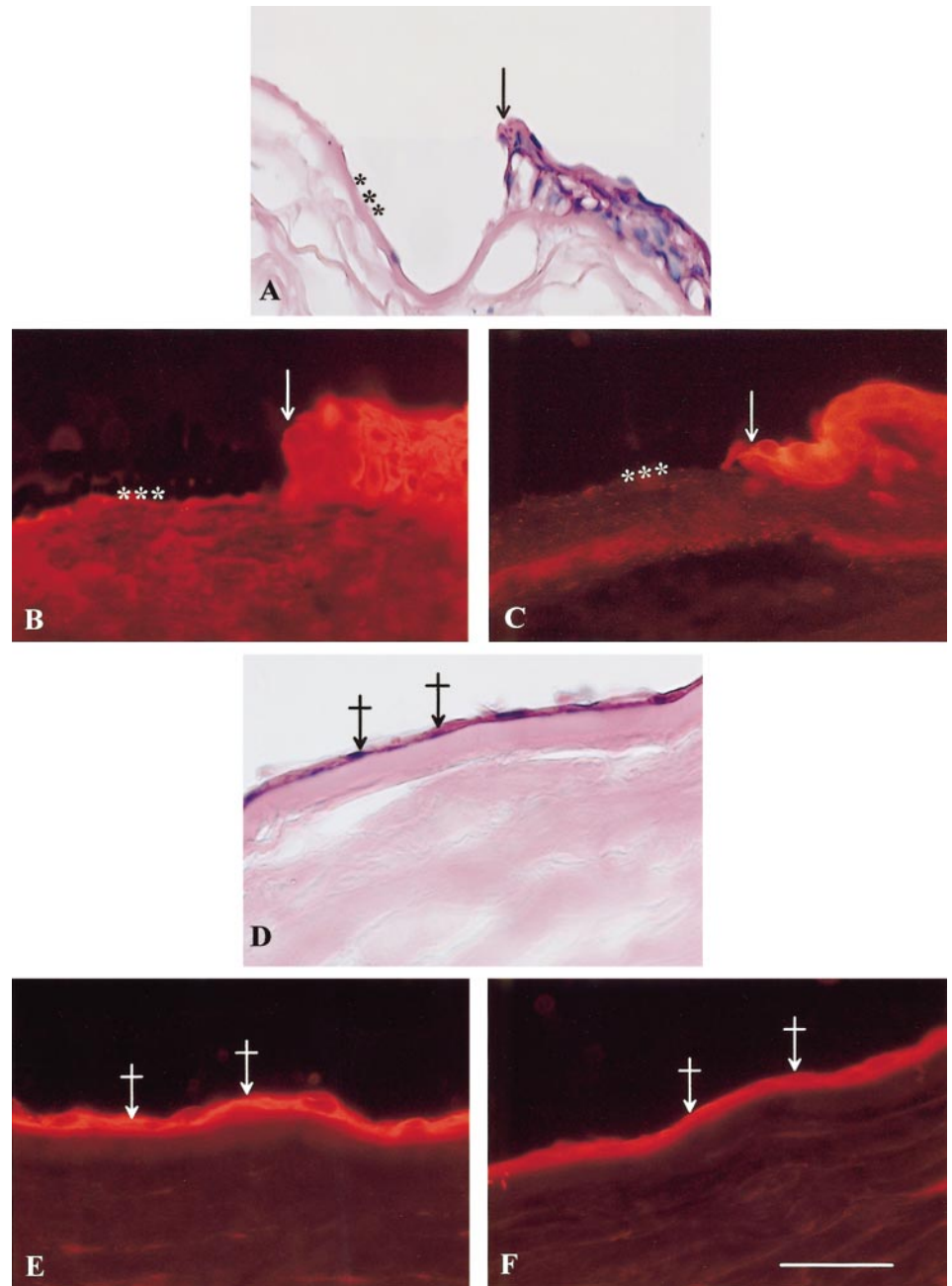


FIGURE 9. Light photomicrographs of the human corneal epithelium at 1 hour (**A** through **C**) and 24 hours (**D** through **F**) after abrasion, and stained with hematoxylin and eosin (**A**, **D**), or antibodies to OGF (1:150) (**B**, **E**) or ζ opioid receptor (**C**, **F**); rhodamine-conjugated IgG (1:100) was used as the secondary antibody. At 1 hour both the edge of the wound (*arrow*) and denuded surface (*asterisks*) can be observed (**A** through **C**), whereas at 24 hours only the reepithelialized surface (*cross-hatched arrow*) that is 1 to 2 cells thick was recorded (**D** through **F**). Immunoreactivity for both OGF and OGFr was detected in epithelial cells at both 1 hour and 24 hours, with staining of the cytoplasm, but not the nucleoplasm, noted. Bar, 40 μ m.

vantages of both methods of tissue culture have been widely discussed.¹⁶⁻²⁸ In the present study we used a modification of the immersion technique that included placing corneas in culture with the epithelial side facing up to avoid disturbance of the epithelium,^{17,22} exclusion of dextran in the media to avoid detrimental alterations,¹⁹ and media changes daily to ensure a frequent replenishment of nutritive substances and to eliminate waste. Moreover, exclusion criteria for the selection of corneas based on such factors as patient history, infectious diseases, and biomicroscopic examination were chosen to optimize and standardize the investigations, and to enhance the viability and well-being of the corneas. The use of the immersion technique appeared to provide the appropriate environment for experimentation. Thus, immersion of the cornea was not observed to be adverse to corneal architecture, especially the structure of the epithelium, for the 7 days of organ culture,

concurring with the observations of previous reports.²⁸ Cell generative processes were maintained, and DNA synthesis was detected in the central and peripheral cornea as well as the limbus. Human corneas in culture reepithelialized, indicating that such properties as cellular migration and proliferation were not compromised. Additionally, the rates of healing were comparable to those in organ cultures using an air-liquid environment,^{16,27} as well as in patients,^{30,31} suggesting that wound repair processes were intact and similar to that occurring *in vivo*.

