Effect of Leukocytes on Corneal Cellular Proliferation and Wound Healing

Lisha Gan, Per Fagerholm, and Hyung-Joon Kim

PURPOSE. To establish whether fucoidin, by blocking the adhesion of leukocytes on the limbal vascular endothelium, prevents extravasation of the cells from the blood stream into the limbal stroma and the wounded area after corneal injury. Successful leukocyte blocking enabled investigation of the influence of leukocytes on corneal cellular proliferation after corneal wounding.

METHODS. Thirty-two New Zealand White rabbits were used. Photorefractive keratectomy (PRK) and a standardized alkali corneal wound were used as models in two sets of experiments. In half of the injured rabbits fucoidin was used to prevent leukocytes from leaving the local vessels. The efficiency of the blocking technique was evaluated by counting the number of leukocytes in the limbal and wounded corneal areas. Proliferating cell nuclear antigen (PCNA) was used as a marker for proliferative activity.

RESULTS. The infiltration of leukocytes into the limbus and the cornea after PRK and alkali injuries can be blocked by fucoidin. The healing rate of corneal epithelium after alkali burn was retarded in the absence of leukocytes. PCNA expression was enhanced in the presence of leukocytes. Fucoidin per se had no influence on corneal cell proliferation and wound healing.

CONCLUSIONS. Polymorphonuclear leukocytes (PMNs) can be prevented from entering the cornea in vivo by fucoidin after PRK and after alkali burn. The corneal epithelial healing rate is delayed in the absence of PMNs in vivo, and PCNA expression increases in the presence of leukocytes. (Invest Ophthalmol Vis Sci. 1999;40:575-581)

Corneal wound healing involves a series of events in which the regulation of cellular proliferation is important. Infiltration of inflammatory cells into injured tissue is a hallmark of wound repair. Polymorphonuclear leukocytes (PMNs) are the first cells that migrate into the tissues in response to insults. In various corneal injuries, such as superficial epithelial denudation and alkali burns, PMNs are found in the limbus and wounded areas within the first 2 days. By the third day, the number of PMNs begins to decrease, and other cell types, such as mononuclear phagocytes, begin to appear along with signs of new capillaries.

The inflammatory process, in addition to a protective function, may somehow be involved in the initiation and stimulation of cellular proliferation and in scar formation. Tissue destruction, that is, stromal melting and neovascular ingrowth after alkali corneal burn, is characteristically mediated by infiltration of PMNs.

Polymorphonuclear leukocytes have been thought to be terminally differentiated, short-lived cells, incapable of proliferation. It has been suggested that inflammatory cells, probably neutrophils, contain growth-promoting substances, termed trephones, which stimulate fibroblast growth. Other investigators have presented more recent evidence that inflammation promotes the wound repair process. Furthermore, Colotta et al. showed that the survival of PMNs can be greatly extended after exposure to microenvironmental signals.

The infiltrated inflammatory cells exert their influence on resident cells through cytokines, which orchestrate the complex network of cellular interactions during wound healing. It has long been known that monocytes and macrophages produce cytokines. The production of cytokines by PMNs was described by Cassatella et al. and by other investigators. Cytokine production by PMNs has been thought to be cross-regulated between leukocytes. Yet, other studies have revealed that the ability of PMNs to release cytokines depends on the functional state of the cells.

Numerous cytokines have been shown to be secreted by inflammatory cells. On a single-cell basis, the macrophages or the monocytes may produce greater quantities of cytokines than PMNs. However, granulocytes constitute most infiltrating cells after wounding and, therefore, probably represent the most important source of cytokines.

Cellular proliferation is essential for healing wounds. The proliferating potential of corneal cells can be changed by interaction with other cells. The changes can be demonstrated by a currently available marker of cellular proliferation, proliferating cell nuclear antigen (PCNA). PCNA is a protein expressed only in cells committed to DNA synthesis and has been used as a naturally occurring marker for cell proliferation in animal and human corneas. The specificity of PCNA immunostaining and its validity as a proliferating cell marker are supported by several lines of evidence.

