Characterization of Lysosomal Enzymes from Cultured Cynomolgus Monkey Trabecular Meshwork Cells

Shyun Jeng, Robert N. Weinreb, and Arnold L. Miller*

The current study characterizes selected properties of lysosomal enzymes associated with cynomolgus monkey trabecular meshwork (MTM) cells. These proteins may participate in the turnover of macromolecules involved in regulating the aqueous outflow. Intracellular levels of lysosomal enzymes in MTM cells were similar to those found in cultured human fibroblasts. The presence of ammonium chloride increased the secretion rate of certain lysosomal enzymes from 47 to 122% of normal. Column chromatography of the secreted enzymes on the galactose-specific lectin Ricinus communis I demonstrated an increase in the number of accessible galactose residues on lysosomal enzymes secreted in the presence of ammonium chloride. The presence of mannose-6-phosphate receptors on the trabecular meshwork cells was demonstrated by the specific uptake of purified 125I-β-D-glucosidase. This uptake represented 20% of that observed with cultured human fibroblasts and was inhibited only 50% by the presence of mannose-6-phosphate.

Interactions between trabecular meshwork cells and surrounding extracellular matrix may play an important role in regulating aqueous outflow. Increasing evidence suggests that trabecular meshwork cells are capable of synthesizing and degrading macromolecular constituents of the extracellular matrix of the trabecular meshwork.1-4 It has been proposed that the increased resistance to aqueous outflow in glaucoma is the result of deposition of these substances, especially glycosaminoglycans, in the trabecular meshwork. Glycosaminoglycans are synthesized by trabecular meshwork cells and are secreted in a soluble form into the extracellular space, where they are polymerized.1-5 Their accumulation can lead to either an impediment of aqueous humor outflow due to the binding of water molecules, or dysfunction of the trabecular meshwork cells. The role of these complex carbohydrates in aqueous outflow was first investigated by Barany,6 who demonstrated that perfusion of the anterior chamber with testicular hyaluronidase reduced intraocular pressure. Subsequent studies suggested that chondroitin AC lyase and other catabolic enzymes including chondroitin ABC lyase reduced resistance to aqueous outflow.7,8 However, little information is available on the properties of lysosomal enzymes and their functional role in the trabecular meshwork and their pattern of secretion from trabecular meshwork cells. Yue et al9 recently reported the presence of the lysosomal enzymes (acid lipase, cholesterol esterase, and acid esterase) in cultured bovine trabecular meshwork cells through the use of histochemical staining and biochemical assays. The current study represents a characterization of cynomolgus monkey trabecular meshwork (MTM) cells with respect to secretion, receptor-mediated endocytosis, and lectin-specific affinity—selected properties known to be important features of lysosomal enzymes in other biologic systems.

Materials and Methods

Culture Conditions

MTM cells were propagated in cell culture in 25-cm² flasks as described previously.10 For these studies, we used confluent fifth-passage cells, which were verified morphologically by light and electron microscopy.10 The MTM cells were maintained in culture with Dulbecco's modified Eagles medium (DMEM) (Irvine Scientific, Irvine, CA) supplemented with 10% (volume/volume) fetal calf serum (Gemini Bioproducts, Calabasas, CA) (heat-inactivated), 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 8% CO₂ and 92% air.

Normal human fibroblasts were cultured under conditions reported previously.11,12 Briefly, the cells

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were grown in 25-cm² flasks according to Coon's modification of Ham's F-12 medium (Irvine Scientific) supplemented with 10% (volume/volume) fetal bovine serum (heat-inactivated), 3% (volume/volume) penicillin-streptomycin (Irvine Scientific), 2% (volume/volume) Fungizone (Squibb, New Brunswick, NJ), and 0.3% (volume/volume) glutamine in a humidified atmosphere of 8% CO₂ and 92% air. Cell viability was monitored by 0.5% trypan blue exclusion. Plates demonstrating high viability of the cells (> 95%) were used in the study.

**Harvesting of Cells and Preparation of Cell Homogenates**

After medium was removed from confluent MTM cells and human fibroblasts, the cells were washed four times with phosphate-buffered saline (pH 7.4). The cells were harvested in 0.9% (weight/volume) NaCl by scraping in phosphate-buffered saline, pH 7.4, and centrifuging for 10 min at 1500 g for 10 min in a RC5B Sorval centrifuge. The cells were resuspended in a minimal volume of glass-distilled water and disrupted at 4°C by sonication with a Kontes microultrasonic cell disruptor (San Leandro, CA) in three 10-sec bursts (setting 8).

