5,12-DiHETE, suggesting the presence of both 5 and 12 lipoxygenase enzyme activities in this tissue. However, human anterior uvea has considerably less capacity (4-5-fold) than rabbit anterior uvea to synthesize lipoxygenase products from arachidonic acid. Since the present data indicate the presence of 5-lipoxygenase activity and SRS is a 5-lipoxygenase product, we are in the process of determining the formation of SRS-like activity in human anterior uvea using the bioassay technique. Our preliminary data with indomethacin-treated human anterior uvea suggest that these tissues also synthesize SRS-like activity similar to rabbit ocular tissues.

In different experimental animal models such as carageenin-induced inflammation, cervical epithelial denudation, and bovine serum-induced uveitis, low doses of nonsteroidal antiinflammatory drugs such as indomethacin that inhibit only cyclooxygenase pathway potentiate PMN chemotaxis. Higgs et al suggested that low doses of indomethacin inhibit only cyclooxygenase but facilitate the synthesis of lipoxygenase products that are chemotactic for PMNs. If lipoxygenase product synthesis by human anterior uvea plays a role in inducing inflammatory cell infiltration in certain clinical inflammatory conditions, then the use of nonsteroidal antiinflammatory drugs may exacerbate the inflammation.

Key words: human anterior uvea, lipoxygenase pathway, arachidonic acid, leukotrienes

Acknowledgment. The authors wish to thank Mrs. Ann Zaragoza for typing this manuscript.


Reprint requests: Dr. Prasad S. Kulkarni, PhD, Eye Research Division, Columbia University, 630 West 168th Street, New York, NY 10032.

Direct and Indirect Determination of Nonuniform Cell Density Distribution in Human Corneal Endothelium

B. H. Schimmelpfennig

The density distribution of endothelial cells was determined, directly and indirectly, by counting cells and cell nuclei in two separate groups of unpaired human corneas. Four areas, measuring 1 square mm each, were counted in the corneal center as well as in the periphery close to Schwalbe's line. In 19 Orcein-stained corneas, the peripheral density of nuclei was 3632/mm^2 ± 592 (SD) as compared with central counts of 2778 mm^2 ± 284 (SD). The other group of 22 corneas, stained supravitally with Alizarin-red revealed a peripheral cell density of 3696/mm^2 ± 721 (SD), in contrast with a central density of 2811/mm^2 ± 425 (SD). There was also an uneven density distribution in the central endothelium. The average difference between the highest and lowest central square millimeter counts in the two groups was 8.0 ± 7.7%.
The vital role of the human corneal endothelium in maintaining stromal deturgescence, as well as its vulnerability during anterior segment surgery, have made it a target for numerous quantitative investigations. In vivo, specular microscopy is considered a valuable tool to determine endothelial cell density of a cornea. It appears to be accepted generally that a sample of 70–150 cells (approx. 0.02–0.04 mm²) is sufficient to determine endothelial density of a cornea as number of cells per square millimeter. However, it has never been proved conclusively that extrapolation from cell counts in such a small area is representative for the central or even the entire endothelium.

In vitro investigations of human endothelial density distribution have been carried out on fixed and unfixed tissue preparations by counting cells or cell nuclei in small grid fields (0.015–0.036 mm²) of various locations. Their results differ, and a definite conclusion from those studies cannot be drawn. This controversy might well have been caused by the small sample sizes, fixation procedures, as well as different locations of the sampling areas. The purpose of the present study was to determine the cell density distribution of the normal human corneal endothelium. Because of the inconclusive results in the literature, it was decided to perform nuclear as well as cellular counts on fixed and unfixed specimens in two separate groups of corneas. In order to reduce sampling errors, several fields measuring 1 square mm each were counted in the central and peripheral endothelium.

**Materials and Methods.** Staining: Two groups of 19 and 22 unpaired human donor eyes were investigated. In Group 1 (donor age 61 ± 18 years; range 22–93 years), excised corneo-scleral buttons were fixed in a mixture of absolute alcohol and glacial acetic acid (proportions 3.5:1) overnight at 4°C. Endothelial nuclei then were stained with 1% Orcein for 1 hr. The tissue was destained and dehydrated in graded alcohol, impregnated with xylene, and flat-mounted on a slide after four 3-mm radial incisions had been made.

In Group 2 (donor age 45 ± 25 years; range 19–94 years), the corneo-scleral buttons were stained with Alizarin-red without fixation. The supravital dye was dissolved in isotonic saline (0.7 g/100 ml) and applied to the button for 4 min. Finally, the specimens were flattened on a slide by four radial incisions and a cover glass to enable light microscopic examination.

**Photomicrography and counting:** Slide photomicrographs were obtained through a Zeiss light microscope with a 6.3/16 objective, Kodak Ektachrome film (ASA 64) and Zeiss yellow or green filters. Each micrograph represented an endothelial area of 1.17 mm².