Leukocytes are known to invade a corneal wound after injury. To study the influence of leukocytes on corneal...
wound healing, elimination of the leukocytes from injured tissue has been tried by whole-body irradiation and other means. Recent advances in molecular biology have shown that the selectin family of adhesion molecules mediates the initial attachment of leukocytes to venular endothelial (rolling) cells before their firm adhesion at the site of tissue injury and inflammation. By inhibiting leukocyte rolling with administration of fucoidin (a homopolymer of sulfated fucose), it is possible to abolish neutrophil infiltration into a skin lesion almost totally.

The purpose of the present study was to determine whether leukocyte infiltration into the cornea after wounding can be prevented by the in vivo use of fucoidin as a selectin blocker. To study the influence of inflammatory cells on corneal wound healing, the blocking efficiency of fucoidin and then the corneal cellular response after blocking were assessed. Furthermore, the influence of fucoidin per se on corneal wound healing was evaluated.

**MATERIALS AND METHODS**

In this study a technique was evaluated to prevent by selectin inhibition the extravasation of PMNs that results in their leaving blood vessels and, thus, entering a corneal wound. Two types of corneal wound models were used to provoke PMNs to enter the cornea. The efficiency of leukocyte blockage by fucoidin was tested, in photorefractive keratectomy (PRK) and alkali wound models. The influence of PMNs on the epithelial healing rate was evaluated in the alkali wound model. The effect of topically applied fucoidin on the epithelial healing rate was evaluated after standardized mechanical epithelial debridement. All experiments were performed on New Zealand White rabbits weighing approximately 2.5 kg. The animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the local animal research ethics committee.

**Selectin Inhibition in PRK Wounds**

One eye of eight New Zealand White rabbits (2.5 kg) were operated on using an excimer laser (Visx 20/20; Visx, Santa Clara, CA). After mechanical epithelial debridement, a photorefractive keratectomy (PRK) and alkali wound models. The influence of PMNs on the epithelial healing rate was evaluated in the alkali wound model. The effect of topically applied fucoidin on the epithelial healing rate was evaluated after standardized mechanical epithelial debridement. All experiments were performed on New Zealand White rabbits weighing approximately 2.5 kg. The animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the local animal research ethics committee.

In four of the rabbits, the cell-adhesion molecule blocker, 5 mg/kg fucoidin (Lot no. 17H3891, F5631; Sigma, St. Louis, MO), a sulfated fucose-rich polysaccharide, was injected intravenously every 2 hours for 12 hours. The remaining four rabbits served as control subjects. After 12 hours the rabbits were killed with an overdose of pentobarbital. The wounded corneas and the corneas from the fellow eyes in the two groups were excised with a 3-mm scleral rim, fixed in 10% formaldehyde for 12 hours, and processed for paraffin sectioning. The sections were stained with hematoxylin-eosin.

Under a light microscope, the number of PMNs was counted in three sections from each cornea, in fucoidin-treated and untreated animals. The selection of the counting areas was done as follows: For the wounded areas, the center of each wound was determined under a low-magnification (×10) objective. After changing to a higher magnification (×20) objective and moving the field to touch the stromal wound bed, all PMNs in the field were counted. For the limbal area, the first limbal vessel was used to center the microscope for evaluation of PMNs in the limbal stroma. Cells in one microscopic field were counted, with the field touching the superficial part of the stroma on each side of the limbus. The PMNs were identified morphologically by their multilobed nuclei. The nonwounded corneas were evaluated in the corresponding locations. The difference between the two groups in number of PMNs was statistically analyzed.

**Selectin Blockage and Epithelial Healing Rate in Alkali Burn**

Fourteen New Zealand White rabbits (2.5 kg) were used. The central corneas of the right eyes were exposed to a 5.5-mm filter paper soaked in 1 N NaOH solution for 60 seconds. Balanced salt solution (BSS) was then used to rinse the wounded area. The wounds were verified with 2% fluorescein intravenously every 2 hours up to 35 hours to prevent PMNs from leaving the blood stream. The corneal defects were photographed after 0, 6, 16, 24, and 32 hours.

Photographs of the epithelial defects were taken using a camera with a 100-mm objective lens (Model EOS 500; Canon, Lake Success, NY) at every time point until the end of the experiments. A fixed distance was used, and a filter (Wratten 47B; Eastman Kodak, Rochester, NY) was placed on the flash. The fluorescein-stained areas were assessed by planimetry. In each cornea, a linear regression coefficient was determined for the epithelial defect size as a function of time. The difference between the two groups in size of the epithelial defect was statistically analyzed.

