Beta radiation inhibition of corneal healing.  
I. Tensile strength and ultrastructure change

Dennis R. Morrison, Atsushi Kanai, and Antonio R. Gasset

Radiation-induced changes in the corneal repair process have been correlated with a marked reduction in the tensile strength of the wound. These changes are characterized by: a prominent deficiency in the synthetic capability of the fibroblastic cells involved in the wound, abnormal ultrastructural detail of both these fibroblastic cells, and the epithelial cells that recover the incision; a complete lack of collagen synthesis in the stroma of the wound; and a marked absence of endothelial cells attempting to recover the incision and seal the wound posteriorly. Electron microscopic studies show that the pronounced ultrastructural changes in the corneal repair phenomenon are evident at radiation doses of less than half that required to produce any discernible damage or alteration in the intact cornea. The lack of tensile strength of the wound, the associated paucity of cells involved in the wound, and the abnormal ultrastructure of these cells was equally apparent in corneal wounds that had received the radiation either three months prior to surgery or immediately after wounding.

Key words: beta radiation, corneal healing, tensile strength

The repair of corneal wounds has been studied from a variety of approaches as summarized by Maumanee.1 Investigations have been grouped according to superficial versus penetrating-type wounds, and discussions have been subdivided as to repair processes involving lesions of the epithelium, stroma, and endothelium. Morphologic transformations that have been described at the light microscope level2,3 are only a part of the extensive ultrastructural changes that occur in normal corneal healing, as described by Jakus4 and LaTessa and Ross.8

Some basic understanding of the various processes that are involved in the corneal repair phenomenon have made possible studies of the effects of different procedures and agents on corneal healing. These studies have shown that the optimum increase in tensile strength of the wound is dependent on the presence of corneal epithelium6 and eyelid closure6,7 and that...
drugs such as corticosteroids\textsuperscript{5, 9} and idoxuridine (IDU)\textsuperscript{10} affect the healing process. Studies on the effects of beta radiation on corneal healing have indicated a temporary inhibition of mitosis in the epithelium,\textsuperscript{11} a delay in fibroblastic activity,\textsuperscript{12} and inhibition of DNA synthesis in three- and four-day-old wounds.\textsuperscript{13}

Tensile strength measurements have been used as a simple tool to test the effect of many agents on the corneal repair processes. To date this system has not been used to evaluate the effect of beta radiation on corneal healing. It is the purpose of this investigation to describe the effects of beta radiation on the tensile strength of the corneal wound and the ultrastructural changes associated with the radiation-induced inhibition of normal corneal healing, when the radiation is administered either months prior to or immediately after penetrating injury.

Methods

Experimental animals. Albino rabbits weighing between three and four kilograms were used throughout the experiments.

Operative procedure. Preoperatively, four per cent atropine was used to dilate the pupils. Intramuscular injections of pentobarbital sodium was used for anesthesia. With standard lid retractors for exposure, an 8 mm. nonpenetrating incision was made through the center of the cornea. Two 7-0 silk sutures were placed about 5 mm. apart and looped out of the incision. The section was then completed with a cataract knife. The wound was immediately closed with the two peripheral sutures.

Irradiation procedure. In one group the beta radiation was administered immediately after closure of the wound. The other group received the radiation treatment three months prior to surgery.

The lids were held open with standard retractors for exposure, an 8 mm. nonpenetrating incision was made through the center of the cornea. Two 7-0 silk sutures were placed about 5 mm. apart and looped out of the incision. The section was then completed with a cataract knife. The wound was immediately closed with the two peripheral sutures.

Postoperative care. Topical administration of four per cent atropine and Neosporin ophthalmic ointment was maintained on a daily basis throughout the first three days following surgery. Thereafter, Neosporin was administered topically every day for the next four days. Cases of infection or extensive anterior synechiae were excluded from the study.

Measurement of tensile strength. Using the method of Gasset and Dohlman,\textsuperscript{6} 5 mm. wide corneal strips were cut from each cornea, perpendicular to the wound, with a parallel razor blade knife. The strip of cornea was carefully fastened in special clamps and sutures were removed and placed on the tensiometer. When the increasing weight was sufficient to break the wound, the device automatically stopped the addition of the weight and the entire load is weighed.

