A classic dose-response relationship between the width of the zone adjacent to the wound edge of rat corneal buttons. Reports

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Activation of initial wound healing responses in rat corneas in organ culture by mesodermal growth factor. Virginia L. Weimar.

In organ culture, mesodermal growth factor (MGF) from mouse submaxillary glands stimulates fibroblast formation, mitosis, and migration of cells into the dead-cell zone adjacent to the wound edge of rat corneal buttons. A classic dose-response relationship between the width of the stromal fibroblast zone and the dose of MGF suggests that fibroblast zone width may be used as a measure for screening agents which influence the initial stages of corneal wound healing.

Normal corneal wound repair has been accepted as occurring in three stages: the lag, fibroblastic or cellular, and cicatricial phases.\(^1\) The lag phase was so named because it was considered, until recently, to be a time when the wounded cornea waited passively for the invasion of healing cells.\(^2\) Weimar has shown, however, that the lag phase is in fact a phase of explosive activity in which the events determining wound repair are initiated.\(^3\) These earliest stages of corneal wound healing have been reproduced in organ culture.\(^4\) This provides a model system in vitro in which those processes which initiate and regulate the initial stages of corneal wound repair may be studied in a controlled synthetic environment.

The initial stages of corneal wound healing include a gradual transition of quiescent fibrocytes adjacent to the wound edge to spindle-shaped fibroblasts within 24 hr of wounding. This transformation is characterized by cell hypertrophy, increased intensity of staining, and the development of numerous large nucleoli.\(^5\) These cellular changes have been quantified in cultured corneas by means of an image analysis computer\(^6\) and have served as guides in the isolation of two factors (F-2 and mesodermal growth factors (MGF)) with potent growth-stimulating activities for corneal stromal cells from rabbits.\(^7\) One of these factors, MGF, has recently been shown to accelerate corneal wound healing in vivo in the rabbit.\(^8\)

In this report we show that MGF is also a potent growth-stimulating agent for corneal stromal cells of another species, the rat. In addition, MGF also stimulated endothelial cell enlargement and mitosis.

The stromal responses could be rapidly quantified by measuring the width of the zone of activated fibroblasts in coronal sections. This method is discussed with regard to the assay of potential stimulatory agents for wound healing.

Materials and methods. Organ cultures were prepared from the corneas of Sprague-Dawley rats, about 200 gm in weight and of either sex. The animals were sacrificed by decapitation, and the eyes were enucleated into Ringer's solution. The cornea was dissected with a small rim of sclera retained for handling. The iris and lens were excised, and 3 mm corneal buttons were cut with a trephine. The buttons were soaked for 20 min in sterile organ culture medium prior to culturing.\(^8\)

The composition of the organ culture medium was as follows: Eagle's minimum essential medium (Earle's base) containing phenol red and supplemented with glutamine, 2 mM/L; Eagle's nonessential amino acids, each 0.1 mM/L; penicillin, 100 U/ml; streptomycin, 100 /&/mu;g/ml; fungizone, 0.25 /&/mu;g/ml; FeSO\(_4\)-7H\(_2\)O, 0.426 mg/mL; CuSO\(_4\)-5H\(_2\)O, 0.238 mg/L; ZnSO\(_4\)-7H\(_2\)O, 0.143 mg/L; MnCl\(_2\)-4H\(_2\)O, 0.053 mg/L; CoCl\(_2\)-6H\(_2\)O, 0.105 mg/L; and (NH\(_4\))\(_6\) Mo\(_7\)O\(_24\)-4H\(_2\)O, 0.114 mg/L. For culture, the buttons were transferred by loop to 2 ml of organ culture medium in Falcon cluster dishes. The buttons (3 buttons/well) were floated on the medium which contained 0 to 10 /&/mu;g/ml MGF added in a volume of 10 /&/mu;l of distilled water. All buttons were incubated for 48 hr at 39°C in a humidified air-CO\(_2\) incubator.

At the end of the culture period, the buttons were fixed in Carnoy's solution overnight, rehydrated by passage through successively lower concentrations of alcohols, water, and then frozen in liquid nitrogen where they may be stored indefinitely. Frozen buttons were serially sectioned tangentially to the surface of the cornea, starting from the anterior surface. Tangential sectioning permits the examination of cellular detail which is not visible in sagittal sections. The sections were stained simultaneously in Giemsa stain (to minimize slide-to-slide variations in stain intensity) and were mounted in Permount.

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Fig. 1. Coronal sections of wound edge responses of rat corneal buttons in organ culture. A, 0 μg/ml MGF. Darkly staining cells in left half of photograph are activated cells. Lightly staining cells in right half of photograph are normal cells. Edge of killed zone (area devoid of cells) is at extreme left of photograph. B, 2.5 μg/ml MGF. Darkly staining cells in left half of photograph are activated cells. Arrow indicates fibroblast which has invaded killed zone. Normal zone right half of photograph. C, 5 μg/ml MGF. Zone of cell activation (darkly staining cells) occupies center two thirds of photograph. Part of zone of cell death (devoid of cells) is seen at extreme left. Arrow indicates dividing cell. D, 10.0 μg/ml MGF. Zone of cell activation occupies entire photograph. Fibroblasts are invading zone of cell death at extreme left. (Giemsa stain; ×330.)

