A Donor-Age-Dependent Change in the Activity of alpha-Mannosidase in Human Cultured RPE Cells

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Six acidic glycosidase activities in cultured human retinal pigment epithelium (RPE) cells from donors of different ages (19 to 80 years) were studied with regard to pH optimum, $K_m$, $V_{max}$, and specific activity. For alpha-mannosidase we found significant age-dependent decreases in specific activity and $V_{max}$ but not in $K_m$. The other glycosidases and acid phosphatase, lactate dehydrogenase (LDH) and citrate synthase showed no change in these parameters with donor age. The alpha-mannosidase activity of older donor cells could be activated almost 2-fold by the addition of zinc. This is the first report of age-dependent change in a human RPE lysosomal enzyme. Since alpha-mannosidase is probably required for the degradation of rhodopsin in the phagolysosomal system of the RPE, decrease in this enzyme activity may lead to accumulation of undigested rod outer segments (ROS) and drusen, both of which are associated with age-related macular degeneration (AMD). Invest Ophthal Vis Sci 30:2341–2347, 1989

It has become increasingly evident that the normal functioning of the retina depends upon its intimate and highly complex interaction with the retinal pigment epithelium (RPE). It has been estimated that the average RPE cell digests about 7500 disc segments per day. This impressive metabolic feat is punctuated by the fact that the RPE cells have a very low mitotic rate; each cell remains active for decades, in contrast to other phagocytic cells which generally show high mitotic rates and relatively short lives. These facts imply an efficient phagolysosomal system within the RPE cell that is maintained for long periods of time. Therefore, small decreases in degradative enzyme content or efficiency might be expected to result in devastating visual consequences secondary to the accumulation of metabolic waste products.

Several pathologic conditions in humans and animals have been shown to be associated with abnormalities in RPE lysosomal enzymes. It has been suggested that lysosomal enzymes may be the primary abnormalities in some retinal pathologies. However, there have been few investigations of possible lysosomal enzyme abnormalities in some of the more common retinal and macular degenerations or possible alterations of enzyme activities with increasing age in ocular tissues. Indeed, there are relatively few studies concerning lysosomal enzymes in the normal human RPE cell.

This report summarizes our studies of the activities, kinetic properties and pH optima of selected enzymes in normal cultured human RPE cells established from patients of various ages.

Materials and Methods

Cell Culturing

Retinal pigment epithelial cell cultures were established from postmortem human eyes obtained from the Georgia Lions Eye Bank. The eyes were received in the Atlanta laboratory within 24 hr of the donor's death. The globes were dipped in Prepodyne solution for 5 min and then allowed to air-dry. An incision was made through the sclera approximately 1 mm posterior to the ora serrata for 360° around the eye. The vitreous was then aspirated and the retina separated from the RPE and cut at the optic nerve head. The eye cups were then washed with Ca$^{++}$ and Mg$^{++}$ free balanced salt solution and treated with 0.25% trypsin for 1 hr at 37°C. The trypsin solution was then aspirated and replaced with minimal essential medium with 20% fetal bovine serum (Gibco, Grand Island, NY). The RPE cells were released by gentle pipeting and transferred to a Primaria tissue culture flask containing culture medium. The flasks were in-

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cubated at 37°C and under 5% CO₂. The medium was changed every 4 to 5 days thereafter. When the cells reached confluence, they were sent overnight at ambient temperatures to the biochemistry laboratory in Cleveland.

Cell Harvesting

Cell flasks were checked for media clarity and cell confluence prior to cell harvest. Decanted medium was routinely cultured onto blood agar plates at the time of cell harvest. The cell layer was washed in several volumes of Dulbecco’s Phosphate Buffered Saline solution (Gibco) to remove remaining medium, and then cells were removed with 5 cc calcium and magnesium free trypsin-EDTA (Gibco) and by gentle shaking for 3 min. Cells from three to six flasks were collected and centrifuged at 900 g for 10 min at 4°C. The cell pellet was washed three times by resuspension in 15 cc ice-cold SVE (0.25 M sucrose, 1 mM EDTA, 1 mM ethanol; pH 7.4), followed by centrifugation at 900 g. The final centrifugation was in a preweighed tube so that pellet weight could be determined. The pellet was then suspended in 3–6 cc (dependent on pellet weight) SVE and homogenized by two passes through a loosely fitting Teflon pestle rotating at 1500 RPM. The homogenate was centrifuged, the supernatant collected and the pellet resuspended as above in an additional 2 cc of SVE. The supernatants were then combined. The combined supernatants were frozen and thawed several times.

Enzyme Assays

Five millimolar solutions of six 4-methyl-umbelliferyl glycosides (Sigma, St. Louis, MO) were prepared in triple distilled water and used as substrate for their respective glycosidases: α-glucoside, β-glucoside, α-galactoside, β-glucosaminide, β-glucuronide, α-mannoside.