Electron microscopy. Biopsy specimens were immediately fixed in cold two per cent osmium tetroxide with phosphate buffer for a period of two hours. Dehydration through a series of ethyl alcohol treatments was followed by embedding in Epon.\textsuperscript{14}

Thick sections were stained with toluidine blue and studied with the light microscope for electron microscopy orientation. The embedded tissue was cut with a Porter-Blum microtome and stained with uranyl acetate-lead citrate. Specific collagen staining was carried out with the use of phosphotungstic acid (PTA).\textsuperscript{14}

Electron microphotographs were taken with a Hitachi 11-C microscope.

Results

Tensile strength of corneal wounds. The tensile strengths of irradiated and non-irradiated corneal wounds at three weeks after penetrating surgery are compared in Tables I and II. The mean and standard deviation of the control eyes were within the ranges previously described by Gasset and Dohlman.\textsuperscript{6}

Paired statistics\textsuperscript{14} showed a significant difference between the tensile strengths of the control wounds and the contralateral wounds irradiated at the time of surgery with 99 per cent confidence ($p = 0.01$). Correspondingly, with 93 per cent confidence, there was a significant difference between the tensile strengths of the control wounds and the irradiated wounds in the group that was treated three months prior to penetrating surgery ($p = 0.02$).
Table I. Tensile strength of corneal wounds irradiated at surgery

<table>
<thead>
<tr>
<th>No. of rabbits</th>
<th>Control mean, nonirradiated (Gm./5 mm.)</th>
<th>Standard deviation</th>
<th>Irradiated mean (Gm./5 mm.)</th>
<th>Standard deviation</th>
<th>Paired statistics*</th>
<th>t0.010</th>
<th>t0.010</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>307</td>
<td>126</td>
<td>103</td>
<td>34</td>
<td>3.35, 4.23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Animals killed 20 days postoperatively.
* t Values calculated at 99 per cent confidence.

Table II. Tensile strength of corneal wounds in eyes irradiated 3 months prior to surgery

<table>
<thead>
<tr>
<th>No. of rabbits</th>
<th>Control mean (non-irradiated) (Gm./5 mm.)</th>
<th>Standard deviation</th>
<th>Irradiated mean (Gm./5 mm.)</th>
<th>Standard deviation</th>
<th>Paired statistics*</th>
<th>t0.010</th>
<th>t0.010</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>260</td>
<td>58</td>
<td>60</td>
<td>25</td>
<td>2.82, 2.87</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Animals killed at 20 days postoperatively.
* t Values calculated at 98 per cent confidence.

It should be noted that several wounds from both the group irradiated at surgery and the group irradiated prior to surgery had no measurable tensile strength after three weeks of healing time. These wounds pulled apart with just the weight of the ten-gram clamps used to hold the corneal strip in the tensiometer. None of the non-irradiated control wounds were so obviously fragile after three weeks' healing.

**Histology.** Three weeks after penetrating surgery the irradiated corneas were strikingly different from the nonirradiated controls. The epithelial cells that had overgrown the incision were enlarged and abnormal in gross morphology, even when compared to more peripheral epithelium in the same irradiated eye. Invariably some polymorphonuclear leukocytes were present in the anterior stroma, aligned along the posterior basal epithelial layer. The epithelium adjacent to and covering the incision was only three or four cells thick (Figs. 1A and 1B), whereas in the control eyes the epithelium was eight to ten cell layers deep over the incision.

Notable in all the irradiated wounds was the complete absence of endothelial cells covering the posterior of the wound and the lack of stromal tissue regeneration. Formation of the anterior chamber was achieved only through closure of the incision by the fibrin clot remaining in the wound.

Although the over-all corneal thickness of the control eyes approximated that of a normal cornea, a consistent observation in the irradiated wounds was a marked thinning of the stroma at the wound site. The irradiated wounds were only about one-third the thickness of the control wounds.

**Ultrastructure.**

**Epithelium.** The control corneal epithelium three weeks after surgery was characterized by some interdigitation, enlargement of intercellular spaces, a layering of some eight to ten cell layers deep, some hemidesmosomes, and portions of basement membrane where the epithelium attaches to the stroma. The basal cell cytoplasm was essentially normal; however, clumps of ribosomal granules were scattered throughout, and tonofilaments were dispersed at random throughout the cytoplasm, with occasional bundling of tonofilaments in some scattered areas.

In contrast the irradiated corneal epithelium, in addition to being abnormal in gross appearance, stained much lighter than normal epithelial cells, especially basal epithelial layers. There was a marked
Fig. 1A. Control cornea after three weeks of healing. Epithelium (Ep) has regenerated to full thickness and stromal regeneration (St) is apparent in anterior wound. Endothelium (Ed) has covered posterior wound. (Epon, toluidine blue. Original magnification ×200.)

