The Mechanism of Accelerated Corneal Epithelial Healing by Human Epidermal Growth Factor

Toshiki Kirazowa,* Shigeru Kinoshita,† Kazuhiko Fujita,* Keiko Araki,* Hiroshi Watanabe,† Yuichi Ohashi,† and Reizo Manabe†

The effect of biosynthetic human epidermal growth factor (hEGF) was investigated on a 10-mm diameter corneal epithelial defect model in rabbits. Topical application of over 10 μg/ml of hEGF five times a day significantly enhanced the epithelial healing rate, in a dose-dependent manner. The maximum healing rate was observed in eyes treated with 20 μg/ml of hEGF (1.59 ± 0.26 mm²/h), whereas application of less than 5 μg/ml of hEGF did not increase the rate of epithelial regeneration compared statistically with control vehicles (1.03 ± 0.24 mm²/h). S-phase analysis indicated that hEGF treatment induced a high rate of epithelial replication, particularly near the limbal region, during 12 to approximately 24 hours after wounding, followed by massive cell replication from 1 mm behind the leading edge through the limbus during 24-48 hours. The change in number and distribution of S-phase cells thereafter did not essentially differ between hEGF-treated and control groups. In concordance with the S-phase analysis, there was a statistically significant increase in the DNA content in regenerating epithelium at 48 and 72 hours in the hEGF-treated group. These findings indicate that hEGF-induced acceleration of large corneal epithelial wound healing is associated with about twofold cell replication in the regenerating epithelium during 24 to approximately 48 hours after wounding. It is concluded that cell proliferation induced by hEGF, particularly that in limbal and peripheral corneal epithelial cells, may play an important role in accelerating epithelial healing. Invest Ophthalmol Vis Sci 31:1773-1778, 1990

Since Cohen isolated epidermal growth factor (EGF) from the submaxillary gland and elucidated the primary structure of mouse EGF (mEGF) as composed of 53 amino acids. EGF has been known to have a potent stimulating effect on epidermal cell proliferation. Human EGF (hEGF), which shows great similarity to mEGF in primary structure and biologic activities, has been found in human urine and is now obtained for use as a biosynthetic substance with a recombinant DNA technique. Since our recent investigations revealed that hEGF is also found in human tears, possibly secreted by lacrimal duct cells, this substance may be essential in maintaining corneal epithelial integrity in epithelial wound healing and under normal conditions.

Previous studies using corneas showed that mEGF stimulates proliferation and migration of epithelial cells in vitro and that topical application of mEGF or hEGF can promote in vivo corneal wound healing in rabbits, primates, and humans. Recently, Soong et al found that EGF did not promote motility of corneal epithelial cells and proposed that acceleration of corneal epithelial wound healing by EGF is entirely due to stimulation of cellular mitosis, not to increased cell motility. However, the precise mechanisms by which hEGF accelerates epithelial wound healing in vivo are not yet known. For instance, to our knowledge, the distribution of S-phase cells in the hEGF-treated cornea in vivo has not been well documented. Such information is essential before topical application of hEGF clinically in indicated corneal disorders.

Therefore, we first reinvestigated the relationship between topical hEGF concentration and epithelial healing rate; then we studied S-phase cell distribution in regenerating epithelium, using bromodeoxyuridine (BrdU) immunohistochemistry, to see whether regenerating epithelium in a specific area responded to hEGF. We also checked the DNA content in regenerating epithelium to determine whether or not there was an increase in the number of regenerating epithelial cells involved in epithelial wound healing. The results showed that topically applied hEGF stimulated corneal epithelial wound healing in a dose-dependent manner and induced a high rate of epithelial healing.
replication, particularly in the limbal and peripheral corneal epithelium. This stimulus to cell proliferation was marked compared with controls.

Materials and Methods

Epithelial Defects

The use of rabbits in this study conformed to the ARVO Resolution on the Use of Animals in Research. One hundred five rabbits were used.

Albino rabbits weighing 2–3 kg were anesthetized by intramuscular injection of ketamine hydrochloride (200 mg per animal). After a 50-μl topical instillation of 0.4% oxybuprocaine hydrochloride, a 10-mm diameter central area of corneal epithelium was marked compared with controls. In all experiments, both hEGF and control vehicle were applied from 0–84 hr after epithelial wounding.

Topical Application of hEGF

According to the method described previously, topical concentrations of hEGF applied topically five times a day (8 AM and 8 PM) were performed between 6 AM and 9 AM.

