Binding of Anionic Phospholipids to Retinal Pigment Epithelium May Be Mediated by the Scavenger Receptor CD36*

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The specific recognition of negatively charged phospholipids in cell membranes has been suggested to play an important role in a variety of physiological and pathophysiological processes. Recent work (Rigotti, A., Acton, S. L., and Krieger, M. (1995) J. Biol. Chem. 270, 16221–16224) has described specific and tight binding of anionic phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolaminol (PI), to the class B scavenger receptors, CD36 and SR-B1. We have previously reported that CD36 is present on retinal pigment epithelium (RPE) and plays a role in the phagocytosis of photoreceptor outer segments (ROS), a function critical to the normal visual process (Ryeom, S. W., Sparrow, J. R., and Silverstein, R. L. (1996) J. Cell Sci. 109, 387–395). We now report that phospholipid liposomes PS and PI, but not phosphatidylethanolaminol, bind specifically to RPE. Cross-competition experiments suggest that PS and PI recognize the same receptor on RPE, while immunoinhibition studies indicate that the receptor is CD36. RPE cells isolated from a mutant rat strain, the RPE cells, recognize the same receptor on RPE, while immunoinhibition studies indicate that the receptor is CD36. RPE of which does not express CD36 (Sparrow, J. R., Ryeom, S. W., Abumrad, N., Ibrahim, A., and Silverstein, R. L. (1996) Exp. Eye Res., in press), did not bind PS or PI, further confirming the role of CD36. We also showed that purified ROS blocked binding and uptake of anionic phospholipid liposomes by RPE and that PS and PI liposomes blocked ROS uptake by RPE, suggesting that PS and PI on the ROS membrane may be the ligands on ROS recognized by CD36. This is the first demonstration that CD36-phospholipid interactions may play a role in normal physiology.

Phospholipids in the plasma membrane of cells are distributed asymmetrically between the inside and outside of the membrane bilayer (5). The outer leaflet of the cell membrane contains mostly neutral phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolaminol (PE), while negatively charged phospholipids are confined exclusively to the inner leaflet in normal cells (6). However, in various physiological and pathophysiological situations, the phospholipid distribution breaks down, and anionic phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolaminol (PI), are exposed on the outer membrane (7). This breakdown of membrane asymmetry occurs in activated platelets (8), in sickle erythrocytes (9), and in apoptotic leukocytes (10). The abnormal external exposure of PS in these conditions allows specific recognition by circulating monocytes or macrophages and thus may participate in phagocytosis of cells by mononuclear phagocytes.

Although it is known that macrophages specifically take up cells when their membranes expose anionic phospholipids (11), only recently have the specific cell surface receptors involved in the binding of these negatively charged phospholipids been identified. Rigotti et al. (1) found that CD36 and SR-B1, members of the class B scavenger receptor family which on macrophages have been previously characterized to phagocytose apoptotic cells (12) and modified lipoproteins (13–15), specifically bound PS and PI liposomes with high affinity. PS and PI liposomes competed effectively with labeled PS liposomes and modified lipoproteins for binding to CD36.

We have recently shown that CD36 is expressed on the retinal pigment epithelium (RPE) and plays a role in the phagocytosis of photoreceptor outer segments (ROS) (2). The phagocytosis of ROS by RPE is critical to the normal visual process. When this process is impaired in experimental animals, the retina degenerates, leading eventually to blindness (3). For many years, it was known that ROS ingestion was mediated by a specific ligand-receptor interaction (16), and recently we reported CD36 as a candidate receptor on the RPE (2). However, the ligand on ROS membranes is yet to be identified. Photoreceptor outer segments are composed of stacks of double-layered circular disks formed continuously at the base of the outer segment of rod and cone cells (17). During the normal process of outer segment disk renewal, packets of disks are intermittently shed from the apical ends of the rod outer segments and are then immediately phagocytosed by the RPE (18). Outer segments consist predominantly of protein combined with phospholipid. Approximately 75% of the membrane protein is rhodopsin, while the phospholipid content of ROS membranes is close to 25%. The phospholipid composition is made up of about 80% PC and PE, while PS accounts for 13% and PI – 7% (19).

The report that CD36 binds PS and PI in vitro and the observation that PS and PI are present on ROS membranes prompted us to investigate the possibility that PS and PI were the ligands involved in the recognition of ROS by CD36 on the RPE surface. Using artificial phospholipid vesicles as models of ROS membrane, we found that phospholipid vesicles containing negatively charged phospholipids such as PS and PI competed for ROS phagocytosis by RPE. Furthermore, PS and PI liposomes bound specifically to and were internalized by RPE, and the binding and uptake were blocked by anti-CD36 IgG. Taken together, these data suggest that anionic phospholipids are the ligands on ROS membranes that are specifically recognized by CD36 on the RPE cell surface. This is the first dem-

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† The abbreviations used are: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylethanolaminol; PE, phosphatidylethanolaminol; RPE, retinal pigment epithelium; ROS, photoreceptor outer segments; LE, Long Evans; RCS, Royal College of Surgeons.
onstration that anionic phospholipids may be physiological ligands for CD36 and participate in normal scavenger receptor recognition.

