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Mannose 6-Phosphate Receptors on the Plasma Membrane on Rat Retinal Pigment Epithelial Cells

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The retinal pigment epithelium (RPE) phagocytizes the tips of photoreceptor outer segments (OS) during normal eye function. It is not known what ligand on OS is recognized by the RPE for removal from the interphotoreceptor matrix. It is possible that a sugar residue on a cell surface glycoconjugate of either the OS or RPE mediates the phagocytic interaction. Pinocytic experiments with a soluble mannose 6-phosphate ligand (125I-labelled mannosidase) showed that similar quantities of ligand were bound by RPE explants from Long Evans rat retinas and from Royal College of Surgeons (RCS/p') rat retinas known to be defective in the phagocytosis of OS. The addition of mannose 6-phosphate reduced the total counts of bound alpha-mannosidase by 23% in both normal and dystrophic RPE explants. Mannose 6-phosphate receptors were visualized on normal and dystrophic RPE plasma membranes by immunocytochemical techniques. Further, phagocytosis was studied using phosphomannan-coated beads as phagocytic particles. Dystrophic RPE phagocytized phosphomannan-coated beads by a mannose 6-phosphate specific mechanism as shown by a significant reduction in the number of these coated beads taken up in the presence of the competing sugar. In contrast, normal RPE showed no uptake of phosphomannan-coated beads. Our findings indicate that a mannose 6-phosphate receptor is on the apical plasma membrane of rat RPE. This receptor may not be involved in normal OS phagocytic recognition, but may function in the trafficking of lysosomal enzymes by RPE cells. Invest Ophthalmol Vis Sci 29:291-297, 1988

The retinal pigment epithelium (RPE) is a highly specialized phagocytic cell which internalizes and digests rod outer segment tips (OS) shed during the maintenance of photoreceptor cells. Recognition sites are presumed to be present on the OS surface. O'Brien suggested that changes in glycosylation of outer segment membranes trigger OS phagocytosis. Several groups have presented evidence to suggest that glycoconjugates may be involved in OS recognition by the RPE. When OS were bound to the lectins, wheat germ agglutinin or Ricinus communis agglutinin, phagocytosis by RPE cells was decreased by 25-40% in contrast to a slight decrease when these same lectins were bound to the RPE cells. Similarly, concanavalin A (Con A) bound to OS phagocytosis by 50% while RPE-bound Con A had little effect. Sugars have also been shown to be inhibitory in the uptake of OS by RPE. The addition of galactose to the OS membrane by enzymatic galactosylation of rhodopsin did not enhance OS binding to RPE cells. Mannose and mannan, but not galactose, inhibited phagocytosis of OS when added to RPE explant cultures. Furthermore, when the sugars N-acetyl glucosamine, L-fucose, D-mannose, or methyl-alpha-D-mannoside were added to rat RPE explants, OS uptake was significantly inhibited while D-glucose, D-fucose, or D-galactose had little effect. Despite these numerous studies, it is still not clear which, if any, sugar interaction between RPE and OS mediates phagocytosis of OS.

We have been studying the role of RPE carbohydrate receptors in mediating the uptake of latex beads which we are using as phagocytic particles. We have compared phagocytic recognition of various ligands on these beads by RPE explants from normal retinas and RPE explants from retinas with a known phagocytic defect. In the present study, we have demonstrated the presence of a phosphomannosyl receptor on the RPE cell surface by the binding of a soluble ligand and by immunocytochemical localization, and have studied the role of this receptor in the
phagocytic uptake of particulate mannose 6-phosphate.

**Materials and Methods**

**Animals**

Rats used in this study were 10–13 day postnatal Long Evans rats (normal retina) and Royal College of Surgeons (RCS/p+) rats (dystrophic retina model). Eight to 12 animals were used for each experimental condition. The rats were maintained on a 12 hr light:12 hr dark cycle with light intensities not exceeding 10 foot-candles (ft-cd) in the cages. The treatment of experimental animals in this study was in compliance with the ARVO Resolution on the Use of Animals in Research.

**Eye Dissection**

Explants were prepared as previously described. Briefly, eyes were enucleated during the end of the light cycle, soaked in RPMI 1640 with Hepes at 4°C during the dark cycle. At the beginning of the light cycle, the anterior eye was cut at the ora serrata. The cornea and lens were discarded. The eye was quartered and the neural retina allowed to detach.