Four central photomicrographs were taken within a 5-mm diameter trephine mark, which had been made during staining. Peripherally, another four micrographs were taken up to 1.6 mm from Schwalbe’s line (Fig. 1, inset). The slides were projected on a screen at a magnification of X220, and cells or nuclei were counted. In order to count 1 mm² out of 1.17 mm², a marginal strip of 0.17 mm² was omitted. In total, 8 mm² per cornea were counted, which is approximately 7% of the entire endothelial surface. The centrally counted area of 4 mm² represented 18.4% of the area, enclosed by the 5-mm trephine mark.

**Investigations on possible corneal shrinkage:** In 11 unpaired corneas (donor age: 63 ± 25 years), three 2.5-mm diameter buttons were punched out by a trephine centrally as well as peripherally close to Schwalbe’s line. The three pairs per cornea, consisting...
Fig. 2. Embryonic cell nuclei of a 74-day-old donor (Omicron staining). Central region (A, C). Periphery (B, D) with nuclear division figures (arrow). Nuclear density in the center.

30-40/μm² and in the periphery, 90-100/μm² (Calibration bar in A, B: 50 μm; in C, D: 100 μm).
of a central and a peripheral button were: (1) exposed to isotonic saline (4 min); (2) stained with 0.7% Alizarin-red (4 min); (3) fixed with a mixture of absolute alcohol and glacial acetic acid and stained with orcein, as described above. After the procedure, the diameter of the buttons was measured by means of an ocular micrometer under the light microscope.

**Results.** In both groups the counts clearly demonstrated a nonuniform distribution of the endothelial cells or their nuclei (Fig. 1). The higher density in the corneal periphery was very similar in the two groups and averaged 3632 ± 592 (SD) cells/mm² in Group 1 and 3696 ± 721 (SD) nuclei/mm² in Group 2. Compared with the central endothelium, there were about 30% more cells within the region that had been examined up to 1.6 mm from Schwalbe’s line.

Based on an inner corneal diameter of 11.7 mm, that region may represent over 40% of the whole endothelial surface. The cell dense zone near Schwalbe’s line was not equally wide and varied from 0.5 mm to almost 2 mm. The cell nuclei in the periphery were of oval shape and, occasionally, nuclear division figures could be observed (Fig. 2B). The arrangement of the rounded central nuclei was very regular in contrast to the pattern in the periphery (Fig. 2A, C). The average density was 2778 nuclei/mm² ± 284 (SD).

The supravital staining revealed a similar distribution. Small cells of undetermined shape could be seen in the periphery (Fig. 3B). Very often, they were separated from Schwalbe’s line by groups of large cells with markedly stained cell borders (Fig. 4B). The central endothelial cells appeared to be regularly arranged in a predominantly hexagonal mosaic (Fig. 3A, 4A). Their average density was 2811 cells/mm² ± 425 (SD). Surprisingly, even the central endothelium was not uniformly distributed. Substantial variations existed among the four counted square millimeter areas in each cornea. In the two groups, the maximal difference within a cornea was expressed in percent. The average difference in Group 1 was 8.0 ± 7.7% (SD) (range: 2–36%) and 9.0 ± 3.6% (SD) (range: 2–18%) in Group 2.

The diameter of the trephined 2.5-mm corneal buttons after the procedure were as follows:

- Group 1: 2.49 ± 0.01 mm (SD) [2.50 ± 0.02 mm (SD)]
- Group 2: 2.50 ± 0.03 mm (SD) [2.50 ± 0.02 mm (SD)]
- Group 3: 2.51 ± 0.02 mm (SD) [2.50 ± 0.01 mm (SD)]

(The diameter of the buttons before the procedure are given in brackets.) The data obviously show that shrinkage was insignificant.

**Discussion.** The inhomogeneous, endothelial cell density distribution in human corneas that could be demonstrated by counting sampled endothelial areas of larger size than in previous investigations may have several implications. So far, in vivo specular micrographic cell density determinations in healthy corneas have been based on clinical reports that endothelial cells are homogeneously distributed. The disparity of those findings and the present data is explained by the considerable difference in sample size, as well as the selection of central and peripheral endothelial areas for sampling. It should be noted that the cell dense region near Schwalbe’s line cannot be visualized by current specular microscopic techniques. It should be mentioned additionally, that in the present study, no obvious age related differences, neither in the central nor in the peripheral counts, could be observed. However, the number of counts is still too small to draw final conclusions. Since the corneas not only revealed an inhomogeneity between central and peripheral endothelial regions, but also showed density variations of nearly 10% in its central parts, the question arises as to what extent 70–150 specular micrographically counted cells represent the entire endothelium. At present, the regulation of cell size and density distribution in the central endothelium of human corneas is not understood. Continuous cell desquamation during life would reduce the number of cells per unit area that probably could result ultimately in decreased endothelial function and stromal edema. Simple local cell enlargement would be finally insufficient to compensate for the loss.