After 35 hours all rabbits were killed. The corneas were excised with a 3-mm scleral rim and cut through the center into two parts. The specimens were fixed in 10% formaldehyde for 12 hours and then embedded in paraffin. Histologic and immunohistologic evaluations were performed in 4-μm sections.

**Leukocytes in Alkali Burns**

The corneas excised after alkali burns were sectioned and stained with hematoxylin-eosin, as described earlier. The sections contained the corneal wound areas. The number of leukocytes was counted in the same way as in the first experiment.

**PCNA Staining and Corneal Cell Proliferative Activity**

PCNA was used to study epithelial and keratocyte proliferative activity during wound healing after alkali burn and after selectin blockage. The corneas, excised after the alkali burns, were sectioned and stained for PCNA. Immunocytochemical staining for PCNA was performed as follows: After dewaxing and rehydrating the sections, the slides were immersed in 1% hydrogen peroxide in 50% methanol for 30 minutes to quench endogenous peroxidase activity. This step was critical for reduction of background staining. All incubations were performed at room temperature in a humid chamber, making sure at each step that
all sections were covered by the reagents. The sections were washed in Tris-buffered saline for half an hour and then incubated with 4% bovine serum for 20 minutes to block nonspecific antibody binding. The primary antibody used to detect PCNA was a mouse monoclonal antibody (PC10; Dako, Glostrup, Denmark) diluted 1:20 with Tris-buffered saline plus serum. The incubation time for the antibody was 1 hour. After rinsing, the sections were incubated with biotinylated goat anti-mouse IgG at a dilution of 1:10 for 1 hour. Binding was detected with the biotin-avidin technique.

The PCNA staining pattern was analyzed under a light microscope. The density of PCNA staining was graded as strong (++), moderate (+), or weak (±), and only the strong and moderate staining were considered to be positive. The epithelium, keratocytes, and endothelium were evaluated.

Topical Application of Fucoidin in Mechanical Epithelial Debridement

To evaluate whether fucoidin per se has an influence on the epithelial healing rate, it was administered topically after epithelial debridement. A 5.5-mm manual epithelial debridement was performed with animals under general anesthesia (ketamine and xylazine) in one eye in each of 10 rabbits. A marker 5.5 mm in diameter was used to outline the area. Five rabbits received a drop of fucoidin (5 mg/ml in BSS) every 2 hours for 48 hours in the ablated eye. The other rabbits served as control subjects and received a drop of BSS in the ablated eye every 2 hours. The epithelial defects were photographically documented as described previously after 0, 8, 24, 32, and 48 hours. The epithelial defects were evaluated by planimetry in the same way as in the alkali burn wounds. After 48 hours the rabbits were killed, and the corneas were excised and fixed in 10% formaldehyde. After sectioning, the specimens were stained with hematoxylin-eosin and examined under the light microscope.

**Statistical Methods**

The statistical significance of differences between groups was evaluated in all cases using the Wilcoxon signed-rank test for the number of PMNs and the Wilcoxon rank-sum test for the epithelial defects in two independent samples.18

**RESULTS**

**Leukocyte Blocking in PRK Wounds**

No difference was found in the number of leukocytes in the limbal area when noninjured corneas and injured corneas in the blocking group were compared. The number of leukocytes was lower in the limbus of the noninjured corneas (<5/microscopic field) compared with the injured non-fucoidin-treated corneas (15-25/microscopic field). In the wound area, the median of the leukocytes was approximately 35 (range, 21-39; n = 12) per microscopic field for non-fucoidin-treated rabbit corneas (Fig. 1A), whereas no leukocytes were found in the wound area of fucoidin-treated corneas (Fig. 1B; P < 0.05). Furthermore, no leukocytes were found in the stroma between the limbus and the wound in the fucoidin-treated or in the non-fucoidin-treated corneas.

**Epithelial Healing Rate in Alkali-Wounded Corneas**

At the end of the experiment, the corneal epithelial defects in the fucoidin-treated group were significantly (P < 0.05) larger than those in the control group (Fig. 2). Therefore, the healing
rate of corneal epithelium in the control group was higher than in the fucoidin-treated group. A difference of epithelial defect size began to appear 16 hours after injury and increased with time (Fig. 2).