After wounding, the epithelial defect was stained with 0.5% methylene blue to confirm that a 10-mm diameter area of central corneal epithelium had been removed. Although methylene blue may have a minor effect upon the epithelial healing rate, as suggested by Ubels et al., this staining yields photographs of higher epithelial-defect resolution than does fluorescein. Since we stained with methylene blue in both control and hEGF-treated eyes at every point examined, the epithelial healing rate could be compared between the two groups.

Topical Application of hEGF

The hEGF produced in Escherichia coli by recombinant technology was purified to homogeneity according to the method described previously. The preparation used in this study contained over 99% authentic hEGF; no significant amounts of proteins derived from host E. coli (<1.0 ppm) or endotoxins (0.01 ppm) were observed on analysis by high-performance liquid chromatography, enzyme-linked immunosorbent assay, and Limulus test. The purified hEGF was dissolved in Dulbecco’s phosphate-buffered saline (PBS) at concentrations of 2, 5, 10, 20, and 50 μg/ml. In the study of epithelial healing rate, paired eyes were used, with 100 μl of various concentrations of hEGF applied topically five times a day (8 AM through 12 midnight) to one eye, and a PBS vehicle to the contralateral eye as a control. For S-phase analysis and DNA content measurement, 20 μg/ml of hEGF was used in the manner previously described. In all experiments, both hEGF and control vehicle were applied from 0–84 hr after epithelial wounding.

Determination of Healing Rate

After staining with 0.5% methylene blue, all eyes were photographed at 8 AM and 8 PM until complete epithelial closure was observed. The areas of photographed corneal epithelial defects were measured by computerized planimeter. For each eye, the best-fit straight line was determined by least-squares regression analysis, using the area of defects measured over time until complete epithelial closure. The converted slope of the straight line was expressed as the healing rate (mm²/hr).

S-Phase Cell Analysis

Proliferation of regenerating corneal epithelial cells was detected immunohistochemically using BrdU (Sigma, St. Louis, MO), an analogue of thymidine, and its specific monoclonal antibody. Briefly: after rabbits were killed at 12, 24, and 48 hr, and 4, 7, and 14 days by an overdose of sodium pentobarbital, the eyes were enucleated. The entire corneas, with 2 ~ 3 mm of scleral rim, were then excised and incubated in Medium-199 (GIBCO, Grand Island, NY) containing 10 μM BrdU at 3 atmospheric pressures of 95% O₂:5% CO₂ at 37°C for 2 hr. After fixation with ethanol:acetic acid (95:5, v/v) for 12 hr, the corneoscleral specimens were embedded in paraffin and cut into 4-μm sections through the greatest corneal diameter. The deparaftinized specimen, treated with 4N HCl for 1 hr to denature the DNA, was incubated in 50-fold diluted anti-BrdU (Becton-Dickinson, San Jose, CA) at 4°C for 12–24 hr. The specimen was then reacted with 100-fold diluted biotinylated antimouse IgG (Vector, Burlington, CA) for 1 hr, and stained with Vectastain ABC-AP kit (Vector, Burlingame, CA) and hematoxylin as a counterstain. S-phase cells were counted in seven 1000-μm fields across the cornea, from 1 mm beyond the limbus to the center; the average number from three adjacent sections was then calculated by a method similar to that reported by Danjo et al.

DNA Content

Rabbits were killed at 1, 2, 3, 4, 7, 10, and 20 days after wounding by an overdose of sodium pentobarbital. After the eye was gently rinsed with saline and the central cornea was marked with an 8-mm diameter trephine, the epithelium within the mark was scraped off completely with a Beaver blade, frozen immediately in liquid nitrogen, lyophilized overnight, and weighed. The DNA content in the scraped-off epithelium was measured according to the method of Heingardner, using calf-thymus DNA as a standard. Briefly, the lyophilized epithelium was dissolved in 500 μl of 2% sodium dodecyl sulfate solution containing 0.1 mg/ml of glycerogen, and pre-
cipitated by the addition of two volumes of absolute ethanol. The precipitate was then dried and treated with 100 µl of 3,5-diaminobenzoic acid (400 mg/ml) (Nacalai tesque, Kyoto, Japan) at 60°C for 45 min; 1.5 ml of 1N HCL was then added. After centrifugation, supernatant fluorescence was measured by spectrofluorometer (Hitachi 650-60, Tokyo, Japan). Excitation and emission wavelengths were 415 nm and 515 nm, respectively.