Absence of any basement membrane along the stromal border, although many cytoplasmic processes extended into the stroma (Figs. 2A and 2B).

The epithelial cytoplasm contained very little endoplasmic reticulum, scattered ribosomal granules, and tonofilaments that were found only in bundles. Mitochondria and other cellular organelles were poorly developed and few in number. No hemidesmosomes connected the basal epithelial cells in the wound area. Basement membrane was found only in basal epithelial layers peripheral from the wound area.
Fig. 1B. Histologic section of wound in contralateral, irradiated, cornea three weeks after surgery (10,000 rads of beta radiation administered at the time of surgery). Note that epithelial cells (Ep) are enlarged and only a few layers thick, also the lack of stromal regeneration (St). The endothelium has not covered the wound posteriorly. AC = anterior chamber. (Epon, toluidine blue. Original magnification x200.)
Stroma. Proliferative collagen fiber formation was evident in the control stromal wound areas immediately adjacent to the epithelial layers after the three-week healing period. There was a large number of fibroblasts in the anterior stroma but very few polymorphonuclear leukocytes.

The fibroblastic cells observed in these investigations were grouped into two different classifications based on distinct morphologic differences. The first type, which we shall henceforth refer to simply as a typical fibroblast, was characterized by extensive, distended, endoplasmic reticulum, with extensive ribosomal granules lining the endoplasmic reticular membrane, large numerous mitochondria, well-developed Golgi complex, and increased flocculent filaments aggregated near the cell membrane (Fig. 3). The second cell type, which we shall refer to as a fibroblast-like cell, was characterized by poorly developed cellular organelles, a small amount of poorly developed endoplasmic reticulum, few ribosomal granules associated with the endoplasmic reticular membrane, few mitochondria, and yet an extensive appearance of intracellular flocculent filaments aggregated near the cell membrane (Fig. 4) quite similar to the intracellular filaments found in a mature fibroblast.

The fibroblasts found in the anterior stromal areas of the control wounds were elongated, flattened, and closely adjoining each other in an interlacing, almost interconnecting pattern. Collagen fibers filled the intercellular spaces (Fig. 5). Some fibroblast-like cells were noted in the anterior stromal area, but more were apparent in the deeper portions of the wound.

In the central and posterior stromal areas of the control (three weeks) wounds, collagen fibers were dispersed quite randomly in a fibrin matrix amid scattered fibroblasts and fibroblast-like cells. The collagen fibers were found to have a large

Fig. 2A. Electron photomicrograph of basal epithelial cells in normal cornea after three weeks of healing. Note polymorphonuclear cell (P) and difference between normal basement membrane (bm) and amorphous substance (am) adjacent to epithelial cell. (Original magnification x6,000.)
Fig. 2B. Electron photomicrograph of basal epithelial cells in irradiated corneal wound after three weeks of healing. Note absence of basement membrane, amorphous substance (am) in area where basement membrane should appear, paucity of mitochondria, and bundled tonofilaments (arrows). St = stromal area. (Original magnification x6,200.)

variation in the diameter of separate fibers. The collagen fibers in the posterior wound were consistently found to be aggregated near the fibroblasts and fibroblast-like cells rather than scattered randomly throughout the intercellular spaces.

Irradiated stromal wound areas after three weeks of healing were characterized by very few cells in the anterior stroma and no cells in posterior portion of the wound (Fig. 1). Almost all of the few cells that were found in the wound were of the fibroblast-like type cell and only an occasional mature fibroblast was found in the healing stroma. The fibroblast-like cells that were present were not elongated or flattened but were roughly globular in shape (Fig. 4) and quite different from the fibroblasts found in the control wounds. Collagen fibers were conspicuously absent from all areas of the wound. Only an occasional collagen fiber was found among the dense aggregates of fibrin that extended from the anterior chamber to the epithelium (Figs. 6 and 7). These fibrin filaments were often clumped into bundles and very closely entwined. This dense interweaving pattern became more compact as one scanned more posteriorly in the wound.