EXPERIMENTAL PROCEDURES

Materials—Reagents (and sources) were: egg phosphatidylcholine, liver phosphatidylinositol, brain phosphatidylserine, egg phosphatidylethanolamine, and cholesterol (Avanti Polar Lipids, Inc., Alabaster, AL); polycarbonate membrane filters (Nuclepore Corp., Pleasanton, CA); sodium [3H]iodide and 1,2-dipalmitoyl-sn-3-phosphatidyl[N-methyl-3H]choline (Amersham Life Sciences Inc.); Dulbecco's modified Eagle's medium, glutamine, minimum essential medium nonessential amino acid solution (Life Technologies, Inc.); fetal calf serum (HyClone, Logan, UT); gentamycin sulfate (Scherck Corp., Kenilworth, NJ); trypsin (Difco); BCA protein assay (Pierce); lactoperoxidase (Pierce); glucose oxidase (Sigma); polycarbonate membrane filters (Nuclepore Corp., Pleasanton, CA); polyethylene glycol (PEG 2000) (Barnes & Noble, New York, NY); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Pierce); chloroform (ACS reagent grade, Fisher Scientific Co.); methanol (ACS reagent grade, Fisher Scientific Co.); 2-mercaptoethanol (Sigma); and 3-5-dinitrosalicylic acid (Sigma). 2-Deoxy-D-glucose (Sigma). Mouse mAb to rhodopsin, Rho-4D2, was a generous gift from Dr. Robert Molday (University of British Columbia, Vancouver, British Columbia, Canada). Rabbit anti-rat CD36 IgG was prepared as described by Ryem et al. (2).

Phospholipid Liposome Preparation—Unilamellar liposomes were made by extrusion through polycarbonate membranes (20, 21). Phospholipid liposomes were prepared containing the indicated phospholipid, phosphatidylcholine, and free cholesterol in a molar ratio of 1:1:1. Liposomes made with all four phospholipids and cholesterol had a molar ratio of 2:1 with PC-PE-PS-PI (50:25:10:1). The lipids were dissolved in benzene and lyophilized overnight. For radiolabeled liposomes, 50 µCi of 1,2-dipalmitoyl-sn-3-phosphatidyl[N-methyl-3H]choline (69 Ci/mmol) was added to each liposome mixture before drying. The dried lipids were resuspended in 150 mM NaCl, 0.1 mM EDTA, 10 mM Hepes, pH 7.5. Once the samples were fully hydrated, they were extruded first through a 1.0-µm pore size polycarbonate membrane, and then one-half of the sample was extruded through a second, 0.1-µm pore size polycarbonate membrane using an extruder device (Lipex Biomembranes, Inc., Vancouver, British Columbia, Canada). The final phospholipid concentrations of each liposome were determined by the method of Dittmer and Wells (22). Liposomes were then stored under argon at 4°C and were used within 2 weeks of preparation. All experiments were performed with both 1.0-µm and 0.1-µm liposomes.

Cell Culture—Primary cultures of Long Evans (LE) and Royal College of Surgeons (RCS) rat RPE were established as described previously (2) from 2–4-week-old rats. The cultures were maintained in a humidified environment at 37°C and 10% CO2, with feeding twice weekly, and were not passaged. The cells were grown for up to 3 weeks.

ROS Phagocytosis Assays—ROS were isolated as described previously (2) from bovine eyes obtained from the slaughterhouse within 4 h of death. Isolated, intact ROS were radiolabeled according to the method of Clark and Hall (23). LE or RCS rat RPE cells were cultured to confluence in 96-well plates or chamber slides. Preincubation of cells with CD36 mAb or different concentrations of phospholipid liposomes was carried out in growth medium for 30 min at 37°C. Subsequently, equal concentrations of [3H]labeled ROS were added to the cells in the presence of the different treatments, and the 96-well plates were centrifuged at 1000 rpm for 4 min and incubated for 2 h in a humidified environment at 37°C and 10% CO2. Following this incubation, the cells were washed thoroughly with culture medium and treated with 100 µl of solubilization buffer (1% SDS, 0.5 N NaOH, 10 mM EDTA) for 30 min at 37°C. Cell-associated radioactivity was measured in a gamma counter, and protein concentrations were determined by the BCA protein assay. The data (cpm/mg protein) were analyzed by analysis of variance to establish significant differences among treatment and control groups.