**Leukocytes in Alkali-Wounded Corneas**

At 35 hours, all corneas from the non-fucoidin-treated group showed massive infiltration of leukocytes (PMNs and macrophages) in the stroma, from the limbus (>100 per microscopic field) to the wound margin (Fig. 3). The density of leukocytes gradually decreased from the limbus to the wound edge. In the fucoidin-treated corneas, few (<5) leukocytes were seen per microscopic field in the limbal area (Fig. 4). The difference between the two groups was significant ($P < 0.05$). No leukocytes were found on the surfaces of wounds in either group.

**Proliferative Activity and PCNA Expression in Alkali-Wounded Corneas**

Positive PCNA was detected in the epithelium, the keratocytes, and the endothelium in the wounded corneas in the control group and in the fucoidin-treated group. PCNA expression was much more pronounced in the three cell types in the non-fucoidin-treated corneas in which leukocytes were present (Figs. 5A, 5C) than in fucoidin-treated corneas (Figs. 5B, 5D). A typical pattern of PCNA expression in the non-fucoidin-treated, alkali wounded corneas emerged. In the epithelium, PCNA was expressed not only in the limbal area in the basal layer but also in the wing layers and extended from the periphery of the corneal epithelium to the regenerating central epithelial cells. In the presence of leukocytes the epithelium appeared thicker, and more positive cells (Figs. 5A, 5C) were seen in all three corneal cell types than were seen in the fucoidin-treated specimen (Figs. 5B, 5D). In the stroma, PCNA was expressed in the limbal area and at the wound edge (Figs. 5A, 5C). Cells expressing PCNA in these areas seemed to be a mixture of different cell types. In the endothelium PCNA expression was found only at the wound edge where the endothelium became multilayered and a retrocorneal fibrous membrane formed (Fig. 5E).

**Topically Applied Fucoidin and the Epithelial Healing Rate**

There was no significant difference in the size of epithelial defect between the corneas that received fucoidin and those that received the solute BSS topically during the first 48 hours after injury. In the anterior one third to one half of the stroma, the keratocytes disappeared beneath the manually debrided epithelium in the wounded corneas (Fig. 6). No leukocytes were found in the central wound at this time point in either group. In the limbal area leukocytes were found in the stroma, but there was no difference in the the number of PMNs between the topically fucoidin-treated or solute-treated group.

**DISCUSSION**

Leukocytes have long been known to be involved in host defense. Recruitment of leukocytes to the site of injury is an essential feature of the inflammatory response in all types of wounds. Although the regulatory role of these cells and their cytokines to the resident tissue cells are closely involved in wound healing, the exact roles of these cells in different stages of corneal wound repair are far from clear.

One of the most important aspects of the inflammatory process after wounding involves cell adhesion events in the nearby vascular endothelium. After leukocyte activation by inflammatory mediators, selectins are released in the wound area from leukocytes and vascular endothelial cells. This process is thought to initiate a series of events that lead to leukocyte adhesion to the blood vessel endothelial cells. All leukocytes with the exception of memory T cells express selectins, which are the ligand of the endothelial cells. Fucoidin has previously been shown to block the leukocyte attachment events in vessels of the skin. Fucoidin acts as a selectin blocker and prevents leukocytes from attaching to vessel walls. The attachment precedes the actual migration from the vessels by the leukocytes. The success of our experiments in blocking leukocytes with fucoidin made it possible to investigate further the role of these cells in corneal wound healing.

In our experiments, the results of PRK and alkali wounding showed that leukocytes infiltrated the limbus and the wound during the first few days after injury. The leukocytes...
accumulated in the limbal and conjunctival vessels were then found in the limbal stroma and in the tears. In the tears, the cells derived from conjunctival vessels are mixed with the leukocytes from the lacrimal gland and are finally brought into the wound surface.\textsuperscript{32-34} In the PRK wounds no leukocytes were found in the stroma between the limbus and wounds in the first experiment. This suggests that the leukocytes first reached the wound through the tears after PRK.

Intravenous administration of the selectin blocker fucoidin effectively prevented leukocytes from entering the limbal and wound areas after PRK injury in this study. Absence of leukocytes on the surface in fucoidin-blocked corneas after
was used as a wound model. The efficiency of the block was appear to stimulate the directed locomotion of leukocytes.7

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ies, 2324  which have shown that PMNs leave the vessels at the limbus during the first day after trauma and are found in a gradually diminishing concentration surrounding the wound. In the early stages, no PMNs are found on the wound surface, which suggests that the major route of the leukocyte invasion into the wound is through the corneal stroma from the limbus in alkali burns. This pattern of leukocyte invasion was found in our non-fucoidin-treated alkali-injured corneas. The different wound models used in the present study showed the different patterns of leukocyte invasion, through tears after PRK or from the the stroma after alkali injury.

