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Corneal Endothelial Changes Following Minor Trauma

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The healing processes that occur when corneal endothelial cells are subjected to only mild trauma are not known. To study these processes we have developed a system that enables only a few endothelial cells to be traumatized or destroyed under continuous specular microscopic observation in vitro. Experiments in which a small group of cells were traumatized by gentle wounding with a microglass tip produced an immediate and distinct dark area having the same size as the tip. Using this method we have produced and observed two types of wound by controlling the force of wounding. The first type of wound, produced by a gentle touch, recovered within 1 hr. The second type of wound, produced by moderate touch, took about 24 hr to recover completely from the trauma. In the second type of wound, we observed migration, elongation, coalescence and mitosis during the healing process. Histological examination revealed that in the first type of wound, the cells remained intact with no apparent damage seen by vital staining and light microscopy. For the second type of wound, the cells were completely missing although there was no apparent damage to Descemet's membrane. Invest Ophthalmol Vis Sci 29:1677–1682, 1988

The various wound healing processes of the rabbit corneal endothelium following mechanical,[1-4] chemical,[5,6] cryothermal[7,8] and ultrasound[9] injuries have been studied by light and scanning electron microscopy. More recently, specular microscopy, which allows sequential observations of the same cornea, has been used to study the dynamic processes that occur during endothelial healing in vivo.[1,10]

It is generally believed that a major wound in which a large number of cells is damaged is primarily repaired by a process of sloughing of the damaged cells followed by migration and possibly coalescence and proliferation of the neighbouring cells.[1]

If the wound is less severe and only a single cell is damaged, the healing process can result in a rosette formation,[9] presumably also due to cell death and sloughing. This article describes the nature of the wound healing process when a small number of cells are subjected to either mild trauma that does not kill the cells or to more severe trauma that kills the cells but does not damage the underlying Descemet's membrane.

Materials and Methods

Corneal Preparation

New Zealand albino rabbits weighting 2–3 kg were sacrificed with an overdose of intravenously administered sodium phenobarbital and the eyes were immediately enucleated and prepared using the method described by Neubauer et al, 1984.[11] After removal of the conjunctiva, a full-thickness circular incision was made in the sclera approximately 2 mm posterior to the limbus using a surgical blade and fine scissors. Great care was taken to avoid incision of the underlying ciliary body and to maintain a formed anterior chamber. The globe then was submerged in a container filled with the appropriate storage medium and further manipulations were performed on the submerged tissue with the aid of a dissecting microscope. The cornea and scleral ring were grasped at the wound margin with forceps and removed by slowly and carefully tearing the tissue from the underlying attachments. The isolated cornea with its scleral rim was submerged and gently agitated in a second and third container of storage medium to provide irrigation and then placed endothelial side up in a specular microscope examination chamber. During this manipulation the cornea retained its shape and, in most cases, folding of the cornea and contact of the endothelium with iris and/or lens was not noticed.

Wounding of the Endothelium

The examination chamber was placed under an eyebank specular microscope (Bio-Optics LSM 2100C, Arlington, MA) that used either a W×10 water immersion or a ×13 noncontact objective lens and either a ×5.0 or a ×3.3 eyepiece for observation and photography. For the initial experiments, a 27 gauge stainless steel needle was bent, using pliers, so that the needle could be inserted at an appropriate angle along the side of objective lens into the eye