A sample of enzyme (5–20 μl) was added to a mixture of 100 μl of citrate-phosphate buffer (Gomori), and 100 μl of substrate and distilled water in a 7.5 X 1 cm Pyrex test tube to a total volume of 0.3 cc. The pH used for each glycoside was the optimum determined in this study. Acid phosphatase activity was measured using 0.1 mM 4-methyl-umbelliferyl phosphate (Sigma) as substrate and using the method described for the glycosidases. The buffer was 0.1 M sodium acetate (pH 4.6). Dilutions, when required, were made with SVE solution. All the measurements were performed in duplicate or triplicate and under conditions of linearity of substrate conversion with incubation time and protein concentration. Incubations were carried out for 15–30 min at 37°C. The reaction was stopped by adding 1.0 ml of 0.4 M glycine-NaOH buffer at pH 10.3.

Glycosidase and phosphatase activities were quantitated by determining the amount of umbelliferone released by the enzyme. Free umbelliferone was measured using an American Instruments Company (AMINCO, Silver Springs, MD) fluorometer (model J4-7439) with a primary filter of Corning 7-60 (360 nm) and a secondary filter of Wratten 2A (415 nm). Umbelliferone standard curves using crystalline 4-methyl-umbelliferone (Eastman Chemical Co., Rochester, NY) were performed for each experiment over a range of 0.08 to 0.3 M. Nonenzymatic hydrolysis and extraneous fluorescence were monitored by zero-time controls accompanying each incubation. In these controls, the tubes were incubated without enzyme which was then added after the addition of glycine buffer.

Mixing experiments were performed by combining equal volumes of homogenates from young and old donors and assaying the mixture as described above at optimum pH. Results were compared to those expected from summing the values obtained from samples incubated separately.

The effect of zinc on α-mannosidase activity was studied by the addition of either 0.5 mM ZnSO₄ or 5.0 mM ZnSO₄ (final concentration) to the incubation mixture.

Citrate synthase activity was determined using DTNB (Ellman’s Reagent, Sigma) at 37°C in a Gilford (Oberlin, OH) recording spectrophotometer.

Lactate dehydrogenase (LDH) activity was determined by the rate of NADH (Sigma) oxidation at 37°C. Activity is expressed in IU with appropriate correction made for temperature. The assays were performed at pH 7.5.

Protein was quantitated by the method of Lowry using bovine albumin (Sigma) as the standard.

All chemicals used were of reagent grade.

Statistical analysis was performed using simple linear regression and calculation of beta coefficients of the independent variable. Paired t-testing was used to compare results of zinc addition. P values of less than 0.05 were considered significant.

Results

The first 14 of 24 sets of RPE cell cultures received were studied in detail for pH and kinetic data. The average age of the donors was 51 years, and the range was 19 to 80 years.

pH Optima

The pH curves for the six glycosidases are shown in Figure 1. The pH optima lie in the acidic range which is typical of lysosomal enzymes.
Fig. 1. Normalized activity of glycosidases plotted as a function of pH. The curve is the average of the data. Each data point represents the average of two or three measurements. The values were from donor ages 19, 29, 36, 48, 78 and 80.
α-Mannosidase (Fig. 1A) exhibited the most acidic pH optimum of the six glycosidases studied. A single pH optimum was found and there was no apparent variation with age. The value of 4.2 is similar to previous reports for the acidic form of this enzyme in extraocular sources.\textsuperscript{12,13} pH optima for α-mannosidase range from 3.2 in bovine RPE\textsuperscript{1} to 7.3 in human lens.\textsuperscript{14} Our optimum pH is similar to that reported by Hayasaka and Shiono for bovine RPE.\textsuperscript{15}

Three α-mannosidase isoenzymes have been demonstrated in the human liver\textsuperscript{13} and fibroblasts\textsuperscript{12} and at least two distinct forms exist in plasma samples.\textsuperscript{16} Isolates of α-mannosidase display an acidic (pH 4.0–4.5), a neutral (pH 6.5–7.0) and an intermediate form (pH 5.4–5.7).\textsuperscript{12,13,16} The acidic form is believed to be the lysosomal form and has been demonstrated to be deficient in mannosidosis.\textsuperscript{12,13} Our findings of a single pH optima of 4.2 suggest that we are measuring predominately a single lysosomal enzyme.

