Connective Tissue Remodeling in Corneal and Scleral Wounds

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The fluorescent dye dichlorotriazinyl aminofluorescein will bind to amino groups of proteins covalently under physiological conditions. It has been used to dye the connective tissue around an ulcer or non-penetrating, linear incision in the rabbit cornea and sclera, and the healing of the tissue has been examined up to 1 yr later. Sagittal sections were stained for light microscopy, and adjacent unstained sections were examined in the fluorescence microscope. The stained sections showed the reestablishment of the lamellar organization of the stromal collagen across the site of the incision; the fluorescence showed where the connective tissue that was present when the wound was made persisted, and thereby defined the limits of remodeling in the healing process. In Bowman’s layer and the adjacent stroma, there was often an abrupt transition from fluorescent to new, undyed connective tissue. Deeper in the scar, and particularly around the ulcer, dark streaks were observed between the fluorescent lamellae, showing apparently new (non-fluorescent) tissue interdigitating with the old. These observations are discussed in relation to the mechanism of healing and the residual mechanical weakness that persists across scars in the cornea and sclera. Invest Ophthalmol Vis Sci 27:1478-1484, 1986

Wounds such as incisions and ulcers in the cornea and sclera usually heal slowly. Although remodeling occurs in the scar, even changing transparency in the human cornea over years, the scar may never restore the original tensile strength to the tissue. A residual weakness in the incised cornea is a matter of special concern in view of the popularity of radial keratotomy, but any prospect of interventions to improve on normal healing is impeded by our lack of understanding of the factors determining tissue strength in the cornea and sclera. It seems probable that the ultimate tensile elements of most connective tissues in vertebrates are the collagen fibrils, but these occur embedded in a proteoglycan-rich matrix as a composite structure. We speculate that the determinants of the tensile strength of the cornea and sclera, and dense connective tissue in general, are the length and organization of the polymerized collagen within the fibrils, the weave and length of the fibrils, and the covalent and non-covalent interactions in the fibrils and between the fibrils and the matrix. The deficits that determine the weakness of a scar could be assessed more objectively if it were possible to analyze the structural features that distinguish a healed scar from normal tissue. We describe here a technique for labeling with a covalently bound fluorescent dye the components of normal sclera and corneal tissue before or at the time of wounding. This procedure clearly defines the limits of the region in which remodeling occurs, and will permit the identification and examination of the transitional region between the scar and the original tissue. We report observations on healing after a series of linear incisions were made part way through the corneas and across the limbus of rabbits. In addition, we have examined the healing of ulcers in the corneas of rabbits. We also discuss the advantages of this dyeing technique over radioisotope labeling, the limitations of the procedure, and some possible applications. We have briefly reported on these studies earlier.

Materials and Methods

Nine white New Zealand rabbits were used in these studies. They were anesthetized with Nembutal (Abbott Laboratories, Chicago, IL) and the local anesthetic proparacaine hydrochloride was applied to the eye. Incisions were made with a diamond knife, either radially or parallel to the limbus. In each eye of two rabbits, two incisions were made, one on each side of the cornea. To minimize the risk of perforating the cornea, the incisions were limited in depth. On sectioning, most incisions turned out to be about one-third the thickness of the stroma. In one animal, the cornea was perforated in one position; the wound rapidly was plugged with fibrin and healed normally. In most experiments, the
wound was immediately dyed by instilling a few drops of a 0.5 percent solution of dichlorotriazinyl amino-fluorescein (DTAF) (Molecular Probes, Inc., Junction City, OR) dissolved in 0.2 M sodium bicarbonate into the incision as it was held open by the knife. After 30 sec, the excess dye was washed out of the wound with buffered saline, the eye was swabbed, one drop of atropine was instilled, and an antibiotic ointment was applied. In three rabbits, instead of applying the dye to the wound, the cornea was dyed before the incision was made. Because the dye cannot penetrate the epithelium, this procedure required the scraping of the epithelium from part of the corneal surface; the scraped, dyed cornea was allowed to re-epithelialize and 2 days later an incision was made.

To produce a corneal ulcer, one cornea of one animal was scraped and dyed and 2 days later 0.5 μg of Worthington (Freehold, NJ) bacterial collagenase in 1 μl of phosphate-buffered saline was injected through a No. 32 hypodermic needle into the corneal stroma. The same quantity of Worthington trypsin was injected into the dyed cornea of a second rabbit. Ulcers developed within 2 days, and were treated with daily topical antibiotics and steroids.

In one animal, three incisions along three sides of a square were made in the cornea, a lamellar flap was lifted, and the superior and inferior surfaces were dyed respectively with DTAF and tetramethylrhodamine isothiocyanate. The latter dye was applied in the same way as DTAF. The flap was sutured back in place with 10-0 nylon and the wound was allowed to heal.