**Enzymatic Assays**

With these homogenates, the lysosomal enzymes N-acetyl-β-D-hexosaminidase, β-D-glucosidase, α-D-mannosidase, β-D-glucuronidase, β-D-galactosidase, and α-L-fucosidase were assayed fluorometrically with the corresponding 4-methylumbelliferyl substrates (Koch-Light, Colnbrook Bucks, United Kingdom). Lactate dehydrogenase was assayed using a diagnostic kit from Sigma (St. Louis, MO); protein was determined according to the method of Lowry et al with bovine serum albumin (Sigma) as a standard.

**Secretion of Lysosomal Enzymes in the Presence of Ammonium Chloride**

MTM cells were cultured as described above to near confluency in 25-cm² flasks. The cells were washed twice with phosphate-buffered saline (pH 7.4) followed by the addition of 2 ml DMEM containing 10 mM NH₄Cl without fetal bovine serum and antibiotics. The MTM cells then were incubated for 24, 48, and 72 hr at 37°C. At the appropriate times the medium was collected and centrifuged at 1000 g for 10 min. The cells were harvested and assayed for protein as described above. After the supernatant fluids were assayed for their respective lysosomal enzyme activities and the cytoplasmic lact dehydrogenase as described above, the 72-hr samples were concentrated against polyethylene glycol (Aquadex III; Calbiochem, La Jolla, CA) for 5 hr at 0–4°C prior to column chromatography studies with the galactose-specific lectin R. communis.

**Column Chromatography on R. communis Agglutinin I (RCA₁)**

The secreted concentrated enzymes from MTM cells, collected both in the presence and in the absence of NH₄Cl, were applied separately to a column (1 × 3 cm) of RCA₁ (E-Y Laboratories, San Mateo, CA), bound to agarose that had been equilibrated previously in 15 mM sodium phosphate buffer, pH 6.0, containing human serum albumin (1 mg/ml), 0.02% (weight/volume) sodium azide, and 0.15 M NaCl. After 15 min, the lectin agarose was washed with several column volumes of this buffer until the unadsorbed lysosomal enzyme activities could no longer be detected in the effluent by fluorescent assays (described in the section on enzymatic assays, above). The lectin-bound lysosomal enzymes then were eluted with 20 ml of 100 mM galactose prepared in the equilibration buffer.

Samples of the secreted enzymes were also incubated for 5 hr at 37°C with 0.5 units of Clostridium perfringens neuraminidase type IX (Sigma, St. Louis, MO) (1 unit will liberate 1.0 nmol N-acetylneuraminic acid per minute), prepared in 100 mM sodium acetate buffer at pH 5.0. The incubation mixture also contained 1 mg/ml of human serum albumin and 0.02% (weight/volume) sodium azide. The neuraminidase-treated samples also were subjected to column chromatography on RCA₁ as described above.

**Uptake of 125I-β-D-Glucosidase**

MTM cells were grown to confluency in media in 24-well multi-test dishes. Each well contained 30–40 mg cell protein. Growth medium, including DMEM, 10% (volume/volume) fetal bovine serum (heat-inactivated), and antibiotics, was removed and replaced with 250 μl uptake medium containing minimal essential medium (9.5 g/l) supplemented with 0.02 M 2-[N-morpholino]-ethanesulfonic acid and 1 mg/ml enzyme-free bovine serum albumin (Sigma) (final pH 6.0). The preparation of iodinated-β-D-glucosidase and its uptake was carried out as described previously. Uptake was allowed to proceed as indicated in Figures 4 and 5 for each experiment and the unabsorbed 125I-ligand was removed by aspiration, followed by 6 1-ml washes with phosphate-buffered saline (pH 7.4 at 0–4°C).
Table 1. Intracellular hydrolase activities* of cynomolgus MTM cells and cultured human fibroblasts