Results

Gross Observation

All 88 of the hEGF-treated eyes developed no unusual corneal clouding or neovascularization and remained clear in all experiments.

Epithelial Healing Rate

The epithelial healing rate is summarized in Figure 1. Neither 2 nor 5 µg/ml of hEGF accelerated epithelial healing, whereas the healing rate increased significantly when 10 µg/ml (P < 0.01), 20 µg/ml (P < 0.05), or 50 µg/ml (P < 0.05) of hEGF solution was topically applied. This acceleration occurred in a dose-dependent manner, plateauing at a concentration of 20 µg/ml. The contralateral eyes, which received PBS only, also tended to show accelerated epithelial healing with increased hEGF concentration, although the difference in healing rate from that in the initial control was not statistically significant.

S-Phase Cell Analysis

In the untouched normal corneal epithelium, S-phase cells were sparse but uniformly distributed all over the corneal epithelium; the average number of S-phase cells per 1 mm was 1.06 ± 0.26. The number of S-phase cells in 20 µg/ml hEGF-treated corneas showed a roughly twofold higher level than that in controls from 24–48 hr postwounding, the number then decreasing to the level of controls at 4 days. As shown in Figure 2, analysis of S-phase cell distribution showed a fair amount of cell proliferation in the limbal epithelium, but not in the regenerating corneal epithelium, as early as 12 hr postwounding in both hEGF-treated and control groups. At 24 hr after wounding, however, the hEGF-treated group showed vigorous cell proliferation around the limbal and peripheral corneal epithelium, with the usual pattern of cell proliferation in the regenerating epithelium; the control group did not show such a dramatic proliferation in the comparable limbal area. In addition, the leading edge of regenerating epithelium showed rather sparse cell proliferation in the hEGF-treated eyes (Fig. 3). At 48 hr, relatively uniform cell proliferation was observed up to the limbus in both hEGF-treated and control groups, together with a higher level of perilimbal cell proliferation in the hEGF-treated eyes. The subsequent change in distribution

Fig. 1. Healing rate of 10 mm diameter corneal epithelial wound closure. Paired eyes were used for measuring healing rate; solid circle indicates hEGF-treated group, open circle control (PBS) group. Circle and bar represent average and standard deviation, respectively. *: P < 0.05. **: P < 0.01 (paired t-test).

Fig. 2. S-phase cell distribution in regenerating corneal epithelium. Number of S-phase cells shows average of three different samples. Arrows indicate limbo-corneal junction; solid columns represent limbal epithelium.
of S-phase cells did not essentially differ between hEGF-treated and control groups.

**DNA Content**

The DNA content in the 8-mm diameter regenerating epithelium in the hEGF-treated group showed statistically significant increase at 2 and 3 days after wounding compared with the control group ($P < 0.01$) (Fig. 4), but it did not exceed the normal level thereafter.

**Discussion**

Although it is well known that EGF stimulates epithelial cells in vitro, and it has been partially proven to do so in vivo by healing-rate measurement,9-17 the present study, using S-phase cell analysis and DNA measurement, is the first conclusive in vivo demonstration of actual hEGF proliferative stimulation of regenerating corneal epithelium to our knowledge.

The interesting findings are that limbal epithelial-cell proliferation was first induced by large epithelial wounding in both hEGF-treated and control groups and that limbal and peripheral corneal epithelial proliferation was enormously amplified by hEGF at 24 to approximately 48 hours after wounding, the latter phenomenon not being observed in controls.

---

**Fig. 3.** Immunohistochemical staining of BrdU-incorporated cells around peripheral cornea at 24 hr after wounding. Upper: untouched normal cornea. Few cells incorporate BrdU. Middle: control (PBS) eye. BrdU-incorporated cells are mainly located 0 ~ 1 mm behind leading edge. Limbal epithelium also shows some response. Lower: hEGF-treated eye. Many BrdU-incorporated cells are observed in limbal and peripheral corneal epithelium. Leading edge of regenerating epithelium can be seen in right margin. Arrows indicate limbo-corneal junction.

