Effects of growth factors on corneal wound healing

Richard S. Smith, Linda A. Smith, Larry Rich,* and Virginia Weimar*

Rabbits received nonpenetrating central corneal knife wounds. Immediately after wounding, a single 2.5 or 5.0 µg dose of mesodermal growth factor (MGF) was applied topically to the wound. Controls were treated with saline. Electron microscopic studies compared the healing responses of the control and treated animals over a 7-day period. MGF was a potent mitogen for keratocytes in rabbit corneas. The single dose enhanced fibroblast proliferation and metabolic activity. Control wounds were not healed after 7 days, whereas those of treated animals showed complete healing.

Key words: cornea, growth factors, wound healing, electron microscopy, fibroblasts, epithelium, collagen

Growth factors are a heterogeneous group of proteins capable of stimulating growth and multiplication of various cell types. The concept of specific growth factors grew out of tissue culture research when it was discovered that certain biologic products (e.g., serum) were needed to stimulate cell proliferation in explants. Although in vitro testing is useful, Gospodarowicz and Moran1 emphasized the importance of in vivo testing to establish the mitogenic activity of growth factors in cell growth and wound healing.

Nerve growth factor was the first of these proteins extracted from the submaxillary glands of adult male mice.2 Other compounds derived from the same source include epidermal growth factor,3 muscle dedifferentiating factor,4 and thymocyte-transforming fac-

Fig. 1. MGF-treated rabbit 48 hr after wounding. A large epithelial plug (E) is present. A few enlarged fibroblasts (arrows) are present at the wound margins. Epithelium and stroma away from the wound are undisturbed. (x175.)

From the Department of Ophthalmology, Albany Medical College of Union University, Albany, N. Y., and the *Department of Ophthalmology, University of Oregon Health Sciences Center, Portland.

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Reprint requests: Richard Smith, M.D., Department of Ophthalmology, Albany Medical College, Albany, N. Y. 12208.
Fig. 2. Control rabbit 48 hr after wounding. The epithelial plug (E) and wound (W) are artifactually disrupted. Neutrophils (N) are evident near the site of injury. Fibroblasts are absent. (x14,500.)

Fig. 3. MCF-treated rabbit 4 days after wounding. The epithelial plug (E) has nearly disappeared. Many enlarged fibroblasts (arrows) are present in the wound. (x140.)
Fig. 4. MGF-treated rabbit 4 days after wounding. In an area close to the wound, an enlarged fibroblast with dilated rough endoplasmic reticulum (E) is seen. Another fibroblast (arrow) is undergoing mitosis. (×15,500.)

Weimar and Haraguchi found two additional substances (mesodermal growth factor (MGF) and F2) which had a potent effect on the growth of mesenchymal cells of rabbit cornea in organ culture. MGF subsequently was shown to accelerate the healing of rabbit and human corneal endothelium in organ culture and to activate wound healing responses in rat corneas.

Light microscopic studies of nonperforating central corneal wounds in rabbits treated with 1 to 5 μg of MGF showed increased fibroblast activity, stromal cell division, and accelerated stromal healing 72 hr after wounding. A modified version of these studies was repeated and continued for 1 week to evaluate the evolution of healing. Ultrastructural studies were performed to assess in greater detail the cellular changes which took place as the wounds healed.

Methods

Adult Dutch belted rabbits were anesthetized with intramuscular ketamine. A central nonpenetrating corneal wound 4 mm in length and 0.5 mm deep was made in each eye. Immediately after wounding, each animal received a single 2.5 or 5.0 μg dose of MGF dissolved in 20 μl of Dulbecco’s phosphate-buffered saline. The MGF was dropped directly on the wound surface with a sterile 30-gauge needle attached to a 100 μl syringe. The operating microscope was used to control wound production and to ensure uniform application of MGF along the length of the wound. In contrast to earlier studies, the MGF was applied to the surface of the wound only, to avoid additional trauma from the hypodermic needle. Control animals received a single application of 20 μl of buffered saline. The wounds were examined daily with a slit lamp, and findings were similar to those previously described. Thirty rabbits were divided into groups of 10. In each group seven animals received MGF, and three served as controls. One group was killed at 48 hr, and one group each at 4 and 7 days.

The excised whole corneas were placed in cold phosphate-buffered glutaraldehyde and paraformaldehyde mixture and fixed for 96 hr. Under the dissecting microscope 1.5 by 5 mm corneal blocks containing the entire wound were washed...
Fig. 5. MGF-treated rabbit 4 days after wounding. In the wounded area (W), there are numerous plump fibroblasts (arrows) which contain abundant, dilated rough endoplasmic reticulum. (×15,500.)

Fig. 6. MGF-treated rabbit 7 days after wounding. The epithelial plug has disappeared and there is a dense, cellular scar in the wound area (arrows). (×140.)
in 0.2M phosphate buffer for 12 hr. Small blocks suitable for electron microscopy were cut at right angles to the wound, postfixed in osmium, and embedded in Araldite. Thick sections for light microscopy localized the wounded area, after which thin sections were cut and stained with lead citrate and uranyl acetate.

Observations

The effects of 2.5 and 5.0 g doses of MGF were identical and are discussed together. When the tissue was cut into blocks for final fixation, each cornea was identified only by number and not by type of treatment. Under the dissecting microscope, the 4- and 7-day material fell into two obvious classes: either a broad epithelial plug and gaping wound or a wound so thoroughly healed as to be nearly invisible. By reference to the number/treatment key, it was found that the nearly invisible wounds were MGF-treated.