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MTM cells†</th>
<th>Fibroblasts‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>N-acetyl-β-D-hexosaminidase</td>
<td>7040</td>
<td>4200–9240</td>
</tr>
<tr>
<td>α-L-fucosidase</td>
<td>1014</td>
<td>840–1200</td>
</tr>
<tr>
<td>β-D-galactosidase</td>
<td>944</td>
<td>714–1066</td>
</tr>
<tr>
<td>α-D-mannosidase</td>
<td>201</td>
<td>108–270</td>
</tr>
<tr>
<td>β-D-glucuronidase</td>
<td>328</td>
<td>175–438</td>
</tr>
</tbody>
</table>

* Expressed as nanomoles per milligram cell protein per hour.
† Results are the average of ten independent experiments with MTM cells. Assays were run in duplicate.
‡ Results are the average of five independent experiments with cultured human fibroblasts. Assays were carried out in duplicate.

After washing, the cells were lysed by adding 200 µl lysis buffer (0.25% [weight/volume] sodium deoxycholate in 15 mM sodium phosphate, pH 6.5) to each well. After 15–30 min at room temperature, 50 µl was removed for protein determination and an additional 100 µl was counted in a gamma counter (TM Analytic, Elk Grove Village, IL). After subtraction of background (40–50 cpm), the uptake was calculated as counts per minute per milligram cell protein. The uptake was carried out in the presence of mannose-6-phosphate, or mannose, or N-acetyl-glucosamine, or L-fucose, or mannose-1-phosphate to identify the receptors involved in the uptake of the labeled β-D-glucosidase.

Results

Intracellular Acid Hydrolase Activities

Intracellular lysosomal enzyme activities of MTM cells were similar to those found in cultured normal human fibroblasts obtained from normal patients. These results are summarized in Table 1. Enzyme activities present in cells grown in the presence of ammonium chloride were not significantly different from those grown in the absence of the amine (data not shown).

Extracellular Release of Acid Hydrolases

N-acetyl-β-D-hexosaminidase, α-L-fucosidase, β-D-galactosidase, α-D-mannosidase, and β-D-glucuronidase were released into the growth medium at rates linear with time (Figs. 1, 2, Table 2). The presence of 10 mM NH₄Cl resulted in an increase in the secretion rate α-D-mannosidase, β-D-glucuronidase and β-D-galactosidase that ranged from 47 to 122%, when compared to the same enzymes from MTM cells grown in the absence of ammonium chloride (Fig. 1). However, the rates of secretion of N-acetyl-β-D-hexosaminidase (Fig. 2) and α-L-fucosidase (data not shown) were not affected by the addition of 10 mM NH₄Cl.
NH₄Cl. This result is in contrast to the secretion pattern seen for N-acetyl-β-D-hexosaminidase from human fibroblasts (Fig. 2B). The lack of significant cell death was ascertained by trypan blue exclusion and by the absence of β-D-glucosidase and lactic acid dehydrogenase activity in the culture medium.

Column Chromatography on RCA₁

Column chromatography of N-acetyl-β-D-hexosaminidase on RCA₁ demonstrated that the amount of enzyme adsorbed to the lectin column increased slightly in the presence of NH₄Cl (data not shown). However, β-D-glucuronidase secreted in the presence of ammonium chloride demonstrated a significant increase in affinity for RCA₁ compared to control samples (Fig. 3). After treatment with neuraminidase, a 2-fold increase in the adsorption of β-D-glucuronidase to the galactose specific lectin was obtained (Fig. 3). There was a corresponding decrease in the quantity of unadsorbed enzyme activity, indicating that incubations with neuraminidase had removed sialic acid from the enzyme.

Table 2. Effect of 10 mM NH₄Cl on enzyme secretion* from cynomolgus MTM cells

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>α-D-mannosidase</th>
<th>β-D-glucuronidase</th>
<th>β-D-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>16</td>
<td>65</td>
<td>53</td>
</tr>
<tr>
<td>48</td>
<td>17</td>
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<tr>
<td>72</td>
<td>47</td>
<td>84</td>
<td>122</td>
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</tbody>
</table>

* Expressed as percent increase over control.

Data represent average of five independent experiments from MTM cells. Each experiment was carried out in duplicate.

10 mM NH₄Cl did not affect the secretion rate of N-acetyl-β-D-hexosaminidase and α-L-fucosidase (data not shown).
Fig. 4. Time course of uptake of $^{125}$I-$\beta$-D-glucosidase. Cultured cynomolgus MTM cells were incubated with 4 units/ml $\beta$-D-glucosidase for various times at 37°C in the absence (open circles) or the presence (filled circles) of 4 mM mannose-6-phosphate.

