Subretinal fluid: Isoenzymes and cytologic studies

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Samples of subretinal fluid from patients with retinal detachment of various duration were studied. Acid phosphatase and nonspecific esterase activity increased with the duration of detachment. The isoenzyme patterns of both enzymes in the subretinal fluid were quite different from those of serum or retinal cells. One acid phosphatase isoenzyme and two esterases were observed in the subretinal fluid. Lactic dehydrogenase in the subretinal fluid of eyes with recent retinal detachment was identical to serum lactic dehydrogenase. A very small amount of retinal lactic dehydrogenase was observed in subretinal fluid of eyes with retinal detachment of long duration. Cytologic examination showed at least three types of cells in the subretinal fluid: Macrophages, pigment epithelial cells filled with ellipsoidal granules, and an unidentified nongranulated cell.

Key words: subretinal fluid, acid phosphatase, esterase, lactic dehydrogenase, pigment epithelial cells, macrophages.

Subretinal fluid that accumulates with rhegmatogenous retinal detachment has been shown to include both serum and vitreous specific proteins. Both the total protein concentration and the size of protein molecules present correlate with the duration of retinal detachment. It is thus generally postulated that fluid vitreous passes through the retinal breaks, and that the increasing permeability of the choriocapillaries then allows serum proteins to accumulate in the subretinal fluid.

Since retinal detachment causes degeneration of the rods and cones and is accompanied by low grade inflammation, subretinal fluid proteins should be derived partly from lysosomal and other enzymes of the damaged retina and partly from inflammatory cells.

This study demonstrates the presence of unique hydrolytic enzymes and tissue enzymes and describes the cytologic profile in 50 samples of subretinal fluid collected from eyes with retinal detachment of varying durations. Duration is defined as the time between the patient's first notice of visual defect ascribable to retinal detachment and the sampling.
Material and methods

Subretinal fluid and blood were collected from patients undergoing first retinal detachment surgery without previous surgical treatment. The patients selected for this study had idiopathic rhexias, i.e., the patients do not have any known diseases of the eye other than retinal detachment. We made this selection in order to collect basic information as a reference for future studies on different retinal diseases.

The subretinal fluid was collected by carefully aspirating the liquid that collected below the perforation site. Slight blood contamination was usually present; heavily contaminated samples were discarded. Each sample was centrifuged for 20 minutes at 2,000 r.p.m.

The supernatant was analyzed for total protein concentration, using bovine serum albumin as standard. Total acid phosphatase activity in subretinal fluid and serum was analyzed using p-nitrophenylphosphate as substrate. Esterase activity was estimated in 1 ml of reaction mixture containing 100 μmoles of phosphate buffer (pH 7.5), 1 μmole of p-nitrophenylacetate, and 10 μl of subretinal fluid or serum. Production of p-nitrophenol was then estimated from the change in absorbance at 410 nm. Activity of both enzymes was expressed as micromoles or nanomoles substrate hydrolyzed per minute per milliliter of sample.

Each sample of subretinal fluid and serum was also examined for isoenzymes after polyacrylamide gel electrophoresis. Acid phosphatase and esterase were analyzed in a 7.5 per cent polyacrylamide gel system (pH 4.5) for 75 minutes with a 4 ma. current per sample. The direction of electrophoretic mobility is shown by the arrow on the left-hand side of each figure. The sample gel and spacer gels are not shown. The gel columns were equilibrated in 0.1 M phosphate buffer (pH 7.5) for 10 minutes or 0.1 M citrate buffer (pH 5) for acid phosphatase staining. Acid phosphatase activity was stained in a medium of 0.1 M phosphate buffer (pH 7.5), 10 mg. naphthyl phosphoric acid and 10 mg. fast garnet GBC in 10 ml. of 0.1 M phosphate buffer (pH 7.5), 10 mg. fast RR salt. The electrophoresis and staining procedures were established in recent studies on leukocyte esterases.

Esterase activity was determined by incubating the gel column at 37° C. with the staining reagent kit containing lactic acid, NAD, and nitroblue tetrazolium, supplied by Canalco, Rockville, Md.