The findings at gross examination were confirmed in thick plastic sections cut for light microscopy. At 48 hr both control and treated animals showed a broad, deep epithelial plug and minimal cellular activity in the adjacent stroma (Fig. 1). The number of fibroblasts in the deep aspect of the wound was slightly increased in the treated animals. At this time and at subsequent stages, the untreated wounds frequently showed artificial separation due to lack of tensile strength of the wound. The 48 hr wound showed stromal irregularity and absence of keratocyte activity in the control animals. A few neutrophils were evident beneath the epithelial plug (Fig. 2).

In the treated 4-day animals the epithelial plug was smaller than in the controls, and the fibroblasts were enlarged and more numerous (Fig. 3). At 4 days the epithelial plug of the controls showed only a slight decrease in size from the 48 hr animals (Fig. 1). At higher magnification (Figs. 4 and 5), fibroblasts in the wound area showed abundant cytoplasm and loss of their fine processes. Mitotic activity was seen. The rough-surfaced endoplasmic reticulum was more abundant than that in the controls and often showed dilated cisternae.

By 7 days the epithelial plug had disappeared in the treated wound, and cellular ac-
tivity was a prominent feature (Fig. 6). In contrast, the controls showed a persistent epithelial plug, artifactual wound disruption, and less striking proliferation of fibroblasts (Fig. 7). Electron microscopy revealed fewer fibroblasts than in the treated animals. The condensed cytoplasm of these cells showed smaller amounts of endoplasmic reticulum. The treated animals showed plump fibroblasts with abundant, dilated rough endoplasmic reticulum (Fig. 8). The overlying corneal epithelium was normal and showed early reconstruction of hemidesmosomes and the epithelial basal lamina (Fig. 8, inset). Collagen fibrils surrounded many of the fibroblasts. This activity was centered in the wound area. Less than 1 mm away the stroma was normal, and keratocytes were characterized by elongated cytoplasmic processes with scanty condensed endoplasmic reticulum (Fig. 9) similar to that seen in the untreated wounds. This emphasizes the focal nature of the process induced by MGF treatment.

**Discussion**

MGF, derived from mouse submaxillary glands, is a protein with a molecular weight of about 26,000 and prominent esteropeptidase activity (Haraguchi et al., unpublished observations). Further biochemical and structural characterization is not yet available. MGF acts as a mitogen in organ culture for fibroblasts and endothelial cells. A single dose of 1 to 5 μg is capable of accelerating wound healing, as evidenced by narrowing of the epithelial plug and production of a deeper, more intense fibroblast response. A single dose of 1 to 5 μg is capable of accelerating wound healing, as evidenced by narrowing of the epithelial plug and production of a deeper, more intense fibroblast response. A single dose of 1 to 5 μg is capable of accelerating wound healing, as evidenced by narrowing of the epithelial plug and production of a deeper, more intense fibroblast response.
morphology after MGF are similar to those observed during healing of normal wounds, but they occur at an accelerated rate. Several authors\textsuperscript{12-15} have observed that the most striking feature of fibroblasts during active wound healing is the presence of large amounts of dilated rough endoplasmic reticulum. After administration of tritiated proline, there was rapid labeling of the rough endoplasmic reticulum in odontoblasts,\textsuperscript{15} indicating that this was the site of synthesis of collagen precursors. In the same experiment,\textsuperscript{15} the radioactive label was followed through the Golgi apparatus to cytoplasmic secretory granules and ultimately to the extracellular space where collagen was being deposited.\textsuperscript{13} This evidence supports the conclusion that the prominent rough endoplasmic reticulum in MGF-treated animals was associated with metabolic activation of fibroblasts, collagen synthesis, and subsequent wound healing.

The biochemical synthesis of procollagen occurs within the cell. Procollagen molecules are secreted into the extracellular space where final assembly into stable, crosslinked collagen fibrils takes place.\textsuperscript{16} Only when this latter event is completed can newly synthesized collagen be visualized with the electron microscope. The endoplasmic reticulum activity and the presence of collagen fibrils closely associated with the activated fibroblasts in treated animals strongly imply that new collagen is produced by these cells. Treatment with MGF does not alter the morphologic changes observed in healing of corneal wounds but does produce a more rapid evolution of the healing process.

Although doses of 2.5 and 5.0 $\mu$g were given in our experiments, these actually represent the maximum possible total dose. The actual effective dose was much lower because much of the 20 $\mu$l of solution flowed off the surface of the cornea at the time of irrigation.
This surface application also explains the slightly slower rate of wound healing at 48 hr than that observed in our earlier studies. It should also be emphasized that the easily observed effects in the present experiment were produced by a single exposure to MGF immediately after corneal wounding. This implies either a rapid alteration of the affected fibroblasts which lasts for up to 7 days or persistence of physiologically significant quantities of MGF over the duration of the experiment.

On the basis of our ultrastructural studies, it is apparent that MGF is a potent mitogen for keratocytes in the rabbit cornea. Exposure to a single minute dose of this protein results in enhanced fibroblastic proliferation and metabolic activity, with consequent acceleration of wound healing. The ability of a specific protein to alter the rate of wound healing is a useful tool for enhancing our understanding of this complex process.

REFERENCES