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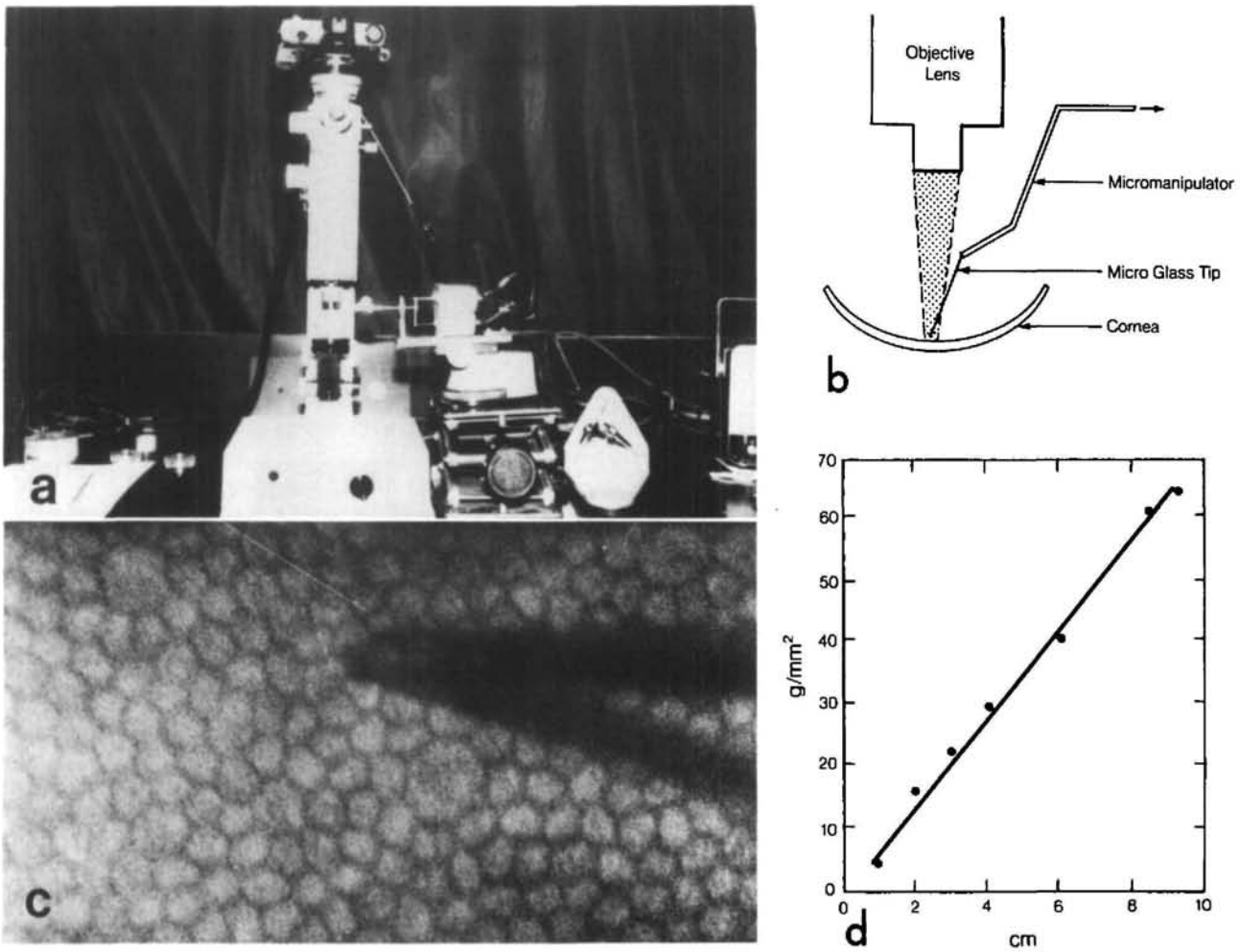

Fig. 1. (a) The experimental system showing the specular microscope and micromanipulator. The cornea with endothelium side up is set into the observing chamber and put under the objective lens. (b) The method used to produce trauma to the endothelium. (c) Specular photomicrograph showing the special microglass tip on the corneal endothelium (×285). (d) The relationship between the distance the handpiece of the micromanipulator was moved in cm and the force per unit area/pressure produced by the tip of microglass, measured using a Mettler balance.

holder without hitting the side of the objective lens. In the later experiments, a fine piece of microglass with a tip diameter of 50–100 μm was made by a specially designed micropipette puller and affixed onto the tip of the stainless steel needle. The glass tip was inserted from the side of the chamber into the eye cup and positioned under the objective lens. Using a micromanipulator, the needle tip was carefully guided while being observed under the specular microscope. An endothelial wound was made with the tip of the glass by a gentle and controlled touch to the endothelial surface. The force with which the tip wounded the endothelium was measured by using a Mettler balance. This system and the microglass tip are shown in Figure 1a–c.

Vital Staining and Histology

After the damage was produced, some corneas were stained with trypan blue and alizarin red and examined as flat sections under the biological microscope for endothelial changes. Other corneas were fixed with 10% formalin, dehydrated through ethanol, and embedded in glycomethacrylate. Three micron sections were stained with toluidine blue for light microscopic observation.

Morphometric Analysis

Computer-assisted analysis was done using a Computer Analysis System (Bio-Optics). Each corner of each of approximately 30 cells was digitized for each photograph. The cells digitized were those within the largest dark area (3600 μm^2) seen during the wound recovery process. The computer then calculated the mean cell area, hexagonality, mean perimeter, mean sidelength and histograms of each cornea.