β-Glucuronidase (Fig. 1B) showed a pH optimum of 5.3. Lentrichia et al report an optimum of 3.5 for this enzyme in bovine RPE; however, an acetate buffer system was employed. Data presented by these same authors indicate that in a citrate buffer system, an optimum of approximately 4.3 is found, which is consistent with the presence of isoenzymes.\textsuperscript{5}

The pH curve for α-glucosidase (Fig. 1C) demonstrates apparent optima of 4.5 and 7.5. This is probable evidence for at least two isoenzymes. Acidic lysosomal and neutral microsomal forms of this enzyme have been previously reported.\textsuperscript{17,18}

β-Glucosidase (Fig. 1D) and α-galactosidase (Fig. 1E) each demonstrate a single optimum pH and are similar to values reported for bovine RPE.\textsuperscript{5}

A pH optimum of 5.3 was found for β-N-acetylglucosaminidase (Fig. 1F). Values of 4.2,\textsuperscript{5} 4.5,\textsuperscript{19} and 4.9\textsuperscript{13} all have been reported for bovine RPE.

**Table 1.** Specific activities and $V_{\text{max}}$ of enzyme activities in human cultured RPE cells of different ages (19 to 80 years); values are mean ± SD of 14 measurements

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity ± SD (µmol/mg protein/hr)</th>
<th>$V_{\text{max}}$ ± SD (µmol/mg protein/hr)</th>
<th>pH optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Mannosidase</td>
<td>0.106 ± 0.071</td>
<td>0.214 ± 0.122</td>
<td>4.2</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>0.059 ± 0.036</td>
<td>0.100 ± 0.062</td>
<td>5.3</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>0.039 ± 0.017</td>
<td>0.130 ± 0.116</td>
<td>4.5, 7.5</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>0.038 ± 0.021</td>
<td>0.152 ± 0.196</td>
<td>4.8</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>0.032 ± 0.019</td>
<td>0.058 ± 0.030</td>
<td>4.4</td>
</tr>
<tr>
<td>β-NAGase</td>
<td>2.408 ± 0.928</td>
<td>3.441 ± 2.043</td>
<td>5.3</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>0.149 ± 0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>46.32 ± 18.83</td>
<td></td>
<td></td>
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<tr>
<td>LDH</td>
<td>50.35 ± 18.89</td>
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</table>

**Kinetic Studies**

Specific activities and $V_{\text{max}}$ values are listed in Table 1 for each of the six glycosidases. The apparent relative order of activities ($V_{\text{max}}$) of cultured RPE cells is: β-N-acetyl-glucosaminidase > α-mannosidase > β-glucosidase = α-glucosidase > β-glucuronidase > α-galactosidase.

Specific activity and $V_{\text{max}}$ of α-mannosidase are plotted against patient age (Fig. 2). α-Mannosidase shows a donor age-dependent decrease in specific activity and $V_{\text{max}}$, both of which are statistically significant ($P = 0.02$ and 0.009, respectively). Our average $K_m$ value of 1.16 mM is comparable to that of Hirani et al\textsuperscript{16} who found a $K_m$ of 0.86 mM in the acidic form of plasma α-mannosidase. No change of $K_m$ with donor age was observed for α-mannosidase.

No significant age related change of specific activity, $V_{\text{max}}$, or $K_m$ was seen with the other glycosidases studied, LDH, citrate synthase or acid phosphatase.

The results of the mixing experiment using supernatants of different age donors are shown in Figure 3; no evidence for an inhibitor was found.

To determine the effect of zinc on α-mannosidase activity, two groups of were studied. The first group contained supernatants of young donors (less than 35 years) and the second contained supernatants of old donors (greater than 65 years). The results are shown in Figure 4. Young and old samples showed significant increases of activity over control values for both 0.5 mM and 5.0 mM ZnSO$_4$.

**Discussion**

This study has demonstrated that cultured human RPE cells show a decrease in the specific activity and velocity of α-mannosidase with increased donor age. This age-dependent change was not found in other
glycosidases studied, LDH, citrate synthase, acid phosphatase or other glycosidases studied.

Clinical and histopathologic changes in age-related macular degeneration (AMD) include pigmentary disturbances, drusen, thickening and degenerative changes of Bruch’s membrane and laminar deposits between Bruch’s membrane and the RPE. These findings are most prominent in the macular area. These same changes, albeit to a lesser degree, are seen as part of the normal aging process of the retina, particularly the macula. The above-mentioned observations have led to the speculation that AMD represents an advanced or accelerated stage of a degenerative process common to all eyes. However, it is has been suggested that a primary abnormality of RPE metabolism may exist and lead to secondary choroid and neurosensory retina changes. It is unknown to what extent abnormalities in metabolism of the RPE results in the clinical findings of AMD.