Endothelium. The control corneal endothelium had re-covered the wound posteriorly very early in the three-week healing period. The endothelium at three weeks was characterized by large intercellular spaces, which were rather straight passages between the cells rather than indiginated. Tight terminal bars appeared at the posterior surface of the endothelial layer. The endothelial cell cytoplasm possessed normal Golgi and typical number and sized...
Fig. 3. Electron photomicrograph of cells found in normal regenerating corneal stroma three weeks after surgical wounding. Mature fibroblasts (Fb) have normal complement of mitochondria, well-developed endoplasmic reticula, and Golgi complex. Note aggregation of epithelial cells (Ep) are enlarged and only a few layers thick, also the lack of stromal flocculent filaments (arrows) along cell membrane. Fibroblast-like cell (Fb-L) also had flocculent filaments (arrows) along cell membrane, however, this type cell had a poor complement of organelles, obvious lack of endoplasmic reticulum, and only a few poorly developed mitochondria. The appearance of flocculent filaments aggregated along the cell membrane was a characteristic of mature fibroblasts and fibroblast-like cells but was not found in normal stromal keratocytes. (Original magnification ×14,000.)
mitochondria, however, the cells were thickened in over-all diameter. There was an increased amount of endoplasmic reticulum lined with ribosomes and notably distended, consistent with some synthetic function. The cell membrane of the endothelial cells was undulated posteriorly along the anterior chamber. Although Descemet’s membrane had not been re-formed, there was a dense amorphous substance that had been formed along the anterior endothelial cell borders, suggesting that synthesis of the membrane was perhaps in progress (Fig. 8).

The endothelium in the irradiated corneas had not re-covered the posterior wound at this time. The endothelium peripheral to the wound area appeared normal all the way out to the limbus.

Again the anterior chamber was apparently maintained only by the epithelium anteriorly and the fibrin clot which closed the wound posteriorly.

Discussion

Simple tensile strength measurements of healing corneal wounds have shown that doses of 10,000 rads of beta radiation cause a significant inhibition of normal repair. Paired sample statistics illustrate this effect significantly better than comparisons of mean tensile strengths and standard deviations. These radiation effects are equally demonstrable when the beta radiation is administered either prior to or immediately after penetrating surgery.

The lack of tensile strength appears to be a result of an inhibition of collagen syn-
thesis in the stromal areas and a failure of the endothelium to close the wound posteriorly.

Ultrastructural observations have shown that other more subtle changes in epithelial regrowth and fibroblastic cellular activities are part of the over-all radiation modification of normal corneal repair. Poorly developed cellular organelles and a notable lack of hemidesmosomes and basement membrane were the most prominent changes observed in basal epithelial cells that re-cover the incision.

Fibroblasts and fibroblast-like cells were...
demonstrated in the normal healing stromas, however, fewer cells were observed in the irradiated corneal wounds. The large majority of these cells were of the fibroblast-like type of cell, while only an occasional mature fibroblast was apparent. The fibroblast-like cells lacked well-developed cellular organelles and distended, ribosomal-lined endoplasmic reticulum typical of synthesizing fibroblasts as described by Chapman. Even some of the few mature fibroblasts that were observed had endoplasmic reticulum that were not distended and enlarged as found in a

Fig. 6. Anterior stromal portion of irradiated corneal wound showing the continued presence of fibrin filaments and a few isolated collagen fibers (arrows). (Original magnification ×56,000.)
normal synthesizing fibroblast. In addition, the fibroblast-like cells were not flattened, elongated, or functionally arranged in the almost interconnecting fashion that was observed in the juxtaposition of the mature fibroblasts found in the healing control corneas. Whether these two basic types of fibroblastic cells are intermediate forms of developing fibroblasts, as described in corneal healing previously by Uehida and Matsumura 17 or whether they are functionally different fibroblasts as shown in tendon regeneration 18 is not well understood at this time.

The irradiation treatment apparently prohibited or interfered with the appearance of the mature, synthesizing fibroblast in the stromal areas of the wound. Collagen synthesis was also inhibited, either as a result of fibroblast inhibition, or concomitant with the lack of mature fibroblasts normally found in the healing cornea. The fibroblast-like cells that were found in the irradiated corneas were morphologically and probably functionally different from connective tissue cells capable of collagen synthesis. This observation is further supported by the total absence of normal collagen in the irradiated corneas even after three weeks of healing.

The importance of the varied and extensive ultrastructural changes induced by beta radiation in normal corneal healing could best be studied from a time sequence basis beginning with the early stages of tissue repair.

The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.
Fig. 8. Posterior portion of control corneal wound after three weeks of healing. High density amorphous substance (arrows) is observed along the perimeter of the active endothelial cells that have covered the wound posteriorly. (Original magnification x12,100.)

REFERENCES
15. Snedecor, C. W.: Statistical methods ap-
plied to experiments in agriculture and biology, ed. 5, Ames, Iowa, 1965, Iowa State University Press, p. 49.