**Bead Coating**

Bead coating followed the procedures of Seyfried-Williams and McLaughlin and Seyfried-Williams et al. Briefly, polybead-carboxylated monodispersed microspheres (1.0 μm diameter, 4.55 x 10^10 beads/ml) (Polysciences, Warrington, PA) were reacted with N-ethyl-5-phenyl-isoxozolium 3' sulfonate (Woodward's reagent) (Sigma, St. Louis, MO) at 22°C for 30 min, then rinsed with PBS. The activated beads were coupled with the large molecular weight fragment of phosphomannan prepared by acid hydrolysis of native Hansenula holstii phosphomannan.

**Pinocytic Experiments**

Alpha-mannosidase from Dictyostelium discoideum was iodinated by the chloramine T method as described by Shepherd et al. Five to seven explants were added per well of a 24 well tissue culture dish. 125I-labelled alpha-mannosidase was added in a total volume of 400 μl of Hanks’ balanced salt solution containing 1% BSA in the presence or absence of 10 mM mannose 6-phosphate. Explants were incubated at 37°C for 2 hr, washed, and bound 125I-alpha-mannosidase was counted. Specific activity of the ligand for experiments with dystrophic RPE explants was 15,000 cpm/ng; for normal explants, 18,000 cpm/ng. Assays were done in triplicate, and results presented are representative of three separate experiments.

**Immunocytochemistry**

RPE explants from normal and dystrophic rats were prepared as described above. The explants were fixed for 4 hr in 2% paraformaldehyde, 0.075 M lysine, 0.01 M periodate in 0.037 M phosphate buffer. The tissue was rinsed and nonspecific binding blocked by incubation in PBS containing 5% normal goat serum (Miles, Naperville, IL). Either rabbit antihistidine liver mannose 6-phosphate receptor or normal rabbit serum was added to the explants at concentrations of 1:10 in PBS/5% normal goat serum/1% BSA and incubated at 4°C overnight. The tissue was washed in Tris-HCl (pH 8.2)/normal goat serum/BSA, and goat anti-rabbit IgG conjugated to 15 nm gold (Janssen, Piscataway, NJ) was added overnight at 4°C. The tissue was rinsed, fixed for 1 hr in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3). The tissue was washed three times in 0.1 M cacodylate buffer, osmicated for 1 hr, uranyl acetate en bloc stained for 1 hr, dehydrated in ethanol, and embedded in Epon/Araldite.

**Phagocytic Experiments**

Explants, consisting of sclera, choroid, and RPE, were incubated for 1 hr in RPMI 1640 medium containing glutamine (Gibco, Grand Island, NY) at 37°C in a 5% CO2 atmosphere. Fresh media was added and phosphomannan-coated beads (50 μl) in the same media were added four times at 30 min intervals at a concentration of 4.55 x 10^10 beads/ml. Specificity of uptake was demonstrated by the addition of 100 mM mannose 6-phosphate (Sigma) together with phosphomannan beads. Uncoated beads and mannose 6-phosphate (100 mM) were added to the medium of RCS/p+ explant cultures as a positive control for phagocytosis. The explants were incubated with the beads for 5 hr and were then washed and fixed for electron microscopy in 2% glutaraldehyde, 2% paraformaldehyde, 0.5% acrolein in a 0.1 M cacodylate buffer (pH 7.3). The tissue was further processed for electron microscopy as described in the methods for immunocytochemistry.

**Statistical Analysis**

The number of beads phagocytized by normal and dystrophic RPE explants was analyzed by electron microscopy. Blocks were randomly selected and sectioned so that beads were counted in an average of 24 cell profiles per animal. The number of cell profiles which contained beads in their cytoplasm was quantitated and expressed as a percent of the total cell profiles counted. Morphological criteria for inclusion of RPE explants in the quantitative analysis were: RPE cells were present as monolayers lying on...
Bruch's membrane, cell junctions between RPE lateral plasma membranes were intact, and beads, but few OS, were seen near the microvilli. The Mann-Whitney U Test was used to compare uptake in the two strains of rats for each bead coating.

**Results**

**Pinocytic Experiments**

Dystrophic RPE explants bound a total of 14.8 ng of ligand added. The addition of 10 mM mannose 6-phosphate blocked binding by approximately 23%, resulting in 3.4 ng recognized by a mannose 6-phosphate specific process. Similarly, normal rat RPE explants bound almost identical amounts of ligand: total binding was 14.2 ng with 3.2 ng specific for mannose 6-phosphate recognition. Background levels (nonspecific binding) were consistently high in all experiments most likely due to the use of tissue explants.