These experiments followed the ARVO Resolution on the Use of Animals in Research.

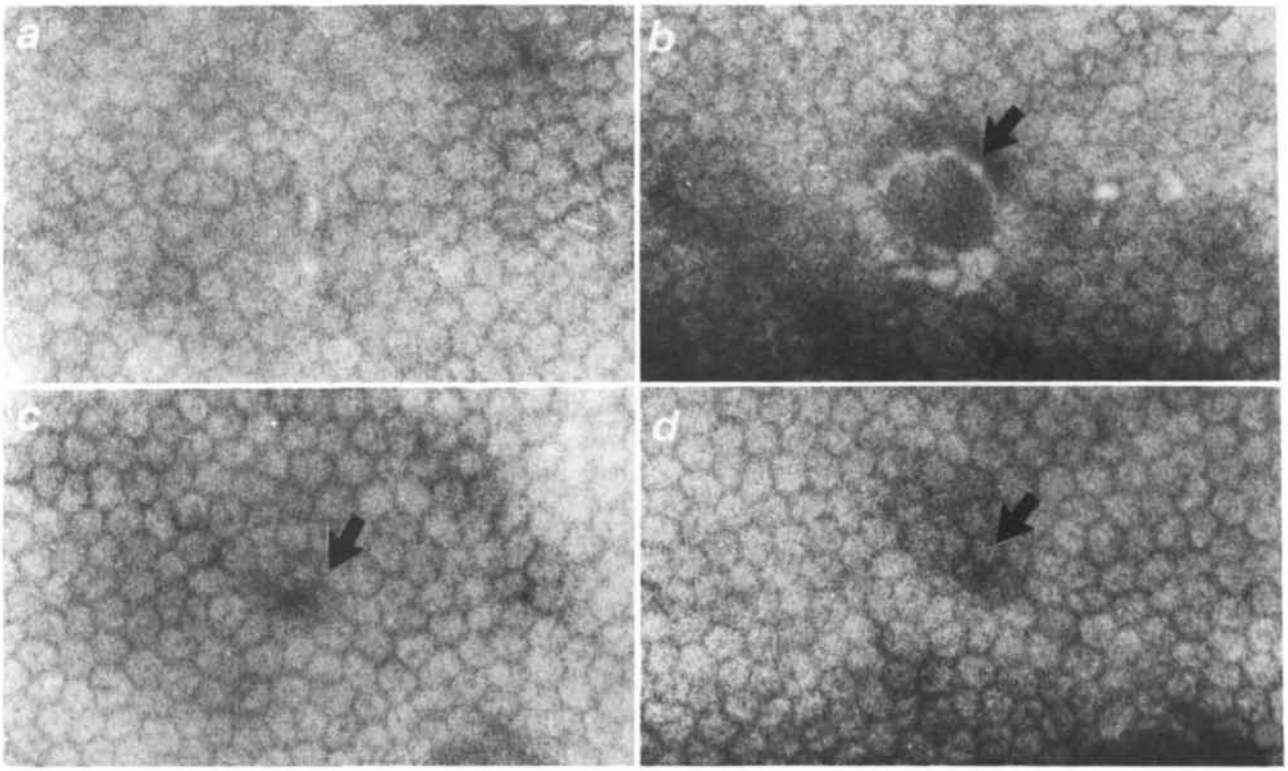

Fig. 2. Specular photomicrographs of type I wound (×335). The black arrow shows the wounded area. (a) Before wounding; (b) just after wounding; (c) 20 min after wounding; (d) 40 min after wounding.

Results

Direct continuous observation of the entire wound healing process with the specular microscope showed that the small group of endothelial cells traumatized by touching with the glass tip produced an immediate and distinct dark area. By varying the wounding force, two reproducible and distinct types of wounds could be produced whose difference depended on the time of recovery to a normal endothelial specular image. The first type of wound recovered within 60 min while the other took a little more than 24 hr to recover to a normal appearance. The wounding force was controlled by the handpiece displacement of the micromanipulator after the tip was seen to just contact the endothelial surface. The relationship between the distance the handpiece of the micromanipulator was moved and the force per unit area is shown in Figure 1d. Using this graph, the force per unit area to produce the first type of damage was around 5 g/mm^2 while that to produce the second type of damage was about 30 g/mm^2. The two types of wound could not be differentiated by the initial appearance of the dark area. However, each type of wound could be reproduced by controlling the force of the glass tip applied to the endothelium.