It is also unknown whether the metabolic abnormality of the RPE is anabolic or catabolic in nature. However, experimental evidence suggests that accumulation of residual material (lipofuscin) secondary to incomplete degradation of phagocytosed membranous discs shed by the adjacent rods and cones may be the primary abnormality in AMD. Lipofuscin is found in greatest quantity overlying areas of abnormal Bruch’s membrane and these granules become more abundant with increasing age. This cellular refuse could accumulate from an interruption in any number of steps required to break down phagocytosed membranous discs, including abnormal lysosomal enzymes which are intimately associated with the degradation of the discs.

To our knowledge, no studies have demonstrated a deficient enzyme in the RPE cell that explains the accumulation of residual material. Previous studies suggest that there is an alteration of substrate (ie, the metabolic waste precursors) that impairs enzyme function, and Young suggests that it is “unlikely” that malformed degradative enzymes are responsible for the accumulation of residual material.

Studies of bovine rhodopsin have demonstrated that mannose and N-acetyl-glucosamine are the major monomers of the oligosaccharide side chains of rhodopsin. The finding of an age-dependent alteration in α-mannosidase activity in RPE cells suggests a mechanism by which disc outer segments may be incompletely degraded, thus leading to accumulation of residual material and impaired visual function.

At least four explanations may be offered for the age-dependent decrease in the activity of α-mannosi-
dase: the presence of an inhibitor in the cells established from older donors, a decrease in the amount of α-mannosidase produced by the older donor cells, a decrease in the efficiency of the enzyme produced by the cells of the older donors, or cell culture artifact.

The presence of an inhibitor of α-mannosidase is unlikely since our mixing experiments failed to show inhibition by the older homogenates. However, these experiments do not rule out the possibility of an inhibitor firmly bound to the α-mannosidase macromolecules of older donor cell cultures.

With other lysosomal enzymes failing to show a similar decrease in activity with increasing donor age, nonspecific extracellular release of lysosomal enzymes seems an unlikely explanation for the decrease in α-mannosidase. Assay of the culture media revealed minimal α-mannosidase levels in young and old cultures (data not shown), so that selective release of α-mannosidase into the medium has not occurred, although such selective release has been described in cultured human RPE cells.37

Age-related alteration of enzyme activities in animal tissues is well documented (see refs. 38–43 for reviews). The two theories proposed to explain this alteration include the production of faulty enzymes due to genetic mutations (Orgel’s Theory39) and post-translational modification of enzymes.40 A pre- or post-translational modification of α-mannosidase resulting in a less efficient enzyme would be expected to result in a change in the $K_m$ value. No age-dependent change in $K_m$ was found in this study; however, additional studies are proceeding to purify α-mannosidase.

We do not believe that our findings are an artifact of cell culture technique since all enzymes studied, with the exception of α-mannosidase, showed no change with in vivo age. We are supported in our contention by Wilcox,44 who in a study of human cultured RPE cells, found no evidence of culture artifact in the age-related accumulation of extracellular cathepsin D.

With regard to α-mannosidase in humans, decreased activities with increasing age have been noted in serum45 and granulocytes,40 while no change in activity is found in brain.45 We know of no quantitative studies that document age changes of α-mannosidase in human ocular tissues. The activity of the acidic forms of human α-mannosidase in extraocular tissues has been shown to be activated up to 190% by elemental zinc.13,16 We have demonstrated a similar activation in cultured human RPE cells in both young and old donors. Zinc serves as a cofactor in a wide variety of enzyme systems, including a number found in ocular tissues,46 and recent clinical studies suggest that dietary zinc supplementation may retard the progression of visual loss in AMD.47 The current study is consistent with the postulate that a defective enzyme (or enzyme system) responsible for age-related degenerative changes of the macula can be augmented by systemic zinc administration.

Difficulties associated with the study of human RPE cells include the timely acquisition of donor material, adequate quantities of donor material and contamination of the desired cells with other tissues and cell types. Therefore, RPE cell culture provides a desirable means to study this cell type. Limitations of cell culture studies include the artificial environment of the cells (specifically the lack of choroidal and ROS/retinal influence and the synthetic milieu of the culture medium), variable phenotypic expression of cells in culture48 and the heterogeneous population of donor cells and lipofuscin (eg, macular, peripheral).34 In this regard, the study of RPE cells from recently enucleated eyes may establish whether the findings of this study may be extrapolated to the native situation, whether the finding is regional and whether the changes in α-mannosidase may correlate to the clinically observed senile changes. Additionally, the use of an in vitro enzyme assay may not accurately represent the conditions of in vivo metabolism.

Despite the above-noted limitations, this study is the first to demonstrate, in human ocular cells, an abnormality of a specific enzyme system that may explain clinically and pathologically observed age-related degeneration of the retina. Clearly, additional studies are warranted.

Key words: aging, α-mannosidase, cell culture, glycosidases, retinal pigment epithelium, zinc

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References