The density of the leukocytes in the limbus was much lower after PRK than after alkali injury in the models we used. The larger number of PMNs in the alkali burn wounds may have been caused by a different stimulus or a different degree of stimulus produced by damaged corneal tissues. Chemotactic agents released from the corneal tissues affected by alkali burn appear to stimulate the directed locomotion of leukocytes.7

The epithelial healing rate was retarded, and even in the short duration of the experiments, the epithelium appeared thinner in the fucoidin-treated alkali-injured group. The presence of leukocytes seems to stimulate the epithelial proliferation, evidenced by PCNA expression. The thicker appearance of the corneal epithelium in the control group seems to be related to the corneal edema. The exact mechanism of these changes is unknown. It seems that the blockade of leukocytes in limbal vessels after wounding delays the response of the tissue to the injury, which may indicate that the leukocytes infiltrating into the wounds play a role as a messenger to stimulate the tissue to react. Determining the consequence of this delay requires experiments of longer duration. Our results are in agreement with previous findings that show that the corneal epithelial healing rate is faster in vivo than that in vitro.

The interactions between PMNs and alkali-injured corneas have been shown to change protein synthesis by corneal cells and to change tissue environments.16 Furthermore, PMNs are well known to be involved in phagocytosis of the cellular debris at the wound area, and thereby to provide a smooth surface of extracellular matrix for epithelial cellular migration. Rapid epithelialization and an even surface are considered positive for wound healing after PRK. Less cellular reactivity and haze, often seen as regeneration, are experienced in fast epithelial healing.

The proliferative state of the corneal cells was further studied by investigating the expression of PCNA in the corneal cells after alkali injury. In this study the expression of PCNA was clearly more extensive in the basal epithelium and throughout the depth and extent of the epithelium when leukocytes were present in the cornea. This result is consistent with the epithelial healing rate result and indicates that the leukocytes that migrated to the limbal area in the early stage after wounding exerted some positive influence on the native corneal cell proliferation.

Leukocytes participate in the wound healing process through their capacity to release cytokines and growth factors, among which the macrophages express basic fibroblast growth factor, transforming growth factor-α, platelet-derived growth factor, and insulin-like growth factor. Polymorphonuclear cells invade the wounds earlier and in greater number than macrophages and produce transforming growth factor-β1, which stimulates proliferation of keratocytes, and other factors that in turn attract macrophages to the area.9 These factors are considered to be stimulators of cell proliferation.35 Depletion of leukocytes markedly delays fibroblast proliferation,36 suggesting the importance of these factors. Leukocytes are a source of growth factors and cytokines, all of which are necessary for initiation and propagation of new tissue formation in wounds.37

The difference in PCNA expression in the corneal cells in the present study may have been influenced by these stimulators secreted by leukocytes. The proliferative response of the epithelium, stroma, and endothelium was found to be increased in areas where leukocytes were present. A similar finding has been described in the retina,38 where leukocytes enhance the proliferative state of retinal pigment epithelial cells after retina detachment.

Leukocytes are known to exert negative influences on wound healing by activating metalloproteases, by damages caused by apoptosis induced by cytokines, or by release of free radicals. These aspects were not specifically addressed in the present study. After injury to the cornea, however, rapid covering of an epithelial defect is advantageous in preventing infections. Similarly, there must be rapid repopulation of the corneal stroma by keratocytes and stimulation of endothelial proliferation, at least in rabbits. Modification of fucoidin may make it possible to study long-term effects of the presence or absence of leukocytes during wound healing.

CONCLUSION

Leukocytes were prevented from entering the injured corneas by blocking the selectins through intravenous administration of fucoidin. The absence of leukocytes in the cornea decreased the epithelial healing rate in injuries caused by alkali. Further-
more, the proliferative potential, seen as PCNA expression, was also reduced in the absence of leukocytes. Fucoidin per se did not influence the proliferative potential of the epithelial cells.

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References