Results

The acid phosphatase activity in the subretinal fluid was dependent on the duration of retinal detachment (Fig. 1). The activity was very low (less than 10 nmoles x min⁻¹ x ml⁻¹) in the subretinal fluid obtained from patients whose retinas were detached for less than two weeks. For longer periods of detachment, the activity increased above 20 nmoles x min⁻¹ x ml⁻¹. The activity in most of the serum samples analyzed had values below 10. Only two subjects had unusually high serum acid phosphatase activity (21 and 25 nmoles x min⁻¹ x ml⁻¹). The activity in the subretinal fluid had no correlation to that of serum, as indicated by the following observations.

Although the total activity of acid phosphatase in the subretinal fluid varied depending on the duration of detachment, the isoenzyme pattern did not change. Only one acid phosphatase isoenzyme was observed in the 48 subretinal fluid samples analyzed. Figs. 2A and B are examples of subretinal fluid samples obtained from recent and prolonged retinal detachment.
patients. The acid phosphatase isoenzyme found had an electrophoretic mobility similar to isoenzyme 5 described in another study on acid phosphatase of leukemic leukocytes.\textsuperscript{15} The retinal (Fig. 2C) and choroid (Fig. 2D) contained two isoenzymes similar to isoenzymes 1 and 3 of normal leukocytes.\textsuperscript{15}

Most of the serum samples analyzed had very low acid phosphatase activity similar to that shown in Fig. 3B\textsubscript{A}. In two subjects, the acid phosphatase activities in the serum were very high (Fig. 3B\textsubscript{B} and B\textsubscript{C}). Fig. 3 shows the isoenzyme pattern of subretinal fluid and serum from three patients and illustrates that the activity in the serum was not related to the activity in the subretinal fluid.
Fig. 5. Substrate specificity of esterase-5λ of subretinal fluids (3 month detachments) were subjected to electrophoresis in triplicate. At the end of electrophoresis, each gel column was stained with a different substrate: A. α-naphthylacetate; B. α-naphthyl butyrate and C. α-naphthyl ASD chloroacetate.

The acid phosphatase activity was barely detectable in the subretinal fluid (Fig. 3A1) of the first patient, whose retina had been detached for one week. However, equivalent amounts of serum (Fig. 3B1) obtained from the same patient showed very strong acid phosphatase in a position slightly lower than that of subretinal fluid acid phosphatase. On the other hand, acid phosphatase activity in the subretinal fluid from the second patient (Fig. 3A2) was very strong because of prolonged detachment, but barely detectable in the serum (Fig. 3B2). Although the third patient had strong acid phosphatase activity in both serum and subretinal fluid, the electrophoretic mobility of serum acid phosphatase was slow and was observed near the top of the acrylamide column (Fig. 3B3). It is obvious, therefore, that the acid phosphatase in the subretinal fluid is not derived from the blood.

Esterase activity in serum samples assayed by spectrophotometric procedure, using p-nitrophenyl acetate as the substrate, was fairly constant among the subjects examined (0.75 to 0.90 μmoles x min⁻¹ x ml⁻¹). Esterase activity in the subretinal fluid obtained from subjects, whose retinas were detached less than one week, was only about 10 per cent of that in the serum (Fig. 4). As the duration of detachment increased, the esterase activity in the subretinal fluid reached the level of activity in the serum. The spectrophotometric method measured the total activity of a number of esterases. Further examination by the isoenzyme technique described below showed that the esterase in the subretinal fluid is completely different from that in the serum.

Two distinct esterase isoenzymes, 1 and 3, were observed in the subretinal fluid. These two esterases are specific for α-naphthyl acetate. When naphthyl butyrate (substrate for macrophages) or naphthyl ASD chloroacetate (substrate for granulocytes) were used, these two isoenzymes were not stained at all, but a weak and diffuse band was seen in position 2 (Fig. 5). Both isoenzymes 1 and 3 were inhibited by fluoride. Fig. 6 shows that the intensity of isoenzymes 1 and 3 was reduced as fluoride concentration increased. However, the weak and diffuse isoenzyme in position 2 was not affected by fluoride.
Fig. 7. Comparison of subretinal fluid esterase to that of serum and eye tissue: A. 5X subretinal fluid; B. 5X serum; C. cetyltrimethyl ammonium bromide (0.1 per cent) extract from 0.5 mg. (wet weight) retina; D. cetyltrimethyl ammonium bromide extract from 0.5 mg. (wet weight) choroid.