The animals were killed with an overdose of Nembutal between 1 and 12 months following wounding, and the eyes were fixed by application of 1% glutaraldehyde in 0.1 M phosphate, pH 7.2, to the epithelial surface, followed by an injection of the same fixative into the anterior chamber via the limbus. The corneas were then dissected out and immersed in the same fixative for 1 hr at room temperature. They were washed in phosphate-buffered saline and then dehydrated through graded alcohol washes before embedding in JB-4 (Polysciences, Inc., Warrington, PA) or Histacryl (LKB Instruments, Inc., Hickersville, NY) resins.

The blocks of embedded tissue were cut with glass knives on an adapted Minot microtome, and 2- or 4-micrometer sections were examined in Olympus (Lake Success, NY) and Zeiss (Carl Zeiss, Inc., Thornwood, NY) fluorescence microscopes with epi-illumination. Similar sections were stained with methylene blue-basic fuschin. Photographs were taken on Kodak (Rochester, NY) Ektachrome 400 or Tri-X film with fluorescent or transmitted light from the same microscopes.

Experiments with animals were done in conformance with the ARVO Resolution on the Use of Animals in Research.

Results

Preliminary experiments established the most favorable conditions and suitable dyes for dyeing dense connective tissues, such as cornea and tendon, to a reasonable depth. Studies over several months showed no evidence of immune reaction against the dyed tissues (Davison, unpublished). Unbound dye in the cornea was rapidly eliminated, presumably in the tears, but the covalently bound dye is stable for at least a year except for slow bleaching in bright light.

The first experimental study on a corneal wound was made on a 2-yr-old rabbit. The cornea was scraped and dyed with DTAF for 30 sec, washed, and allowed to heal. Two days later a radial incision was made. After 3 months, the rabbit was killed with an overdose of Nembutal, the cornea was fixed, dehydrated, embedded in plastic, and sectioned transverse to the incision. Stained sections of the scar showed no apparent discontinuity in the lamellae that bridged the gap where the incision had been made, but the fluorescence microscope showed a dark, undyed wedge where new tissue had replaced the old around the incision. This wedge was narrower under the epithelium, and the boundaries between fluorescent and non-fluorescent tissue were sharper there than deeper in the stroma (Fig. 1). The changes at the greatest depth in the stroma could not be resolved because little of the dye that had been applied to the surface had penetrated that far.

To define better the limits of remodeling within corneal incisions, subsequent studies on other animals were made by applying the dye solution into the open wound instead of wounding the previously dyed cornea. These wounds were examined 1, 3, or 12 months following wounding. Figure 2 shows such a dyed, fixed eye dissected from a rabbit. The scars are brilliantly fluorescent. Micrographs of sections across the wound cut 12 months after dyeing are shown in Figure 3. There was little difference discernable between 3 and 12
The healing of the incised sclera was examined in a 1-yr-old wound that had been extended through the limbus. Here, the appearance of the scar was different, with an apparent interdigitating of the dyed and undyed tissue close to the surface as well as deeper in the scar (Fig. 5).

To determine whether these incised wounds healed in a different manner from those resulting from an ulcer, collagenase or trypsin was injected into the superficial layers of two rabbit corneas which had been previously dyed with DTAF. Despite steroid treatment, the trypsin ulcer progressed to perforate the cornea and a retrocorneal membrane formed before the ulcer healed. After 6 months, the tissue was sectioned and examined. Unfortunately, the DTAF did not penetrate far enough into the stroma to define adequately the

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remodeling around the ulcer. From the collagenase injection, an ulcer formed and healed. Sections of the cornea were examined 1 yr later, when inspection showed a undyed patch in the middle of a dyed area of cornea. The results are shown in Figure 6. Under the epithelium and peripheral to the ulcer, a new layer of tissue had been laid down over stroma that was brightly fluorescent and showing no evidence of erosion. Towards the center of the healed ulcer, there were clear streaks of dyed lamellae projecting between undyed lamellae. At the center of the ulcer, all of the dyed tissue had been replaced. Over this area, the stroma appeared about 10% thinner than the unaffected cornea (not shown), despite the added lamellae at the surface.

Finally, to assess remodeling between the lamellae, we dyed the inner surfaces of a lamellar flap with DTAF and rhodamine isothiocyanate, and after 4 months, when the resutured flap had healed, the sectioned tissue was examined in the fluorescence microscope. There appeared to be no undyed tissue visible between the tetrarhodamine isothiocyanate- and the DTAF-treated stromal layers that were clearly preserved (Fig. 7), so the healing involved no detectable tissue synthesis between the lamellae.