This study used NTX to block opioid receptors from endogenous opioids during reepithelialization of the abraded, cultured, human cornea and showed that healing was markedly accelerated above control levels. The increase in repair was reflected by changes in the size of the defect, rate of healing, and the incidence of corneas with complete reepithelialization.

The action of opioid peptides with regard to corneal restitution was direct and independent of systemic factors, since the effects observed were detected in an organ culture setting. Given reports that the sole action of NTX is to block opioid receptors,^{3,2} these data suggest that native opioid peptides act as receptor-mediated inhibitory growth factors with respect to cell migration and proliferation during restorative processes in the human eye. Moreover, because disruption of opioid peptide-receptor interfacing provoked an acceleration in corneal repair, the endogenous opioid(s) related to growth must be constitutively expressed; these results are consonant with earlier observations in rats and rabbits.^{13,14} The data demonstrate, for the first time, that an endogenous opioid system is involved with epithelial repair in the human cornea.

The prediction, based on the NTX experiments, that an endogenous opioid serves as a negative growth factor in corneal repair was confirmed in studies of the effects of OGF application (in high titer), on the healing of corneal epithelial abrasion. Injured human corneas displayed a subnormal rate of healing and delay in the incidence of complete closure of the defect. Furthermore, we learned that one of the mechanisms underlying this regulatory property is mediation of opioid activity by a receptor, as indicated by the opioid antagonist blockade experiments. Whether OGF is the only opioid peptide associated with wound healing in the human cornea requires clarification. Moreover, the present investigation focused on the initial phases of repair and the covering of the abraded surface—not the full restitution of the corneal epithelium (e.g., junctional complexes).

Another aspect of this study is whether the results from an organ culture setting of the human cornea reflect the situation in vivo. Some information along these lines has been forthcoming from studies in rabbits and rats,^{13,14} where prevention of opioid interaction with receptors was found to enhance healing of the corneal epithelium using topical (rabbits, rats) and systemic (rats) application of NTX. Because a parallel increase in healing occurred both in vitro and in vivo with NTX in rabbits,¹³ one may tentatively conclude that the findings in the present organ culture model are indicative of the human condition.

In the course of these studies we discovered that modulation of OGF activity had a significant impact on DNA synthesis in the corneal epithelium adjacent to the abraded region. OGF depressed DNA synthesis, whereas application of NTX to the injured cornea resulted in an increase in DNA synthesis. It is well known that cell proliferation contributes to the reepithelialization process.^{2-4,7} Therefore, our data suggest that at least one mechanism for the changes in healing observed on addition of OGF or NTX to organ cultures of the human cornea is an alteration in cellular generation. This study also extends earlier observations as to the presence of OGF and its receptor in human homeostatic corneal epithelium¹² and serves as the initial demonstration that this endogenous opioid system functions in the human cornea to regulate DNA synthesis. Finally, because wound healing involves cell migration,^{2-4,7} one can hypothesize that OGF also is targeted to another mechanism: cellular movement needed for reepithelialization. Our findings in the present study suggest for the first time that OGF modulates two major processes of repair in the human cornea: cell proliferation and cell migration.

The demonstration that OGF is a peptide that functions by way of receptors in the human cornea in this investigation

would predict that both the peptide and receptor are present on epithelial cells. Indeed, previous reports have shown that both OGF and OGF_r are located in corneal epithelial cells.^{9,10,12,13} The relationship of OGF and OGF_r in the wound-healing process demonstrates that both peptide and receptor are retained in the region adjacent to the wound 1 hour after injury, and can be observed in the reepithelializing cells 24 hours after abrasion. These results suggest that despite mechanical disruption, OGF and OGF_r are retained in the residual epithelium—and presumably function—during repair of the ocular epithelium. This concept is supported by the fact that alterations in OGF, either by application of an excess of peptide or by decreasing the effects of peptide by receptor blockade, have a marked influence on the course of wound healing.

The present studies have shown that an endogenous opioid system plays an important role in corneal wound healing in humans. Given the difficulty and variability of obtaining human corneas, our strategy was to examine whether such a system was present and functioned in the human cornea, as it does in the rat¹⁴ and rabbit.¹³ Although long-term investigations with human corneas are feasible to address the influence of opioids on the entire course of healing, it might be more advantageous to perform such research in vivo using an animal model. However, even at this juncture, one can offer speculation on the clinical implications of our findings. First, in a therapeutic sense, chronic opioid receptor blockade could be a means to rescue individuals experiencing delays in wound healing. Second, the lessons learned in this study may aid in our understanding of why individuals could have a retardation in corneal reepithelialization. For example, chronic overproduction of OGF, or an increase in binding affinity or capacity of the OGF receptor, may delay wound healing and/or cause a disorganization of the repair process.

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