Morphometric Analysis of the Corneal Endothelium

Specular Microscopy vs. Alizarin Red Staining

Dayle H. Geroski and Henry F. Edelhauser

Computer-assisted analysis of endothelial morphology provides useful indices of cell shape and size which appear to correlate to the monolayer's functional status. In this study morphometric data obtained by wide-field specular microscopy of in situ corneal endothelia are compared to data obtained by alizarin red S staining of excised corneas. Both human donor corneas and rabbit corneas were studied. The results of the study indicate that considerable (14%) cell shrinkage occurs in rabbit endothelia following staining. Associated with this cell shrinkage is a normalization of cell area which is manifest as a significant \( P < 0.001 \) decrease in the coefficient of variation of cell area. The percentage of hexagonal cells, however, remains unchanged. These changes were not mitigated by lowering the osmolality of the saline rinse following staining or by minimizing evaporation by placing a drop of silicone oil on the stained button. In human tissue no significant differences in cell area or cell shape were noted in comparing morphometric data obtained by staining to that obtained from specular microscopy. Useful morphometric data can be obtained by alizarin staining. This technique combined with cell morphometric analysis could provide valuable data in corneas whose lack of clarity limits or precludes specular microscopy. Caution, however, must be exercised in comparing morphometric data by this method to those obtained in situ for rabbit tissue.


Materials and Methods

Rabbit Studies

For the rabbit studies 2- to 3-kg New Zealand white rabbits of either sex were used. Immediately prior to specular microscopy the rabbits were sedated with an intramuscular injection of ketamine (30 mg/mg) and xylazine (6 mg/kg), and two drops of topical proparacaine were applied to each cornea. The central endothelium of each cornea was photographed with a Keeler-Konan wide-field specular microscope. The animals were overdosed with an intracardiac injection of sodium pentobarbital and their eyes were enucleated. The corneas were then carefully excised leaving a 2- to 3-mm ring of sclera.

To minimize endothelial wrinkling and thus optimize the field for light microscopy, a 7-mm diameter button was trephined from central cornea (endothelial side up). The endothelial surface of the button was then stained with the intercellular stain alizarin red S using the method described by Taylor and Hunt.4 Each day prior to its use the pH of the stain was adjusted to 4.2 with dilute (0.1%) ammonium hydroxide. The osmolality of the working stain solution was 309 mOsm/kg. A staining time of 3 min was found to provide optimal demarcation of intercellular borders. After this period of time the stain was rinsed from the endothelial surface using sterile isotonic saline. The endothelium was then promptly

From the Departments of Ophthalmology and Physiology, The Medical College of Wisconsin, Milwaukee, Wisconsin. Supported by EY-05609, National Eye Institute, Bethesda, Maryland. Submitted for publication: April 18, 1988; accepted September 13, 1988. Reprint requests: Dayle H. Geroski, PhD, Department of Physiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.
photographed using light microscopy. A total of 23 eyes were used in this series of experiments.

All animals used in these studies were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Human Studies

Human donor eyes judged to be unsuitable for use in keratoplasty were obtained from the Wisconsin Lions Eye Bank. For the eight eyes used in these studies mean (±SE) donor age was 75.5 ± 4.17 years, mean time from death to enucleation was 2.0 ± 0.5 hr, and mean time from enucleation to use of the tissue was 5.3 ± 0.8 hr. The use of tissue within 12 hr after donor death minimizes folding of Descemet's membrane and enables specular visualization of large fields of cells. Before specular microscopy the donor eyes in their sterile moist chambers were placed into a 37°C water bath and warmed for 15 min. The central endothelium (approximately 28 mm²) was then photographed using wide-field specular microscopy.

The corneas were then excised and processed for alizarin red staining as previously described. Each cornea examined in this study was photographed using both specular microscopy and alizarin red staining.

Endothelial Morphometry

Based on the clarity of cell borders, one specular microscopy and one alizarin staining negative were selected for each eye. The negatives were printed to a final magnification of ×400 and ×300, respectively, using appropriate calibration negatives. Because of the low magnification (×15) of light microscopy used, it was not possible to magnify these negatives to our standard ×400. A group of specular micrographs enlarged to both ×300 and ×400 was used to determine that merely changing the magnification of enlargement had no effect on the morphometric data (data not shown). For each photograph, 100 contiguous cells were traced and digitized as previously described. The computer-assisted analysis provided the parameters of cell area, coefficient of variation of cell
area and the percentage of cells which were hexagonal.