3H-Labeled Liposome Phagocytosis Assays—3H-Liposomes were diluted to the indicated concentrations in RPE growth medium with 2.5% sucrose, added to LE or RCS rat RPE cells in the absence or presence of different treatments, and incubated for 2 h in a humidified environment at 37°C and 10% CO2. Following this incubation, the cells were washed thoroughly with culture medium and treated with 200 µl of solubilization buffer (described above) for 30 min at 37°C. Cell-associated radioactivity was measured in a beta counter, while protein concentrations were determined by the BCA protein assay. In blocking or competition experiments, cells were preincubated for 30 min at 37°C with rabbit anti-rat CD36 IgG, control preimmune IgG, unlabeled liposomes, or ROS which were diluted 3-fold in Hanks’ balanced salt solution, pelleted at 5000 rpm for 20 min at 4°C, and resuspended in growth medium with 2.5% sucrose at a concentration of 5 x 102-1.5 x 105 ROS/ml. The data (cpm/mg protein) were analyzed by analysis of variance to establish significant differences among treatment and control groups.

RESULTS

To determine whether phospholipids could be bound and internalized by normal rat RPE, the binding and/or ingestion of different radiolabeled liposomes was examined at 37°C. RPE isolated from normal LE rats showed a significantly high level (p < 0.01) of PS liposome binding and/or uptake at a concentration of 150 µg of phospholipid/ml (Fig. 1A) compared with liposomes containing other phospholipids. This was dose dependent, because PS liposomes at a concentration of 25 µg/ml demonstrate a significant (p < 0.1) yet much lower level of uptake. Liposome binding and/or uptake by LE RPE was dependent on the phospholipid composition of the liposome. While PC and PI liposomes were taken up by RPE in a concentration-
dependent manner, binding and/or uptake was less than that of PS but significant (p < 0.1) in comparison to PE. In contrast, there was no significant uptake (p > 0.1) of PE liposomes at either concentration. These results indicate that PS phospholipids are the predominant phospholipid bound and/or internalized by LE RPE with a small amount of binding and/or uptake seen with PC and PI phospholipids. Liposomes composed of all four phospholipids in a molar ratio similar to that of isolated bovine ROS also demonstrated significant levels of binding and/or uptake (p < 0.1) by LE RPE.

The specificity of phospholipid binding and/or uptake were further studied by determining whether unlabeled liposomes or anti-CD36 IgG could compete for or block \[^{3}H\]PS, \[^{3}H\]PI, and \[^{3}H\]PC binding and/or uptake by RPE. Table I demonstrates that the unlabeled anionic phospholipids, PS and PI, at 150 \(\mu\)g/ml were effective competitors of both \[^{3}H\]PS and \[^{3}H\]PI binding and/or uptake when PS and PI were preincubated with cells. Meanwhile, PC had no effect on \[^{3}H\]PC binding and/or uptake; similarly, PS did not have a pronounced effect on \[^{3}H\]PC binding and/or uptake. However, \[^{3}H\]PC binding and/or uptake were blocked by unlabeled PC. When LE RPE were preincubated with anti-CD36 IgG, \[^{3}H\]PS and \[^{3}H\]PI binding and/or uptake were significantly inhibited (p < 0.01) compared with \[^{3}H\]PC. Preimmune IgG had no effect on the level of binding or uptake of any of the liposomes, demonstrating specificity. These data indicate a high level of specific binding and/or uptake of PS liposomes by LE RPE and a lower level, but specific, of binding and/or uptake of PI and PC liposomes. The reciprocal ability of PS and PI to inhibit binding and/or uptake implies that they are probably recognized by the same receptor while the specific inhibition seen with anti-CD36 IgG suggests that this receptor is CD36. These results are consistent with the findings of Rigotti et al. (1), who have reported that CD36 is a receptor for anionic phospholipids such as PS and PI, by cells transfected with CD36 cDNA.