The first type of wound studied was produced by a very light touch (about 5 g/mm^2) of the microglass tip on the endothelium. Figure 2 shows the endothelial appearance at various times for the first type of wound. Before the endothelial touch (Fig. 2a), the endothelium had a normal appearance. As the glass tip wounded the endothelium, a dark area immediately appeared at the point of the tip (Fig. 2b). This dark area progressively recovered to a normal specular microscopic appearance within 40 min (Fig. 2c,d). During this recovery time period, we observed no change in the appearance of the cells surrounding the dark area. This was confirmed by computer analysis of the digitized cell pattern, which showed no statistically significant morphological changes of the cells neighboring the dark area during the recovery period. For this type of wound, no cells stained with trypan blue, showing that all cells remained viable and no membrane damage was produced. This and the other histological findings showed no cell damage.

The second type of wound studied was produced by a stronger touch (about 30 g/mm^2) of the microglass tip. The time sequence showing recovery from this type of wound is shown in Figure 3. Before wounding (Fig. 3a), the specular microscopic appearance was normal with cells that were uniform in area

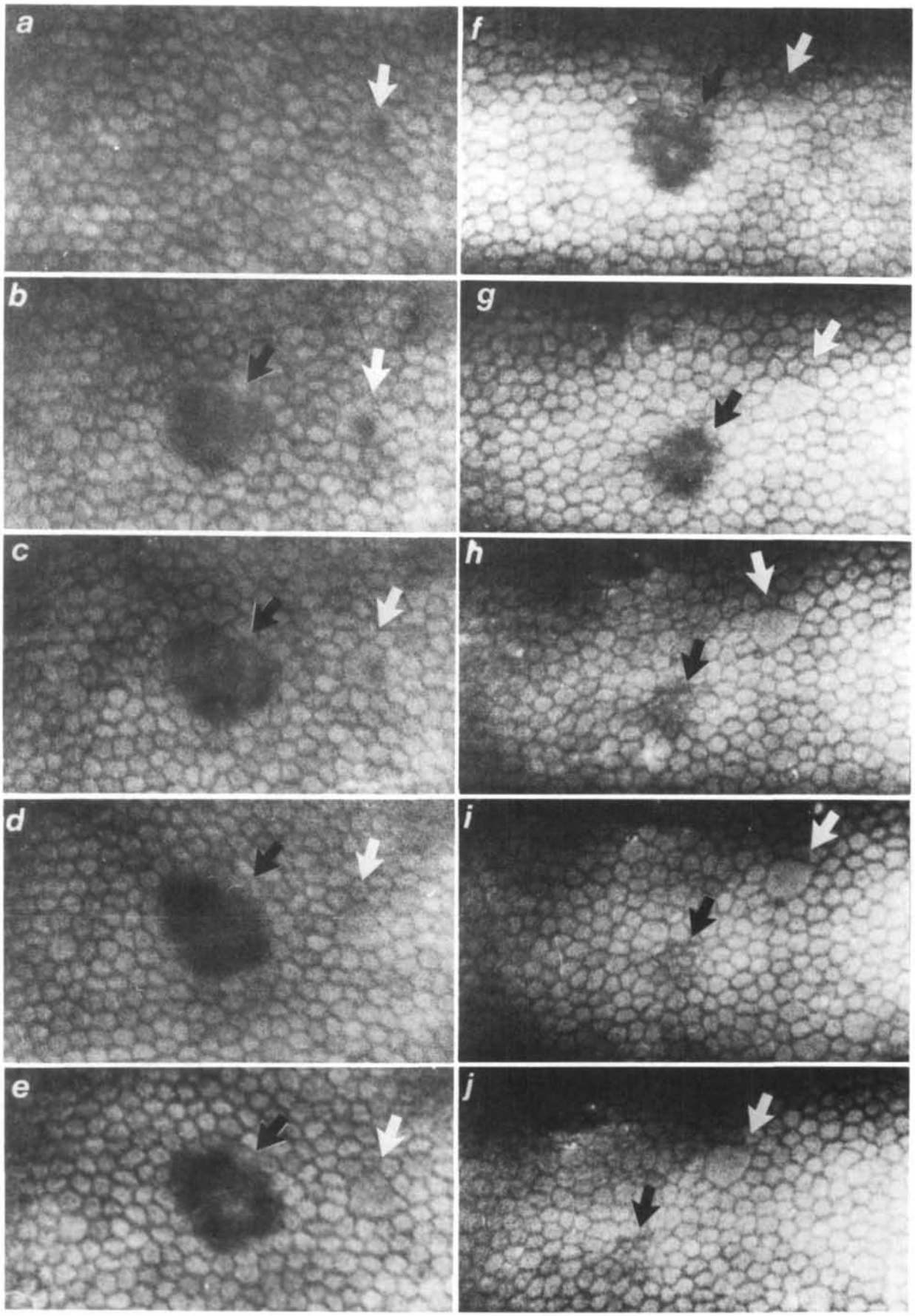

Fig. 3. Specular photomicrographs of type 2 wound (×267). The black arrow shows the wounded area. The white arrow denotes the unique cell used as a reference. **(a)** Before wounding; **(b)** just after wounding; **(c)** 30 min after wounding; **(d)** 1 hr after wounding; **(e)** 2 hr after wounding; **(f)** 4 hr after wounding; **(g)** 6 hr after wounding; **(h)** 12 hr after wounding; **(i)** 18 hr after wounding; **(j)** 24 hr after wounding.

Table 1. Typical changes around the damaged area of a single cornea

N = 1 (mean ± SD)	*Area (μm^2)*	*Perimeter (μm)*	*Sidelength (μm)*	*Hexagonality (%)*
Before wounding	180.8 ± 22.2	51.5 ± 3.9	8.5 ± 2.1	69.0
24 hr after	54.8 ± 26.6	33.6 ± 4.9	7.4 ± 2.3	37.5