Results

Representative specular and stained photomicrographs for one rabbit and one human cornea are shown in Figures 1 and 2, respectively. For the rabbit cornea in situ, mean endothelial cell area was measured as 295 \( \mu m^2/cell \), percent hexagonal cells was 58\%, and the coefficient of variation of endothelial cell area was 0.22. The corresponding values measured after alizarin red staining were: cell area, 228 \( \mu m^2/cell \); percent hexagonal cells, 63\%; and endothelial coefficient of variation, 0.14. For the 65-year-old human donor cornea shown in Figure 2, the morphometric parameters determined by specular microscopy (compared to staining) were as follows: mean endothelial cell area, 357 \( \mu m^2/cell \) (345); percent hexagonal cells, 63\% (65); and coefficient of variation, 0.32 (0.29).

Mean (\( \pm SE \)) endothelial cell area determined by analysis of specular photomicrographs is compared to that obtained from light photomicrographs of alizarin red stained endothelia in Figure 3. These data demonstrate that in rabbit endothelia significant (\( P < 0.001 \)) cell shrinkage occurs when the cells are stained with alizarin red. Endothelial cell area decreased 14\% from 289 \( \pm 7 \mu m^2/cell \) to 248 \( \pm 7 \mu m^2/cell \) in rabbit tissue following staining. By comparison, the mean endothelial cell area for human donor tissue was found to be equivalent using either method (Fig. 3). Mean cell area determined by specular microscopy was 466 \( \pm 35 \mu m^2/cell \). This value is not significantly different from the 454 \( \pm 33 \mu m^2/cell \) determined by staining.

The coefficient of variation (CV) of cell area (standard deviation of cell area divided by the mean cell area) provides a quantitative index of the variability of cell area or polymegathism. This parameter for both human and rabbit tissue is shown in Figure 4. A significant (\( P < 0.001 \)) decrease in the endothelial CV
was noted in rabbit corneas following staining. Thus the shrunken endothelial cells of the stained corneas are more uniform in size. On the other hand, no change in endothelial CV was seen in human corneas following staining (Fig. 4).

Hexagons are the predominant cell shape in the normal endothelium; thus deviations in the percentage of cells which are hexagonal can be used as an index of cell shape variation (pleomorphism). The percentage of hexagonal endothelial cells determined by specular microscopy and alizarin red staining is shown in Figure 5. No significant differences in this index of pleomorphism were found comparing specular microscopy to staining in either rabbit or human tissue.

Since a significant degree of cell shrinkage was observed in rabbit corneal endothelium, but not human, following alizarin red staining, an additional group of experiments was performed to investigate the effects of short-term storage on this cell shrinkage. In the original series of experiments rabbit corneas were stained immediately after enucleation. Human tissue, on the other hand, was stored an average of 5 hr in a sterile moist chamber at 4°C, and then warmed for 15 min at 37°C immediately prior to specular microscopy and staining. To determine whether this difference in tissue processing might account for the differences observed in comparing the rabbit and human endothelial groups, a group of six rabbit eyes was processed in a manner comparable to that used for human tissue. After enucleation, each eye (n = 6) was placed into a sterile moist chamber and stored for five hours at 4°C. The eyes were then warmed for 15 min in a 37°C water bath. Specular microscopy and alizarin staining were then performed exactly as for human tissue. The results of this experiment are shown in Table 1.

Five hours of cold (4°C) moist chamber storage followed by warming had no significant effect on mitigating the shrinkage associated changes noted in rabbit endothelia following alizarin red S staining (Table 1). Significant (P < 0.001) cell shrinkage and normalization of cell area (decreased CV, P < 0.02) were also observed in the stored and rewarmed tissue. Finally, since a significant degree of cell shrinkage was observed in rabbit corneal endothelium following alizarin red staining, one additional group of experiments was conducted to determine whether the shrinkage could be minimized. For these experiments an additional 20 rabbit corneas were examined in four experimental groups (n = 5/group): (1) the osmolality of the sterile saline (305 mOsm) rinse was adjusted by dilution to 200 mOsm; (2) the osmolality
Table 1. Morphometric analysis of 5-hr stored rabbit corneas (n = 6 eyes, mean ± SE values)

<table>
<thead>
<tr>
<th>Specular data</th>
<th>Alizarin red S data</th>
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<tbody>
<tr>
<td>Cell area (μm²)</td>
<td>284 ± 4.1</td>
</tr>
<tr>
<td>Percent hexagons</td>
<td>62.3 ± 2.1</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>0.26 ± 0.02</td>
</tr>
</tbody>
</table>

* P < 0.02 compared to specular value.

of the rinse was further reduced to 100 mOsm; (3) to investigate the possibility that evaporation contributed to the observed shrinkage, a drop of silicone oil was applied to the endothelial surface of the stained button immediately following the rinse (200 mOsm); and (4) the effects of tissue fixation were investigated by immersing the corneal buttons in 2.7% phosphate buffered glutaraldehyde for 10 min immediately after staining.