The RCS rat is an animal model of retinal degeneration with a defect localized to RPE cells that are unable to phagocytose shed photoreceptor outer segments (3, 4). In this work, we have studied the role of phospholipids in the phagocytosis of photoreceptor outer segments by RPE. Scavenger receptors have been reported to recognize the negatively charged surface of a ligand and have been extensively described with respect to their phagocytosis of modified low density lipoprotein, which often has increased net anionic charges (25). A CD36 cDNA-transfected cell model has recently been used to demonstrate that anionic phospholipid liposomes also bind CD36 (1). We have now shown that normal RPE specifically bound and/or internalized liposome vesicles containing PS phospholipids and to a lesser extent, PI and PC. The binding and/or uptake of PS and PI was found to be competed by excess, unlabeled liposomes of either type and inhibited by a specific anti-CD36 antibody. PC uptake, although blocked with cold PC liposomes, was not affected by PS or by the presence of anti-CD36 IgG. These results indicate that PS and PI are recognized by the same receptor on RPE which may be CD36 (1, 2). We have also examined whether isolated ROS could compete for \[^{3}H\]PS and \[^{3}H\]PI phagocytosis by RPE. When normal RPE were preincubated with isolated unlabeled ROS, \[^{3}H\]PS binding and/or internalization were significantly inhibited (p < 0.01) compared with control, untreated \[^{3}H\]PS binding (Fig. 2). \[^{3}H\]PI binding and/or uptake was also significantly blocked (p < 0.05) when LE RPE were preincubated with ROS. Similarly, binding and/or uptake of \[^{3}H\]liposomes containing PC, PE, PS, and PI was significantly inhibited (p < 0.01) in the presence of excess, cold bovine ROS, while RPE were not able to compete for either \[^{3}H\]PC or \[^{3}H\]PI binding or uptake.

Previously, using a quantitative in vitro assay with \[^{125}\]I-labeled ROS, we established that CD36 mediates the phagocytosis of ROS by RPE. In Fig. 3, the same assay was performed with LE RPE by measuring \[^{125}\]I-labeled ROS binding and/or uptake in the presence of unlabeled liposomes of various phospholipid compositions. In the presence of PS liposomes, \[^{125}\]I-labeled ROS binding and/or uptake was significantly inhibited (p < 0.01) to an extent similar to that seen with anti-CD36 IgG (2). To a lesser extent, PI liposomes also significantly inhibited (p < 0.05) levels of ROS binding and/or uptake as compared to PC or PE liposomes. Meanwhile, neither PC nor PE liposomes had a significant effect (p > 0.1) on the levels of ROS binding and/or uptake.

**DISCUSSION**

In this work, we have studied the role of phospholipids in the phagocytosis of photoreceptor outer segments by RPE. Scavenger receptors have been reported to recognize the negatively charged surface of a ligand and have been extensively described with respect to their phagocytosis of modified low density lipoprotein, which often has increased net anionic charges (25). A CD36 cDNA-transfected cell model has recently been used to demonstrate that anionic phospholipid liposomes also bind CD36 (1). We have now shown that normal RPE specifically bound and/or internalized liposome vesicles containing PS phospholipids and to a lesser extent, PI and PC. The binding and/or uptake of PS and PI was found to be competed by excess, unlabeled liposomes of either type and inhibited by a specific anti-CD36 antibody. PC uptake, although blocked with cold PC liposomes, was not affected by PS or by the presence of anti-CD36 IgG. These results indicate that PS and PI are recognized by the same receptor on RPE which may be CD36 (1, 2). The data also suggest that PC is taken up by a different mechanism. Further evidence supporting a role for CD36 in the binding and/or uptake of PS and PI was obtained by examining liposome binding to dystrophic RCS rat RPE. We have reported...
that RCS RPE lack CD36 expression and that the defect in RPE-mediated ROS uptake may be associated with its absence (26). RCS RPE did not bind or internalize either PS or PI liposomes, suggesting that the absence of CD36 prevents binding and/or internalization of these negatively charged phospholipids. On the other hand, since PC liposomes were bound and/or internalized by RCS rat RPE at levels similar to those seen with LE rat RPE, a receptor mediating PC liposome phagocytosis may be present on dystrophic as well as normal RPE.

Our results with RPE are the first description of CD36-specific binding of PS and PI liposomes by cells normally expressing CD36. More importantly, they suggest a physiological role for the CD36-phospholipid interaction in the selective phagocytosis of photoreceptor outer segments by RPE (2), a conclusion critical to the normal visual process (3, 4). This conclusion is based on our demonstration that PS and PI, but not PC or PE, liposomes blocked ROS binding and uptake by RPE and conversely isolated ROS blocked PS and PI binding and/or uptake. Furthermore, we showed that liposomes consisting of phospholipids in molar ratios similar to that for purified, bovine ROS were bound and/or internalized by RPE and that this process was inhibited by excess purified, bovine ROS as well as by anti-CD36 IgG. This report provides evidence that ROS phagocytosis is mediated by the interaction of the scavenger receptor CD36 on the RPE with anionic phospholipids present on the surface of ROS membranes. Since previous work demonstrated a consistent 60% decrease in ROS uptake in the presence of antibodies to CD36, we have suggested that CD36 acts together with a co-receptor to mediate ROS phagocytosis (2). The similar level of inhibition of PS and PI uptake seen by anti-CD36 IgG further confirms this two receptor model. It is possible that the co-receptor may be an unidentified receptor involved in the binding and/or uptake of PC liposomes.

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