Heterogeneity in Ultrastructure and Elemental Composition of Perinuclear Lens Retrodots


Purpose. To unravel the cataractogenic process(es) leading to the birefringent lenticular bodies known as perinuclear retrodots.

Methods. Ten human lenses containing biomicroscopically verified perinuclear retrodots were systematically screened and analyzed using scanning electron microscopy and energy dispersive x-ray microanalysis to verify their ultrastructure and elemental composition.

Results. Three types of retrodots were distinguished, different in size, ultrastructure, and origin. Two of them contained calcium phosphate, the third probably contained calcium oxalate. All three types were separated from surrounding normal fibers and the crystalline inclusions were sequestered within membrane-lined bodies.

Conclusions. Because of these observations and data found in the literature it is postulated that elevated free calcium is the initiating factor in the formation of retrodots, trapped by either oxalate or phosphate and sequestered in the retrodots. It is suggested that the oxalate is derived from ascorbate because of impaired protection against oxidative stress in the older lens. Phosphoric acid is believed to be released by calcium-induced hydrolysis of membrane phospholipids. Invest Ophthalmol Vis Sci. 1994;35:199-206
cataract extraction lenses obtained from the Cornea Bank Amsterdam and the Department of Ophthalmology of the Erasmus University, Rotterdam showing identical inclusions.

MATERIALS AND METHODS

Six lenses obtained after extracapsular cataract extraction and examined at the slit lamp for the presence of retrodots using the Oxford criteria 1 (Fig. 1) were used in this study. In addition, two donor lenses obtained from the Cornea Bank Amsterdam and two extracapsular cataract extraction lenses from Rotterdam were used, which proved to contain identical inclusions on biomicroscopic and SEM screening. The extracapsular cataract extraction lenses had additional cataractous features. The donor lenses were free of these.

The six lenses from Oxford were immediately fixed in a 0.1 M phosphate-buffered solution of 5% glutaraldehyde (P-GL), pH 7.3. The lenses from Rotterdam and the donor lenses were fixed in a 0.08 M cacodylate buffered solution of 1.25% glutaraldehyde and 1% paraformaldehyde (C-GL/P), pH 7.3. Fixation was for several weeks. The lenses were dissected and pieces containing retrodots and minimal other disturbances were selected for further treatment. These pieces were dehydrated in a graded series of ethanols and critically point dried using CO2 as the intermediate. The uncoated specimens were inspected and analyzed in a Philips SEM 505 scanning microscope (Philips Industries, Eindhoven, The Netherlands) equipped with an EDAX PV 9800 microanalysis system using a windowless ECON detector (EDAX, PV 9760/26; EDAX-ItI., Mahwah, NJ). A high tension of 15 kV was used throughout and EDX signals were obtained from spot measurements (spot size 100 to 200 nm). After completion of the EDX analysis the specimens were coated with a thin (5 nm) layer of platinum and the EDX-analyzed sites or identical structures were photographed.

RESULTS

The results of the systematic SEM and EDX inspection of the retrodots from all ten lenses are summarized in Figures 2 to 9. On the basis of these observations three types of retrodots can be distinguished. Apart from size differences no other differences could be observed on biomicroscopy.

Type 1 retrodots (Figs. 2, 3, 4) are characterized by the smooth lining of their dome or discus-shaped cavity studded with ridges that are continuous with membranous elements crisscrossing the cavity (Figs. 2C, 3B, 3C). In addition they contain globular elements of irregular form (Figs. 2B, 4B, 4C). In many instances this globular content is lost during fracturing for SEM. In comparison to EDX spectra of normal control regions (insert Fig. 2A) the Type 1 retrodot spectra (insert Fig. 2B) exhibit enhanced S and Na peaks and a lower O peak. In addition, pronounced P and Ca peaks appear. The smooth lining and the fibers immediately underlying it (insert Fig. 2C) also show increased S and Na peaks and a somewhat lower O peak. A significant Ca peak is present but no P peak was observed. The observations illustrated in Figure 4 tentatively allow the conclusion that Type 1 retrodots originate from wormlike membrane aberrations on individual fibers, which accumulate or form small globular elements that grow into mature retrodots. The EDX spectra of these early Type 1 retrodots are similar to that illustrated in Figure 2B (insert).