The effects of these modifications to the staining procedure on endothelial cell area are shown in Figure 6. Adjusting the osmolality of the rinse from 305 mOsm/kg to 200 or further to 100 was observed to have no mitigating effect on endothelial cell shrinkage. Reducing evaporation from the stained button with a drop of silicone oil was found to be similarly ineffective. Tissue fixation in glutaraldehyde, on the other hand, was observed to effect greater cell shrinkage than the staining procedure alone. The mean endothelial cell area of corneal buttons fixed in glutaraldehyde after staining (211 ± 9 μm²/cell) was reduced (P < 0.001) 18% compared to the unfixed tissue (248 ± 7 μm²/cell).

Discussion

The intercellular stain alizarin red S is a convenient method of visualizing cell borders and provides a rapid technique of screening for endothelial cell damage.6,7 This method is as effective as silver impregnation8 in demarcating cell margins and has the further advantage of speed and simplicity of technique. Both methods have been used extensively to evaluate the integrity of the endothelial monolayer—for example, in wound repair,6,9-10 in inflammation11 and following donor corneal storage.12 Staining has further been used to assess endothelial cell density and cell area during wound healing in rabbit13 and monkey14 corneas. More recently, Landsman et al, using silver nitrate staining, have quantitated endothelial cell shape changes during the early stages of wound repair in the cat cornea.10 Despite their considerable use, the effects of these staining techniques on the morphology of the corneal endothelium have not been previously described. The significance of potential staining artifacts, such as cell shrinkage or swelling, has thus not been previously defined.

The results of the present study demonstrate that the alizarin staining technique can be used to obtain useful endothelial morphometric data. In human donor corneas the morphometric data obtained from alizarin staining are entirely comparable to data obtained using specular microscopy of in situ donor corneas. The morphometric data presented in this study for human donor corneas agree favorably with those described by Matsuda et al for corneas from older donors.15 They are also comparable to in vivo measurements made in individuals comparable in age to that of the donors in this study.15,16

In rabbit corneas a significant degree of cell shrinkage occurs with alizarin staining. The observed 14% decrease in mean cell area is also accompanied by a normalization of cell area, manifest as a decrease in the endothelial CV. The cells are thus smaller and more uniform in size. Despite these significant changes in cell area, no significant alterations in cell shape occur. The percentage of hexagonal endothelial cells remains unchanged. This latter observation reflects the fundamental stability of the hexagonal shape and also suggests that, although endothelial cell size changes may occur quite rapidly (for example, in response to osmotic gradients), alterations in endothelial shape occur more slowly, perhaps because cell shape changes require the disruption and reformation of intercellular junctions.
Trypan blue is commonly used in conjunction with alizarin red to distinguish areas of cell damage. Since the trypan blue stain fades quite rapidly, brief tissue fixation in glutaraldehyde is frequently employed to preserve staining intensity. The results of this study show that this tissue fixation effects further endothelial cell shrinkage. Mean endothelial cell area decreases nearly 30% compared to in situ area as measured by specular microscopy.

Though a tendency toward cell shrinkage and normalization of cell shape following alizarin red S staining is suggested by the data for human tissue (Figs. 3, 4), these changes are not significant, as they are for rabbit tissue. This difference in cell shrinkage between human and rabbit endothelia is apparently unrelated to the effects of cold storage and rewarming on the endothelial cells since similarly stored and rewarmed rabbit corneas showed endothelial cell shrinkage which was entirely comparable to that noted in fresh tissue. It is possible that human tissue may respond to storage/rewarming or the staining procedure differently than rabbit; however, a more likely explanation of the differences noted comparing human and rabbit tissue lies in the variability of the human tissue. The older and more variable human tissue shows greater variability in endothelial morphology—obtained either by specular microscopy or alizarin red staining. Further, it is likely that this tissue would be more variable in its response to a hypertonic stress, as occurs in alizarin red staining.

Useful morphometric data can be obtained by alizarin staining. This technique combined with cell morphometric analysis could provide valuable data in edematous corneas which preclude specular microscopy. Caution, however, must be exercised in comparing morphometric data obtained in this manner to those obtained in situ. Because of the significant effects of tissue fixation on endothelial cell area, it would be preferable to avoid fixation for morphometric studies.

Key words: alizarin red, corneal endothelium, endothelial morphology, morphometric analysis, specular microscopy

References