**Fig. 4.** DNA contents in regenerating epithelium. Shaded area represents value of standard variation of mean in normal corneal epithelium ($n = 8$). Bar indicates standard deviation of mean ($n = 6$). **: $P < 0.01$ (paired t-test).
the control groups always showed a proliferative zone proximal to the site of regenerating epithelium but not distal, it can be concluded that distal proliferative activity involving the limbal and peripheral corneal epithelium is a specific response to hEGF. Moreover, the fact that hEGF did not induce significant cell proliferation in untouched normal corneal epithelium (data not shown) indicates that such proliferation occurs as a result of both hEGF and unknown biologic signals from the wound.

Cotsarelis et al.\(^26\) report that similar preferential stimulation of limbal epithelial proliferation was observed when a tumor promoter, \(0\)-tetra-decanoylphorbol 13-acetate (TPA), was topically applied. These findings, in conjunction with the concept that slow-cycling corneal epithelial stem cells are located in the limbal lesion,\(^27\)\(^28\) lead to the hypothesis that limbal epithelial cells (mainly stem cells) are very sensitive to growth stimulatory substances such as hEGF and TPA. This hypothesis is also supported by the previous finding that regenerated epithelium of limbal origin in total corneal epithelial debridement treated with EGF was thicker than that in the control eye.\(^10\) Whether at 24 hours S-phase cells in the corneal epithelium adjacent to the limbus proliferate mainly in the region or migrate from the limbus remains unknown. However, since corneal epithelium has an EGF receptor and shows mitotic activity in vitro under EGF,\(^9\) it is reasonable to speculate that peripheral corneal epithelium, which is thought to have more mitotic potential in vitro than central corneal epithelium,\(^29\) also responds to EGF. Of considerable interest is the fact that unlike the limbal and peripheral corneal epithelium, the regenerating epithelium near the leading edge showed minimal cell proliferation despite EGF application. Whether strong inhibitory factors (eg, TGF-\(\beta\)) for epithelial growth are involved\(^30\) or the central corneal epithelium is less sensitive to EGF remains to be determined.

The present study also clearly demonstrates that topical application of hEGF has the ability to accelerate epithelial healing if the concentration exceeds 10 \(\mu\)g/ml.\(^6\) This concentration agrees with those reported by other investigators.\(^1\)\(^3\)\(^5\)\(^15\)\(^16\)\(^31\) We further demonstrated that the epithelial healing rate is affected by hEGF concentration in a dose-dependent manner, even in in vivo situations. However, the fact that (1) concentrations over 50 \(\mu\)g/ml did not show any additional acceleration and (2) the report by Ho et al.\(^15\) using 50–2000 \(\mu\)g/ml of mEGF did not show dose-dependent acceleration implies that down regulation may occur even in in vivo topical application.

Since the appropriate concentration of EGF for cultured corneal epithelium or epidermal keratinocytes is thought to be around 10 ng/ml,\(^32\) topically applied hEGF in this study was set at a roughly 1000-fold higher concentration than that in tissue culture media. According to the report by Schults et al.,\(^31\) topically applied EGF rapidly disappeared, only about 10% remaining in the conjunctiva and 0.1% in the cornea after 10 minutes. Of considerable interest is the question of why this dose-dependent acceleration occurs despite the rapid dilution. One possible explanation is that although topically applied hEGF is diluted by tears and washed out rapidly, hEGF penetrating the cornea binds to the EGF receptors of corneal and limbal epithelial cells for an unexpectedly short duration. If so, a twofold difference in initial concentration of hEGF may affect cellular response even in vivo. Since regenerating epithelium is thought to be more permeable by water-soluble molecules than normal epithelium,\(^32\) water-soluble hEGF may penetrate easier in the early stage of epithelial wound healing.

A curious finding is that the eyes contralateral to hEGF-treated eyes also tended to show a slight increase in epithelial healing rate, although the rate was not statistically significant. Since a similar result is reported in a study by Fredj-Reygrobellet et al.\(^18\) who state that the topical application of fibroblast growth factor accelerated epithelial healing in the contralateral eye, our results in this regard may be valuable. If so, the mechanism should be investigated in a future study.

Our experiments strongly indicate that hEGF eye drops are very effective in ocular surface disorders requiring substantial cell proliferation, particularly when limbal stem-cell proliferation is needed to maintain or cure the damaged ocular surface epithelium. Therefore, clinical trials of hEGF topical application in such diseases would seem to hold much promise.

**Key words:** epidermal growth factor (EGF), S-phase cell, corneal epithelium, limbal epithelium, corneal wound healing

**Acknowledgment**

The authors thank Robert Brady for editing this manuscript. They have no financial interest in hEGF products.

**References**

INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE / September 1990