The existence of a peripheral zone with increased cell density suggests the possibility for two regulatory mechanisms: (1) Small peripheral cells migrate towards the center, where they substitute enlarging cells to balance the overall cell size. (2) The peripheral endothelial cells also enlarge, thus contributing additional cell volume, if the central endothelial cells exceed a critical size.

In cataract surgery, the common approach to the anterior chamber is a 170° corneo-scleral incision. In aphakic cadaver eyes, the incision has been shown to interrupt Descemet’s membrane permanently in that zone of increased cell density. Any damage to the peripheral endothelium and its basement membrane could well have an influence on repair mechanisms.
such as cell migration or cell enlargement and, therefore, interfere with central endothelial wound-healing after surgery. It has been demonstrated recently in primates, that a small central endothelial lesion causes almost the entire endothelium to move and enlarge even the distant cells in the periphery.\textsuperscript{11} Shrinkage of the corneas in the plane of their posterior surface did not occur during the staining procedures. Thus, the area of the matrix on which the counted cells and their nuclei are resting was unchanged. A shrinkage of the cells is unlikely to occur, since Alizarin-red is a supravital dye and has been used in similar studies.\textsuperscript{12} Shrinkage of the cell nuclei cannot be excluded, however, and their density per unit area should not be influenced by their size, as long as the underlying matrix does not change its dimensions.

**Key words:** corneal endothelium, corneal endothelial density, cell density, cell counts, endothelial density distribution, in vitro endothelial counts.
Zurich, Switzerland. Portions of this work were reported at the Annual Meeting of the Association for Research in Vision and Ophthalmology, May 2, 1982, Sarasota, Florida. Submitted for publication: March 25, 1983. Reprint requests: B. H. Schimmelpfennig, MD, Department of Ophthalmology, Universitätsspital, 8091 Zurich, Switzerland.

References

Circadian Disc Shedding in Xenopus Retina In Vitro

John G. Flonnery and Steven K. Fisher

To further examine the endogenous rhythm of disc shedding and phagocytosis observed in several species, adult Xenopus were entrained to a 12 hr light/12 hr dark cycle and then placed in constant darkness. At various times during a 3-day period of constant darkness, eyes were explanted and placed into culture medium, then processed for light and electron microscopy. A clear rhythmicity of disc shedding was observed, with pronounced peaks at the times light onset occurred in the original entrainment cycle. Modification of the HCO3- ion concentration in the medium was found to raise the amplitude of the peak of endogenous disc shedding. Explants maintained in culture medium containing deuterium oxide (a compound known to perturb circadian oscillators) were found to shed with a longer interval between peaks. The addition of the protein synthesis inhibitor, anisomycin, to this preparation suppressed the shedding rhythm. The findings suggest the presence of a circadian oscillator for rhythmic disc shedding residing within the amphibian eye. Invest Ophthalmol Vis Sci 25:229-232, 1984

LaVail\textsuperscript{1} was the first to show that most disc shedding from rod photoreceptors occurs within 1 to 2 hrs of the onset of light in animals entrained to a daily lighting cycle. More recently, he has shown that, in rats, this peak in phagosome number within the RPE has characteristics of a circadian rhythm, with light acting as a zeitgeber.\textsuperscript{2} While earlier work showing that reserpine diminished this daily rhythm suggested that input from the pineal may be important in cyclic disc shedding,\textsuperscript{1} more recent studies have shown that the rhythm occurs unimpaired after removal of the pineal, superior cervical ganglia, hypophysis, and thyroid-parathyroid glands.\textsuperscript{3,4} In albino rats in which one eye is patched and the optic nerve is transected, constant light blocks rhythmic shedding only in the unpatched eye,\textsuperscript{5} suggesting that the circadian oscillator controlling rhythmic shedding resides within the eye.

In the African clawed frog (Xenopus laevis), this shedding response is largely evoked by the onset of light; but there is evidence that rhythmic shedding continues in constant darkness, although at a greatly reduced level when compared with animals maintained under cyclic lighting.\textsuperscript{6} We have previously demonstrated that light-induced disc shedding proceeds normally in Xenopus eyes kept in vitro.\textsuperscript{7} It seemed to us that the most direct approach to determining whether a circadian oscillator that controls disc shedding exists within the eye would be to look for evidence of rhythmic disc shedding in Xenopus eyes placed in culture and kept in constant darkness. In this report, we demonstrate that explanted eyes show a rhythmic increase in rod disc shedding that persists in darkness,