Type 2 retrodots (Figs. 5, 6, 7) are dome- or discus-shaped and their cavity lining has a rough texture (Figs. 5B, 6B). They contain numerous (Fig. 6A) complex crystals, varying in size between 15 and 30 μm consisting of mutually anchored platelike elements (Figs. 5B, 6B). EDX spectra (insert Fig. 5A) of these crystals clearly show very pronounced Ca and P peaks, and small Na, O, and C peaks (at high Ca concentrations two Kα-peaks at 3.69 and 4.04 keV were observed). With respect to their pathogenesis Figure 7 allows the conclusion that they probably originate from coalescing, disrupting membranes of a number of stacked fibers (Fig. 7; A, B, and C), which subsequently form a multilamellar pocket within which the crystals are formed (Fig. 7, D and E). The early stages of Type 2 retrodots (Fig. 7C, insert) exhibit pronounced Ca (double peaks) and P peaks but in addi-

FIGURE 1. In vivo retroillumination photograph of a human lens with retrodots. The rounded oval features are the retrodots of variable size. Note the reversal of background illumination within the retrodots, ie, background lighter on the right, retrodots lighter on the left. The bright so-called corneal reflex is an artifact from the flash.
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FIGURE 2. Panel illustrating different ultrastructural aspects of Type 1 retrodots. (A) Survey micrograph illustrating the dome-shaped appearance of unfractured Type 1 retrodots (arrowheads) surrounded by normal fibers. SL = suture line. (90001,P-GL). (B) Intermediate Type 1 retrodot (approximately 30 μm) containing irregularly shaped material surrounded by fibers with normal membranes. (90001,P-GL). (C) Large Type 1 retrodot (approximately 55 μm) the content of which is lost during SEM preparation and which is surrounded by typical membranes of deep cortical fibers studded with grooves and ridges (arrowheads). The retrodot cavity is lined by a smooth membrane, discontinuous with the underlying normal fibers (white arrow) and studded with ridges. (91112,P-GL). The x-ray spectra of normal regions (black and white dots, A and B) show carbon (C), oxygen (O), sodium (Na), and sulphur (S) peaks. The lining of the cavity and the underlying fibers exhibit an enhanced S peak and a Ca peak (asterisks, C). The Type 1 retrodot content has in addition to C, O, Na, S, and Ca peaks a pronounced phosphorous (P) peak, (asterisks, B).

Type 3 retrodots (Fig. 8) are dome-shaped structures lined by smooth radially coursing fiberlike elements (Fig. 8A) most likely pinched off from neighboring fibers. The content of these retrodots, which is also often lost during SEM preparation, consists of large accumulations (Fig. 8, A and B) of irregular pillarlike elements (Fig. 8C). EDX spectra (Fig. 8C, insert) revealed that these elements only have pronounced Ca, O, and C peaks. No ultrastructural bodies representing early stages of this type of retrodots were found.

Incidentally (Fig. 9) one or two closely adjacent retrodots were observed having ultrastructural and EDX characteristics of Type 2 and Type 3 retrodots.

In keeping with the biomicroscopic observations the SEM observations revealed that all three types of retrodots were well-demarcated lenticular bodies separated from the surrounding normal fibers. They are lined by what apparently seem to be membranes that segregate the content from neighboring unaffected fibers. With respect to their size, Type 1 retrodots are the smallest, ranging from 25 to 50 μm. Type 2 retrodots range from 100 > 500 μm and Type 3 retrodots have a maximal size of approximately 300 μm. Of the ten lenses systematically surveyed, five proved to contain only Type 1 retrodots, and two contained only Type 2 retrodots. Types 1 and 2 were observed together in one lens and two lenses contained all three.

FIGURE 3. In cross section (A) Type 1 retrodots proved to be spindle-shaped and its cavity to be filled with irregularly shaped material (arrow, A and C) intermingled with membranous elements (open arrows, A and C). The ridges on the lining of the cavity (arrowheads, B) proved to be continuous with these membranous elements (arrowheads, C). Note that the fibers adjacent to the retrodots have normal groove-and-ridge studded membranes (asterisks, B and C). (A: 90001,P-GL; B: 90001,P-GL; C:88260,C-GL/P).
FIGURE 4. Panel illustrating the possible origin of Type 1 retrodots. In regions of these retrodots fibers are observed with wormlike imprints (arrowheads, A) closely associated with regions containing small irregular elements (asterisks, A). In a further stage small accumulations of irregularly shaped material (asterisks, B and C) lined by smooth surfaced membranes (arrowhead, B) are found. X-ray microanalysis shows peaks identical to that observed in Type 1 retrodots (cf. Fig. 2B). (A: 89205,P-GL; B: 90001,P-GL; C: 90199,C-GL/P).

types of retrodots. All retrodots were located in the intermediate and deep cortex and were not found in the nucleus. Types 1 and 2 retrodots were observed in the posterior, anterior, and equatorial regions of the lens, whereas Type 3 was restricted to the equatorial region.