and shape except for a few giant cells which were used as reference points. Immediately after the glass tip wounded the endothelium, a dark area appeared, as in the case with a light touch. The appearance of this dark area was essentially identical with that seen for the first type of wound produced (Fig. 3b); however, the healing process was considerably different and the recovery time was much longer. For this type of wound, the dark area began to expand gradually just after wounding and reached a maximum size in about 1 hr (Fig. 3c,d). The surrounding cells of the wound began to change their shape in 3 hr (Fig. 3e–g), protruding towards the center of the wound. Over the next several hours the damaged area became progressively smaller and less distinct until it was difficult to distinguish the previously damaged area from the surrounding cells. By 12 hr the dark area had essentially disappeared although a subtle darkness persisted for an additional 4 to 12 hr (Fig. 3h). At this time the recovered lesion was covered by cells whose size was approximately half that of the cells seen before the wound was produced. In addition, large nonhexagonal cells were occasionally seen (Fig. 3i,j).

Computer-assisted morphological analysis showed a large difference between the cells before and after the wounding. The mean area, hexagonality, mean sidelength and mean perimeter showed dramatic changes during the recovery period. The typical changes around the damaged area for a single cornea are shown in Table 1. Comparing of the mean cell area before damaging and after recovery, it appears that normal cells have been replaced by smaller cells.

The changes in size of the dark area are shown in Figure 4. The area expanded at the beginning and decreased gradually.

Figure 5a shows a typical histological section of the first type of wound. The endothelium was intact with no apparent changes from normal. We could not find any damaged endothelium after continuous cross-section. Figure 5b shows a similar histological section of the second type of wound. Several endothelial cells were absent without any damage to the surrounding endothelium, which showed normal thickness and a normal cell surface. Descemet's membrane was intact at the wound.

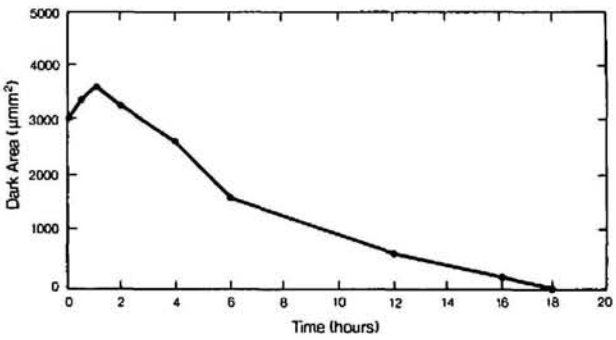

Fig. 4. Typical change in wound area with time for type 2 wound.