**Immunocytochemistry**

Using en bloc immunogold labeling of RPE cells in explants, anti-mannose 6-phosphate receptor reactiv-
Figs. 4, 5. Electron micrographs of RPE explants incubated with phosphomannan-coated beads. Fig. 4. Dystrophic RPE avidly phagocytyzed phosphomannan-coated beads as shown by the presence of numerous beads (arrowheads) in the cytoplasm. Beads are also seen in the external milieu near the microvilli (MV) (arrows). PG, pigment granules (x10,200). Fig. 5. Normal RPE did not engulf phosphomannan-coated beads. Beads (arrowheads) are observed in close apposition to the microvilli (MV) (arrows) but are not inside the cell (x10,710).

ity was observed at the apical plasma membrane of both dystrophic and normal RPE (Figs. 1–3). Gold labelling was seen in coated pits structures and on proximal regions of the microvilli (Fig. 1). No labelling was noted on the plasma membranes of OS (Fig. 1) nor on the sclera or exposed edges of choroidal cells (not shown). Gold particles were most often associated with regions of the plasma membrane resembling coated pits in both dystrophic and normal RPE (Figs. 2, 3). RPE explants incubated with control rabbit sera were negative (not shown).

Phagocytic Experiments

Transmission electron microscopy demonstrated that phosphomannan-coated beads were avidly phagocytyzed by dystrophic RPE explants (Fig. 4). Numerous beads were observed in the cytoplasm and between microvillous membranes. Normal RPE did not phagocytyze the phosphomannan-coated beads as beads were observed in close proximity with microvilli, but were not observed in the cytoplasm (Fig. 5). Specificity of uptake of phosphomannan-coated beads by dystrophic RPE was shown by a significant reduction in the numbers of beads phagocytyzed with the addition of 100 mM mannose 6-phosphate in the explant culture medium (Fig. 6). In the presence of the competing sugar, beads are only seen in an extracellular location. The addition of 100 mM mannose 6-phosphate did not change the ability of the RPE cells to ingest beads with an irrelevant coating (uncoated) as uncoated beads were taken up in similar numbers by dystrophic RPE in the presence (Fig. 7) or absence (not shown) of the competing sugar. The results of bead uptake are summarized in Table 1. Beads were incubated with the RPE explants for 4 hr in RPMI 1640 containing glutamine at 37°C in a 5% CO2 atmosphere. Beads were counted in three or four animals with an average of 21 cell profiles scored per animal. Each experiment was performed two times. Dystrophic RPE phagocytyzed 6.9 (SEM 2.4) phosphomannan-coated beads/cell profile (P < 0.01) as compared to normal RPE which did not phagocytyze any phosphomannan-coated beads. The dystrophic RPE cell profiles which contained beads represented 48.6% of the total number of cell profiles counted. Accordingly, 51.4% of the dystrophic RPE cell profiles counted contained no beads.

Discussion

The phosphomannosyl receptor has been well characterized on many cell types such as macrophages,19,21 fibroblasts,22–24 CHO cells,25,26 hepatocytes,27 lymphocytes,28–30 and natural killer cells31 and has been shown to function primarily in the translocation of newly synthesized lysosomal enzymes from Golgi to lysosomes. Approximately 80%
Figs. 6, 7. Specificity of phosphomannan-coated bead uptake was shown by the addition of mannose 6-phosphate (100 mM) to the incubation medium of dystrophic RPE explants. Fig. 6. Phagocytosis of phosphomannan-coated beads is inhibited in the presence of the competitor. Beads (arrowheads) are seen outside the RPE cells. MV (arrows), microvilli; PG, pigment granules (×10,700). Fig. 7. Uncoated beads (arrowheads) are engulfed in the presence of mannose 6-phosphate demonstrating that the RPE cells are phagocytically competent. MV (arrows), microvilli; PG, pigment granules (×10,700).

of the receptor is inside the cell, with 20% located on the plasma membrane. This plasma membrane component, at least in fibroblasts, may be a normal consequence of the pathway of membrane recycling, and may represent movement of intracellular receptor transiently to the outside.

Several recent reports have suggested the existence of phosphomannosyl receptors on RPE. Wilcox reported that the addition of 20 mM mannose 6-phosphate to the medium of cultured human RPE enhanced release of several lysosomal enzymes. Kean et al have demonstrated the binding of mannose 6-phosphate-BSA to cultured chick embryo RPE cells and Shirakawa and Kean have shown that phosphomannan and mannose 6-phosphate inhibit the binding of rhodopsin-liposomes to RPE cells from cultured chick embryos.

Our study presents, for the first time, direct evidence that RPE cells express a phosphomannan receptor on their surface. Normal and dystrophic rat RPE cells bind similar levels of soluble ligand, suggesting that pinocytic mannose 6-phosphate receptors are present in both animals. These pinocytosis data are confirmed by the immunocytochemical results which show the presence of mannose 6-phosphate receptors on dystrophic and normal RPE. The receptor is localized on the apical surface of the RPE cells in regions resembling coated pits which is similar to the localization of surface mannose 6-phosphate receptors on CHO fibroblasts and hepatocytes.