The uptake of $^{125}$I-$\beta$-D-glucosidase was linear for at least 2 hr (Fig. 4). The uptake was saturable and only partially inhibited by 2 mM (Fig. 5) or 4 mM (data not shown) mannose-6-phosphate. Double reciprocal plots of the inhibition of $\beta$-D-glucosidase by mannose-6-phosphate (Fig. 6) show competitive inhibition with an apparent $K_i$ value of $2.2 \times 10^{-4}$ M. Other sugar or sugar phosphates, including mannose, N-acetyl-glucosamine, fucose, mannose-1-phosphate, and glucose, were found to have no effect on the uptake of the $^{125}$I $\beta$-D-glucosidase by MTM cells.

### Discussion

Basic amines are known to enhance the secretion of newly synthesized lysosomal enzymes from a variety of cell types.\(^{17-21}\) It is believed that these compounds are taken up by lysosomes, altering the binding between the mannose-6-phosphate receptor and lysosomal enzymes. As a result, newly synthesized lysosomal enzymes are secreted into the extracellular space rather than routed to the lysosome. The current studies demonstrate that at least five acid hydrolases were normally released into the extracellular medium over a period of time from cultured MTM cells. The secretion of $\beta$-D-glucuronidase, $\beta$-D-galactosidase, and $\alpha$-D-mannosidase was enhanced by the presence of 10 mM NH$_4$Cl in the culture medium. In contrast to these results, the secretion of N-acetyl-$\beta$-D-hexosaminidase and $\alpha$-L-fucosidase was unaffected by the presence of the ammonium chloride. Prior studies with cultured human fibroblasts and macrophages demonstrated significant increases of the secretion of lysosomal enzymes, including N-acetyl $\beta$-D-hexosaminidase, as the result of exposure to ammonium chloride or other amines.\(^{19-21}\) This suggests that in MTM cells the presence of ammonium chloride may exert selective effects on the secretion of different lysosomal enzymes. The significance of this result is not known at this time.

RCA$_1$ column chromatography of secreted N-acetyl-$\beta$-D-hexosaminidase and $\beta$-D-glucuronidase from MTM cells revealed that in the presence of NH$_4$Cl there was an increase of galactose residues associated with lysosomal enzymes. This increase suggests that the presence of the amine favored the transport of the residues through the trans part of the Golgi apparatus, where they would be exposed to terminal sugar transferases, including galactosyl transferase. This is consistent with previous results obtained with these enzymes from other cell systems.\(^{19,22,23}\)
Lysosomal enzyme receptors on MTM cells were characterized by using exogenous iodinated-β-D-glucosidase from Dictyostelium discoideum. This enzyme has been shown to exhibit receptor-mediated endocytosis by human fibroblasts.15,16 Our studies revealed that the uptake rates of iodinated-β-D-glucosidase by MTM cells were only 20% of that of human fibroblasts. This endocytosis was inhibited 50% by the presence of up to 10 mM mannose-6-phosphate (data not shown). Mannose-6-phosphate receptors have been demonstrated to play an important role in the intracellular transport and uptake of acid hydrolases in several cell types,24 including rat retinal pigment epithelial cells.25,26 However, in MTM cells, they appear to be only partially responsible for the uptake of lysosomal enzymes. Similar experiments carried out in the presence of other competing sugars revealed that the remaining uptake does not involve interactions with receptors that recognize mannose, N-acetylglucosamine, glucose, or L-fucose residues on the lysosomal enzymes (data not shown). These results with MTM are quite different from the reported interactions between lysosomal enzymes and their receptors on human fibroblasts11,24 and mouse macrophages,27,28 where mannose-6-phosphate and mannose or-n-acetylgalcosamine play a significant role in the recognition and transport of lysosomal enzymes.

We have characterized selective properties of lysosomal enzymes from MTM cells. The results indicate many similarities and some differences to properties of lysosomal enzymes isolated from cultured human fibroblasts and macrophages. These studies represent the first step in establishing a functional role for the enzymes in the metabolic operation of the trabecular meshwork system.

**Key words:** lysosomal enzymes, trabecular meshwork cells, endocytosis, lectin, glaucoma

**References**