Discussion

This investigation is the first reported prospective study that permitted continuous observation showing the effects of minor direct mechanical trauma on the corneal endothelium. The method used enables continuous observation of the wound healing process following controlled mechanical trauma in vitro and enables minute and subtle changes that occur during the healing process to be documented. The advan-

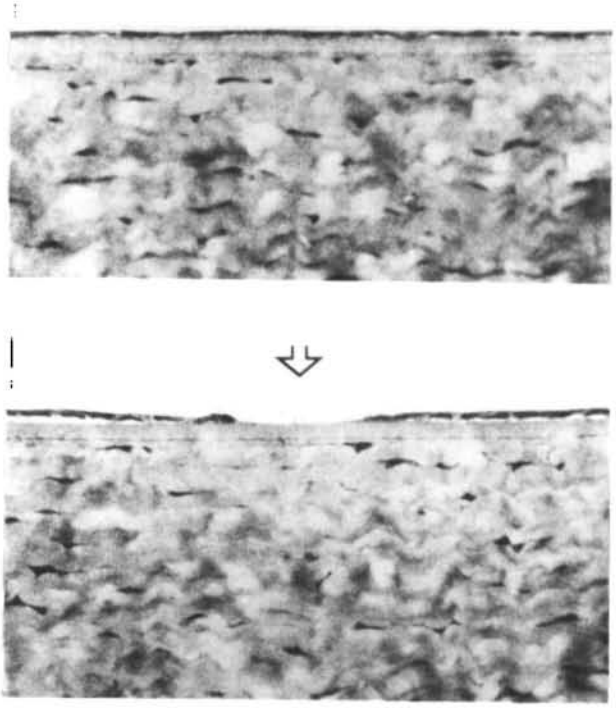

Fig. 5. Corneal sections (3 μm) stained with toluidin blue under light microscope 1 hr after wounding. (**a**) Type 1 wound. (**b**) Type 2 wound. The endothelium at the wound is missing although Descemet's membrane is intact in the type 2 wound (×250).

tages of the technique described are: (1) the entire sequence of the wound healing process can be monitored continuously by specular microscopic observation; (2) the degree of damage can be controlled by the force of touching; and (3) very minor damage to a controlled group of cells can be made without affecting either surrounding cells or Descemet's membrane.

Clinically, many dark areas seen in specular microscopy are thought to represent cells with damaged membranes which are either themselves damaged or dead cells. Our results show that there are at least two types of dark areas, one representing cells having reversible membrane changes that do not lead to cell death and that recover quickly, and another representing irreversible membrane changes that lead to cell death. Two types of dark areas, reversible and irreversible, have been previously reported following corneal storage[11,12] and following experimentally insulted rabbit corneal endothelium.[13]

Neubauer et al observed that transient black areas were also produced by cooling to 4°C. These areas were confined within the borders of an individual cell.[11] These dark areas might be the same as our first type of dark area.

Sherrard also reported specular microscopically dark endothelial cells or small groups of cells of in vitro corneas that represented reversible or irreversible changes.[13] Comparable examples of dark cells seen by scanning electron microscope (SEM) illustrate that those cells which are reversible are either rich in microvilli or the posterior contour of the cell is changed by roughening of and minor inclination of the posterior membranes; irreversibly changed cells are ruptured or fragmented. Both cases resulted in areas of darkness in the specular microscopical image of the endothelium. These findings agree well with our findings.

Olson et al reported that the endothelial repair process occured in two ways that are related to lesion size.[9] Small lesions healed by a process of rosette formation, while large lesions were repaired by a process of endothelial differentiation and migration.

It is likely that when the cell surface is touched it immediately alters its surface shape, which results in the dark appearance seen in specular microscopy. However, as long as the cell is healthy and not irreversibly damaged it will recover its shape or surface to a normal state in a short period of time, (ie, an hour or so).

For the case of the more severe type 2 wound, even though the size of the lesions produced was small, we observed both much smaller as well as larger cells appearing at the end of healing process (Fig. 3, Table 1), suggesting that perhaps both mitosis and coalescence occurred and might be healing mechanisms for small lesions. Whether or not the individual cells were stable or subsequently changed in area was not studied.

These findings suggest that when specular microscopy is used to evaluate the state of cornea, as in eyebank evaluation of the donor cornea, one should determine whether or not the dark areas seen following storage are transient, in which case the cornea is undamaged, or persistent, in which case the cornea is irreversibly damaged.

Key words: cornea, endothelium, wound healing, rabbit, specular microscope

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