Normally, the esterase in position 2 was not obvious because of the strong activity in positions 1 and 3. When esterase activity in positions 1 and 3 was completely inhibited by fluoride, the activity in position 2 became obvious (Fig. 6D). The isoenzyme 2 in subretinal fluid appears to be identical to the major esterase band in the serum (Fig. 7B), which was also insensitive to fluoride inhibition.

Esterase isoenzyme patterns in subretinal fluid, choroid serum, and retina are compared in Fig. 7. The major esterase isoenzyme in serum was diffuse and insensitive to fluoride inhibition. Both choroid and retina had only one esterase. Its electrophoretic mobility was similar to isoenzyme 3 in the subretinal fluid, and was also sensitive to fluoride inhibition.

Isoenzyme patterns of lactic dehydrogenase isoenzymes 3, 4, and 5 were found in the serum of all the subjects analyzed and no significant variation in quantity and quality was observed in the samples. Retina extracts contained all five isoenzymes (Fig. 8). Subretinal fluid collected from eyes with retinal detachments of less than three weeks' duration contained three isoenzymes identical to those found in the serum. In these samples, the intensity of lactic dehydrogenase was always lower than in the serum. In six samples obtained from eyes with retinal detachments of a duration longer than two months, very low amounts of isoenzyme 1 and 2 were found (Fig. 8D, arrows).

Three types of cells not found in normal circulating blood were found in the subretinal fluid (Fig. 9). Many large macrophages (Fig. 9A) were observed in all samples examined. They were similar to those in subretinal fluid described by Laszczzyk.16 Another type of large cell (Fig. 9B) filled with ellipsoidal granules was observed in 80 per cent of the samples examined. These granulated cells were different from macrophages. With the Wright-Giemsa method, the cytophism of these cells stained light blue evenly, in contrast to the macrophages which stained...
irregularly and showed vacuoles. Furthermore, macrophages had very strong activity in nonspecific esterase, while these granulated giant cells had very weak or no esterase activity. The origin of the third type of cell (Fig. 9C) is still unknown. When stained by the Wright-Giemsa stain, its cytoplasm was light blue, the nucleus was red, and the chromatin was highly condensed.

Discussion

Our unpublished data confirms previous reports that the total proteins in subretinal fluid increase with the duration of the retinal detachment. This communication describes our observation that some hydrolytic enzymes (acid phosphatase and esterase) also increased with the duration of the detachment. The random deviations of each analysis from the general trend is probably due to the extent of detachment, hole size, and other unknown clinical factors. However, the activity of these enzymes in subretinal fluid was not dependent on the enzyme activity in blood. Acid phosphatase isoenzyme 5 and esterase isoenzyme 1 were particularly interesting because their electrophoretic mobilities were quite different from the isoenzymes of acid phosphatase and esterase in serum and in retina. Therefore, it is unlikely that these two enzymes simply leak from the circulating blood or from the ruptured tissue cells. It is probable that these enzymes are actively secreted by cells in the immediately surrounding area, and are not accumulated inside normal cells in significant amounts. The increasing hydrolytic enzyme activity in the subretinal fluid could be an important factor in the gradual deterioration of the detached retina.

On the other hand, lactic dehydrogenase probably arises from the blood and ruptured cells. In cases of recent retinal detachment, only serum lactic dehydrogenase isoenzymes were observed in the subretinal fluid. Retinal lactic dehydrogenase isoenzymes were barely detectable after prolonged detachment when retinal degeneration began.

The retina used in this study is an autopsy sample. However, our unpublished data showed that fresh retina removed from owl monkey or rabbit eyes showed isoenzyme patterns identical to those of human retina. Therefore, the data shown in Figs. 2C, 7C, and 8A can be used to represent that of living tissue.

We have also examined these enzymes in the centrifuged vitreous samples from
normal monkey and rabbit eyes. There was no detectable acid phosphatase activity in the vitreous samples. Esterase and lactic dehydrogenase isoenzymes in the vitreous are identical to that of serum, although the activity in the vitreous is much lower than that of serum. Therefore, the results obtained from animal vitreous samples are not described.

Machemer's histologic examination of experimentally detached retinas of owl monkeys showed proliferation of pigment epithelial cells and invasion of macrophages. It is difficult to obtain human eyes for histologic examination. Our observation of large numbers of pigment epithelial cells and macrophages in subretinal fluid obtained from human subjects indicates that Machemer's findings in experimental animals fit with those in human retinal detachment.

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REFERENCES