The pinocytic mannose 6-phosphate receptor on the surface of RPE cells may have a function similar to the mannose 6-phosphate receptor reported on the surface of fibroblasts. In these cells, 80–90% of the receptor is thought to function within the Golgi system by compartmentalizing lysosomal enzymes (bearing mannose 6-phosphate sugars), through the endoplasmic reticulum, peripheral Golgi elements (GERL) and into lysosomes. In some way, a small

Table 1. Number of phosphomannan-coated beads phagocytized by dystrophic* and normal† RPE explants

<table>
<thead>
<tr>
<th>RPE</th>
<th>Competing sugar</th>
<th>Beads/cell profile</th>
</tr>
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<tbody>
<tr>
<td>Dystrophic</td>
<td>None</td>
<td>6.9 (2.4)</td>
</tr>
<tr>
<td>Dystrophic</td>
<td>Mannose 6-phosphate</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

* RCS/p rat retinas.† Long-Evans rat retinas.

Beads were incubated with RPE explants for 4 hr in RPMI 1640 + glutamine medium at 37°C in a 5% CO2 atmosphere. Beads were counted in three or four animals with an average of 21 cell profiles scored per animal from two separate experiments. All values are expressed as the mean ± SE. The number of cell profiles containing beads is expressed as a percentage as compared to the total number of cell profiles counted; dystrophic RPE, 48.7% (8.7).
percentage of receptor reaches the plasma membrane, clusters in coated pit regions, and is internalized through the coated pit-receptosome-Golgi pathway.\textsuperscript{25}

Alternatively the receptor on RPE cells may more closely resemble the receptor on macrophages, which may have additional roles. It has been shown that macrophage cell lines and freshly isolated macrophages secrete phosphorylated enzymes.\textsuperscript{36} In cultures where high levels of enzymes are present extracellularly, phosphomannan receptor levels are low. When extracellular enzymes are low, receptor levels are high. Thus the macrophage and the RPE phosphomannan receptor may be involved in packaging newly synthesized enzymes within lysosomes as well as “recapturing” secreted phosphorylated enzymes for delivery to lysosomes.

The role of mannose phosphate receptors in cell-particle recognition has been well documented by Rosen and his coworkers who have shown that the mannose phosphate receptor on lymphocytes specifically recognizes a mannose 6-phosphate determinant on endothelial cells of post-capillary venules in the course of lymphocyte recirculation through lymphoid organs.\textsuperscript{28} Lymphocytes, but not thymocytes, also bind specifically to beads which had been conjugated to phosphomannan.\textsuperscript{29}

Our pinocytosis data suggest that normal and dystrophic RPE both express cell surface mannose phosphate receptors. However, when RPE explants are exposed to phosphomannan coated beads, only dystrophic RPE phagocytize the beads in a mannose phosphate dependent manner. There are several possible explanations. It has been shown that dystrophic RPE is not deficient in phagocytosis, but in the specific recognition of OS and subsequent internalization. The phagocytic mechanism is intact as evidenced by successful ingestion of carbon particles,\textsuperscript{12} latex beads\textsuperscript{17} and beads coated with Con A, \textit{Ulex europaeus} agglutinin, \textit{Lens culinaris} agglutinin,\textsuperscript{8} succinylated wheat germ agglutinin and N-acetyl glucosamine.\textsuperscript{9} Although both normal and dystrophic RPE express pinocytic mannose phosphate receptors, these may only recognize and internalize soluble ligands. A different receptor or recognition event may be involved in phosphomannan bead uptake, a recognition that is unrelated to (1) the pinocytic receptor and (2) the OS-recognition defect in dystrophic RPE.

The presence of mannose 6-phosphate receptors on RPE explants has been demonstrated in this study, but no connection of that receptor to the recognition of OS in vivo has been established. We are currently pursuing the study of other carbohydrate-dependent cell-particle and cell-protein recognition systems by the RPE.

**Key words:** mannose 6-phosphate receptor, phagocytosis, pinocytosis, retinal pigment epithelium, RCS\textsuperscript{p+} rats

**Acknowledgments**

The authors are grateful to Dr. Leonard H. Rome, UCLA, Los Angeles, California for the gift of the rabbit anti-bovine liver mannose 6-phosphate receptor antibody and to Dr. Morey Slodki, Northern Regional Research Center, USDA, Peoria, Illinois for supplying the phosphomannan used in these experiments. The authors appreciate the excellent technical assistance of Lou Boykins and Marinetta Cooper.

**References**

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