The peripheral region of each button, which was injured by the process of preparing the corneal buttons, was examined for signs of cellular activation and death. This area contains three characteristic zones of cellular activity: at the wound edge, a zone of cell death indicated by a total absence of cells; next to the zone of cell death, a zone of cell activation indicated by cell hypertrophy, increased intensity of staining, and the occurrence of spindle-shaped fibroblasts; and centrally, a zone characterized by smaller, faintly stained fibrocytes similar to those observed in the uninjured tissue. Measurements of zone width for the regions of cell death and cell activation were collected for three sections per cornea. In order to standardize the depth for the measurements, the first serial section was located to contain a portion of the endothelium and the anterior chamber. This and the preceding two sections were used for measurements of zone widths. An area including the wound edge on each of the three sections was photographed at a magnification of 50× to produce a 35 mm color transparency. These were projected onto a screen, and zone widths were measured with a ruler. The slides were coded so that the observer did not know which treatment was being quantified. The true widths of the zones were calculated with factors derived from photographs of a stage micrometer scale. Six corneas were examined for each of four treatment groups which received 0, 2.5, 5, or 10 μg of MGF per milliliter of culture medium.

Counts of stromal mitotic figures were made at 125× for each of the three sections used for measurement of zone widths. These are expressed as mean mitotic figures per section.

MGF was prepared in this laboratory as described elsewhere. The lyophilized salt-free prep-
Fibroblast response and mitotic figures in rat corneas for different doses of MGF following 48 hr of culture. A, Fibroblast (●—●) and dead zone (x—x) widths. The means ± S.E.M. is plotted in all cases. B, Average count of mitotic figures per tissue section in the three sections employed in assessing zone widths for each dose of MGF.

The preparation was dissolved in water twice distilled in Pyrexware. The stock solution (~0.5 mg/ml MGF) was maintained at 0 to 4° C and was sterilized by filtration through a 0.45 μm Millipore filter. The preparation was used within 1 hr.

Results. The cellular changes at the wound edge in a cultured cornea without added growth factor are shown in Fig. 1, A. A zone of activated cells was seen as the group of deeply staining fibroblasts and hypertrophied cells on the left one third of the photomicrograph. Slightly activated and normal cells, staining very lightly, were seen on the right two thirds of the photomicrograph. An edge of the zone of cell death adjacent to the cut edge of the button was seen at the extreme left. The cellular responses to doses of 2.5, 5, and 10 μg/ml MGF are shown in Fig. 1 (B, C, and D). With 2.5 μg/ml MGF, the zone of darkly staining fibroblasts had increased and occupied more than one half of the photomicrograph (left side). Part of the zone of cell death is seen at the extreme left. With 5 μg/ml MGF, the zone of deeply staining fibroblasts and hypertrophied cells occupied all but the extreme right edge of the photograph. At this dose, significant fibroblast migration into the zone of cell death had occurred at 48 hr. With 10 μg/ml MGF, the zone of deeply staining fibroblasts and hypertrophied cells occupied the entire photomicrograph, and fibroblasts had almost completely repopulated the zone of cell death.

The widths of the zones of cell death and cell transformation were measured for all corneas and plotted against the dose of growth factor (Fig. 2, A). The width of the cell death zone decreased sharply at 5 μg/ml MGF as fibroblasts invaded this zone (Fig. 2, A). A standard growth curve was obtained for the width of the cell transformation zone (Fig. 2, A). The curve could be transformed to a linear dose-response curve by plotting the logarithm of the dose vs. the logarithm of the width of the zone of cellular response.

Stromal cell division, as indicated by counts of mitotic figures, was stimulated at all concentrations of MGF tested (Fig. 2, B). The dose-response curve closely paralleled that for fibroblast zone width.

Discussion. We have previously shown that the initial stages of corneal wound healing in vivo can be reproduced in vitro in organ cultures of rabbit corneas. The morphological effects of MGF have been evaluated in vitro on rabbit corneal buttons in organ culture and in vivo in corneal wounds of rabbits. In vitro after 48 hr, MGF stimulated the formation of fibroblasts, resulting in many more fibroblasts than in control corneal buttons. This morphological evidence in vitro of the acceleration by MGF of these initial putative processes in wound healing suggested that MGF might accelerate wound healing in vivo. When tested in vivo in injection wounds and nonperforating knife wounds in rabbits, MGF stimulated fibroblast formation and mitosis and population of the wounds by fibroblasts and accelerated repair of the tissue matrix.

In this report, we have shown that MGF has the...
same effects on rat corneas in organ culture as were observed for rabbit corneas. In addition, during this study which focused primarily on the effects of MGF on stromal morphology, we noted stimulation of the endothelial cell layer. The stimulation of both stromal and endothelial cells in rat corneas by MGF may reflect their common mesothelial origin. However, the spectrum of cell types which are directly stimulated by MGF remains to be established.

The screening and testing of agents such as MGF for their effects on wound healing is ordinarily difficult due to the lack of practical assay methods. Although tissue culture affords one means of assessing growth stimulating or inhibiting effects of test agents, the method is time-consuming, and the relevance of the data can often be questioned. Prolonged culture of cell lines leads to dedifferentiation and the loss of normal morphology and tissue interrelationships. In contrast, the cells in organ culture retain their normal morphology and tissue relationships for several days. Consequently, it has been our experience that more reliable indications of the influence of an agent in vivo, such as for MGF on corneal wounds of the rabbit, can be obtained from organ culture.

Previously, we have quantified cell responses in organ culture by a computerized image analysis method. This technique, although providing a detailed view of morphological changes in test corneal buttons, is extremely time-consuming, and the equipment is generally unavailable. The observations presented here indicate that the width of the fibroblast zone may be used as a simple test parameter for screening of agents which can influence the initial stages of corneal wound healing. This parameter reflects the extent of fibrocyte transformation and cell migration, both of which appear to control subsequent wound healing. The method requires only a source of corneal tissue, an incubator, and equipment used for routine histological procedures.

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REFERENCES


Four subjects who had never worn contact lenses underwent measurement of their corneal thickness by each of three measurement methods: Haag-Streit pachometer with/and without Mishima-Hedby attachment and the Syber specular microscope. Data were collected on three