Discussion

The application of the dye DTAF either before wounding the cornea or into the corneal wound appeared to have no effect on the healing process, because histological sections from undyed, wounded tissues obtained from previous studies could not be differentiated from those sections that were treated with the dye. Healing was not delayed, and eye function appeared normal, although the scars were brilliantly fluorescent for a year or more after dyeing. For the pur-
poses of labeling tissue, the dye has several advantages over biosynthetic labeling with radioisotopes. The dye can be applied to selected regions; it attaches to all accessible amino groups in the area to which it can diffuse, and it is not restricted to those macromolecules that are synthesized after the label is administered. Furthermore, it would seem practical to employ different dyes successively to mark changes occurring with time; and the covalent binding of the dye to any amino-acid would preclude the labeled amino-acid from being reutilized if the protein is catabolized. In contrast, a radiolabeled amino-acid may be reutilized, making analysis and interpretation of the distribution of radioactivity more difficult. We have used DTAF and tetrarhodamine isothiocyanate in our investigations because they react under only slightly alkaline conditions, hydrolyze slowly to allow extensive diffusion into tissue, and the bound dyes bleach only slowly. Undoubtedly, other dyes can be used for covalent labeling. With dyes with appropriate properties, it might be feasible to examine the remodeling that occurs in ectasias, such as keratoconus. Unfortunately, the local high concentrations of dyes cannot be measured quantitatively in situ by fluorescence, so, unless they are also radiolabeled, turnover cannot be quantitatively assessed.

It is known that the scar tissue that is synthesized in wounds is progressively remodeled, yet the lamellar organization in the scar is persistently different from the unwounded cornea. It has also been found that all of the donor collagen in a corneal transplant is not replaced by the host after healing. Our observations are consistent with these reports, but in addition we have now demonstrated the domains in which connective tissue is replaced around the healed wound as a result of the remodeling process. Our experiments on the cornea show: 1) that in the superficial stromal layers there is an abrupt transition from the dyed, original tissue to the non-fluorescent wedge of tissue that, along with a thickened epithelial layer, fills the gap that opened at the surface of the incised wounds; and 2) that deeper in the wound the transition is less abrupt and the fluorescent and non-fluorescent tissues appear to interleave. The inter-penetrating dyed and undyed layers were more obvious in the depths of the healed corneal ulcer than in the scars from the incised wounds.
The histological slides showed that, in the deeper regions of the scar in the cornea, the healing mechanism restored the parallel layers across the region where the incision had interrupted them. In contrast, in the superficial regions of the scar, poorly organized lamellae were seen. These observations do not prove the point, but are compatible with the possibility that some of the connective tissue that was synthesized early in the healing process was laid down with little relationship to, or connectivity with, adjacent lamellae, and this scar tissue persists around the region where Bowman's membrane originally was present.

In the sclera, the lamellar organization is far less orderly than in the cornea. In the scleral scar, the limits of remodeling were clearly defined by the persistent, fluorescent tissue; in contrast to the cornea, interleaving lamellae could be recognized at the surface as well as in the depths of the scar.

In previous studies, we showed that the major component of the cornea that is dyed by DTAF or tetramethyl rhodamine isothiocyanate is type I collagen (type V collagen is also dyed, but it is a minor component). We conclude that the reestablishment of the lamellar organization of the corneal stroma across the region of the incision results from the formation of new layers of collagen fibrils, which apparently weave between, or come into continuity with, fibrils that existed in the cornea before the wound was made. These same conclusions also apply to wounds in the sclera.

We could discern little difference between sections taken 3 months and a year after wounding.

Despite remodeling that can proceed for years and the fact that the tensile strength across the scar increases progressively, a strip of tissue from a cadaver eye under tension was reported always to break at the scar from an incision. What is the reason for the persistent weakness? From our observations, several explanations may be proposed. In the case of our rabbits younger than 1 yr, there was always a thickening of the epithelium and a thinning of the stroma at the region of the incision. The stroma also appeared thinner under the healed ulcer. However, there was less thinning of the stroma beneath the scars in older rabbits' corneas, and we are not aware that human corneas show such changes; thus, it is unlikely that a thinner stroma explains all the weakness of the corneal scar. The more probable cause is either that the fibrils across the scar are less well organized for effective stress bearing, or that the covalent continuity of the fibrils across the scar is not consistently reestablished in the healing process. The non-planar arrangement of the lamellae in the superficial layers of the scar is compatible with the first proposal; the abrupt scar-cornea junction in the same region is compatible with the second. Deeper in the scar, where the fluorescent lamellae taper off into the undyed scar tissue, the appearance of the section resembles the progressive dilution of the fluorescent fibrils that we observed in the dyed, growing rabbit cornea, so perhaps the remodeling process resembles the growth process in the depths of the incision and in the healed ulcer. To investigate the healing process further and to examine more closely the abrupt transitions between dyed and undyed lamellae, we will extend these studies with covalent labels that can be detected in the electron microscope.

Results of studies performed with this dyeing technique may also provide important contributions to the understanding of the refractive changes and healing of corneas of radial keratotomy patients. In those patients,
the incisions extend through 90 percent of the corneal stroma, and the healing in such circumstances may differ from that which we have described in our studies here. Such investigations should use animals with corneas that more closely resemble human corneas in their physical properties.

Key words: corneal wounds, scleral wounds, collagen, fluorescent dyes, remodeling

Acknowledgment

References