DISCUSSION

The current systematic SEM study reveals that the perinuclear retrodots described previously,1 which are identical to the "Sphärolithen" of the German literature7-9 and the calcium containing birefringent bodies of Harding et al,3 may be subdivided into at least three subtypes of varying size: Type 1, 25 to 50 μm; Type 3, 50 up to 300 μm; and Type 2 100 to more than 500 μm. Biomicroscopic observations as illustrated in Figure 1 only show the retrodots to vary in size. They are clearly different with respect to their ultrastructural appearance and two of them may have a different pathogenic origin. In keeping with the biomicroscopic observations all three have a dome- or discus-shaped outline and are well demarcated from the surrounding unaffected lens parts. The SEM observations allow the conclusion that they are segregated by membranes from the neighboring unaffected fibers and that the content is sequestered from the adjacent fibers. The segregation of these aberrant lenticular inclusions is largely analogous to that found in early radial opacities described by Vrensen and Willekens10 and Vrensen et al11 and may be taken as an additional support of the view of Bron and Brown12 that there is a strong tendency in the lens to seal off damaged portions from undamaged portions, i.e., those that have an annealing mechanism.

A number of authors9-13-16 have argued that the main component of these crystalline inclusions is calcium oxalate. Harding et al,3 using the same analytical tools as in the current study, (SEM and EDX analysis), concluded that the birefringent lenticular bodies contain calcium oxalate. Our Type 3 retrodots have similar EDX spectra as those described by Harding et al,3

FIGURE 5. Ultrastructure of Type 2 retrodots. In the survey picture (A) of a partly fractured retrodot the discoid shape can be appreciated. The cavity is partly filled by a rough, textured surface (arrowheads, B) and is surrounded by normal fibers (A, B). In cross section (C) the space crossing appearance of the complex crystals can be seen. The retrodots of this Type 2 range from approximately 100 to more than 500 μm. All x-ray spectra of the crystals exhibit pronounced Ca and P peaks and minor C, O, and Na peaks. At high concentrations Ca exhibits two Ka peaks at 3.69 and 4.04 keV (A: 88260,C-GL/P; B: C-GL/P; C: 90198, C-GL/P).
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FIGURE 6. Ultrastructure of a very large (more than 1 mm) Type 2 retrodot from which the upper lining has been fractured away during the SEM procedure. The rough surfaced lining (arrowheads, B) is occupied by complex crystals (size approximately 15 to 20 nm). SL = suture line. (A: 91089, P-GL; B: 90218, C-GL/P).

ie, high Ca, low S, and no P peaks. Because of the use of a windowless ECON detector we additionally found C and O peaks. However, this elemental composition can be ascribed to a number of other insoluble calcium salts as, for example, calcium carbonate, calcium formate, calcium hydroxide, etc. The microchemical analysis carried out by Harding et al and the laser microprobe analytical observations of Pau and Kaufmann of birefringent spheroliths strongly favor the calcium oxalate nature of this material. Calcium oxalate crystals give similar EDX spectra. The calcium oxalate type of retrodots is rather infrequent and only few were observed in the equators of two lenses.

The type 2 retrodots clearly contain calcium salts of phosphorous. Because a quantitative interpretation of the EDX signals is very complicated, especially for the low energy oxygen signal, it cannot be determined which form of calcium phosphate is present. Crystals of different calcium phosphate salts yield identical EDX spectra. Because of the poor solubility of the dibasic Ca-HPO4, this is the most likely candidate. A critical technical point to be raised here is whether the crystals are artifacts caused by fixation with a phosphate-buffered solution. If this were the case the calcium would have to have been present as free calcium in high concentrations or as a salt, which would be changed to calcium phosphate, e.g., calcium oxalate. Our observations do not favor this view. First, identical calcium phosphate crystals were observed in lenses fixed in a cacodylate-buffered fixative (Rotterdam and donor lenses). Second, the calcium phosphate crystals of Types 1 and 2 retrodots have completely different appearances. Finally, within one lens two or three types occur simultaneously even in one or two adjacent retrodots (Fig. 9).

The nature of the content of Type 1 retrodots is the most complex of all. The pronounced P peak and the enhanced Ca signal indicate that a calcium salt of phosphorous is certainly one of the constituents. However, the relative low Ca peak as compared to the P peak is puzzling because the calcium phosphate of Type 2 crystals have about equally intense peaks for both elements. When taking into account the elevated S peak and the presence of crisscrossing elements in

FIGURE 7. Possible origin of Type 2 retrodots. In regions close to Type 2 retrodots small (approximately 10 μm) circular elements are observed (asterisks, A), which seem to be formed by coalescence of membranes of a group of stacked fibers (B and C) and are covered by disintegrating membranes (asterisks). In a further stage the disintegrating membranes seem to form a cavity in which the typical crystals are formed (arrowheads, D and E). The X-ray spectra of these early Type 2 retrodots show in addition to the pronounced Ca (double peak due to high concentration), P, Na, C, and O Peaks a significant S peak. (A: 88260, C-GL/P; B: 88260, C-GL/P; C: 90198, C-GL/P; D: 90218, C-GL/P; E: 90198, C-GL/P).
FIGURE 8. Type 3 retrodots are characterized by their round shape, their lining by radially running elements (arrowheads, A) and a pillarlike content (asterisks, A, B, and C). Their size varies between 100 and 300 μm. The x-ray spectra of Type 3 retrodots content exhibit peaks only for Ca, C, and O. (A: 90198.C-GL/P; B: 88260, C-GL/P; C: 88260,C-GL/P).

FIGURE 9. Incidentally one retrodot or two closely adjacent retrodots were observed, which reveal the ultrastructural and x-ray characteristics of Type 2 (black and white dots) and Type 3 retrodots (asterisks). Note that the membranes of surrounding fibers have a normal ultrastructural appearance. (901892, P-GL.)

gests that calcium phosphate is the most prevalent form of retrodot. The presence of a calcium oxalate retrodot is more inferential. It seems likely that an elevated calcium level within the fiber is the initiating factor for the pathological formation of retrodots and that the excessive calcium is eliminated either in the form of calcium phosphate or calcium oxalate, which are subsequently sequestered in membrane-bound bodies: the retrodots.

The crucial role of calcium in cataract is now generally accepted and the presence of calcium phosphate and calcium oxalate in Morgagnian and hypermature cataracts has long been noted. Experimentally it has been shown that calcium induces aggregation of crystallins. The observation of Shun-Shin et al, that the presence of retrodots in the human lens is significantly associated with increased nuclear light scatter, is in line with this. Recently Duncan et al showed that the free calcium content of clear human lenses significantly increases with age and could account for the enhanced optical density and light scatter. In addition, there is ample evidence that altered calcium levels stimulate the activity of proteolytic enzymes in the lens and that both lens crystallins and cytoskeletal proteins are substrates for the calcium-activated proteases. In conjunction with the notion that crystallins, cytoskeletal proteins and the lipid bilayer are firmly associated it is not surprising that low (< 0.5 mM) and high (> 1.3 mM) levels of calcium alter the membrane structure and can even lead to complete disruption. It has further been shown that the eye lens exhibits phospholipase activity and that the hydrolysis of phospholipids is a complex process regulated by protein kinase C and stimulated by calcium. In view of this evidence a tentative hypothesis on the origin of the calcium phosphate de-
alate would be able to trap the only slightly increased breakdown of proteins and phospholipids and that oxalate eventually will drop to more physiological levels. As a result of this trapping the calcium content would not be as such that it inevitably leads to a decrease in activity from cortex to nucleus. Furthermore, freeze fracture structure studies\textsuperscript{11,28} allow the conclusion that deep cortical and nuclear membranes have a cholesterol to phospholipid ratio of 5 or more and are impermeable to ions. Moreover, it has been found\textsuperscript{29} that the phospholipase activity in the lens shows a decrease in activity from cortex to nucleus. This would explain the absence of retrodots in the lens nucleus. High calcium levels can lead to the uncoupling of cells ultrastructurally indicated by the presence of square arrays and it has been shown\textsuperscript{10,29} that isolated early opacities in the human lens are surrounded by membranes densely populated with these square arrays. Because of this it is tempting to postulate that the elevated calcium levels locally lead to uncoupling of parts of fibers by formation of square arrays and that therefore the retrodots remain restricted to relative small parts of lens fibers. It should be noted that spheroloths are said to occur in the lens nucleus in Morgagnian cataract.\textsuperscript{14} It may be that retrodots can be formed in the nucleus, but less frequently than in the deep cortex, or that the definition of “nucleus” in some reports, also includes perinuclear cortex.

Finally, two puzzling questions remain to be answered. Why are there two types of retrodots containing calcium phosphate and why is the calcium trapped by phosphate in two types and by oxalate in the other? It has been shown that free calcium is not evenly distributed throughout the normal animal lens\textsuperscript{17,20} but that it is relatively low in the most superficial regions, increases toward the deeper cortex, and is low in the nucleus. If this were the case for the human lens as well, it would mean that most superficially the calcium level would not be as such that it inevitably leads to a breakdown of proteins and phospholipids and that oxalate would be able to trap the only slightly increased calcium. Whether Type 1 retrodots are just early stages of Type 2 retrodots, still containing remnants of lens fibers, remains to be investigated.

**Key Words**

human lens, retrodots, ultrastructure, elemental composition, calcium

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**References**