A Distinct Integrin-Mediated Phagocytic Pathway for Extracellular Matrix Remodeling by RPE Cells

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PURPOSE. To characterize the phagocytosis of extracellular matrix components by retinal pigment epithelial cells and to determine which receptors and signal transduction pathways are involved.

METHODS. Fluorescent latex beads were coated with fibronectin (FN), collagen type I or IV, or thrombospondin and incubated with human retinal pigment epithelial cells for 3 hours. Phagocytosis was quantified by flow cytometry. The effects of adhesion blocking antibodies to cell surface receptors (α1, α3, α5, β1, α5β1, αvβ5, αvβ3 integrins and CD36) and inhibitors of specific intracellular signaling pathways (tyrosine kinase phosphatidylinositol 3-kinase [PI3-kinase], protein kinase C [PKC], and mitogen-activated protein kinase) were determined using FN-coated beads.

RESULTS. Phagocytosis of FN-coated beads was greater than phagocytosis of beads coated with collagen type I, collagen type IV, or thrombospondin or uncoated controls (P < 0.0005). Anti-α5, -β1, and -α5β1 antibodies markedly inhibited FN phagocytosis (P < 0.0005); the inhibitory effects of anti-α5 antibody were stronger in the initial stages (binding) than in the later stages (internalization) of phagocytosis. There was no significant effect on phagocytosis when anti-α1, -α3, -αvβ5, -αvβ3 or -CD36 antibodies were used. Fibronectin phagocytosis was decreased by inhibitors of tyrosine kinase (genistein, 100 μg/ml, P < 0.005) and PI3-kinase (wortmannin, 5 μM, P < 0.01), but these reagents did not affect the uncoated controls. The PKC inhibitor calphostin C (400 nM) nonspecifically increased the phagocytosis of FN-coated (P < 0.05) and uncoated beads (P < 0.01).

CONCLUSIONS. Subconfluent retinal pigment epithelial cells preferentially phagocytose FN over other extracellular matrix components. Phagocytosis of FN utilizes the α5β1 integrin, is mediated in part through tyrosine kinase and PI3-kinase signaling pathways, and is modulated by PKC. Phagocytosis of extracellular matrix by retinal pigment epithelial cells may represent a novel mechanism for remodeling of the provisional extracellular matrix during outer retinal wound healing. (Invest Ophthal Vis Sci. 1999;40:2713–2723)

Phagocytosis of rod outer segments (ROS) is a critical function of the retinal pigment epithelial (RPE) cell.1 The long apical microvilli of the differentiated RPE cell ensheathe the photoreceptor outer segments, facilitating the phagocytosis of large numbers of shed ROS tips each day.4 The mechanisms involved in ROS phagocytosis are specific, receptor-mediated, and analogous in many ways to those involved in the phagocytosis of apoptotic cells by macrophages.2–9 The receptors and ligands involved in ROS phagocytosis by RPE cells are still unclear; however, mannose receptors, CD36, and αvβ5 integrins have each been implicated.5–9 In the normal eye, RPE cells are thought to be highly selective for ROS phagocytosis.5 In vitro studies using confluent cells have shown that RPE cells preferentially choose to phagocytose ROS over other particles such as red blood cells, algae, bacteria, and yeast.3 In vitro studies have also shown that RPE cells can phagocytose uncoated latex beads nonspecifically, although this occurs through different non–receptor mediated mechanisms.10

The possibility that RPE cells might change their phagocytic preference and ingest other materials in the context of an altered pathologic environment has not been previously considered. Proliferative vitreoretinopathy (PVR) is a well-recognized complication of serious ocular trauma and rhegmatogenous retinal detachment, as well as a major cause of failure of retinal reattachment surgery.11 Proliferative vitreoretinopathy can be viewed as a protracted wound-healing response in the outer retina, involving the same processes that are found in wound healing elsewhere, including cell-matrix adhesion, migration, and extracellular matrix (ECM) degradation.11,12 It is characterized by proliferation and migration of cells through retinal holes to form membranes on the retinal surface and within the vitreous.11 Proliferative vitreoretinopathy–affected membranes are composed of a mixed population of cells; however, the RPE cell has been identified, by both light and electron microscopy and by immunohistochemical studies,11,13–15 as a major and critical component. These cells are embedded in a framework of ECM proteins, such as fibronectin, collagen types I and IV, and thrombospondin.16,17 Immuno-
nothern histochemical studies of PVR-affected membranes have revealed a close relationship between ECM components and migrating RPE cells, suggesting the need for careful study of this interaction.

The PVR-affected membrane is a dynamic structure with an ECM composition that changes over time. The amount of fibronectin present in the PVR-affected membrane decreases with age, suggesting that either differential production or degradation of the ECM may be occurring. Different invasive cells (including macrophages, fibroblasts, and cancer cells) use comparable strategies, such as secretion of proteolytic enzymes, to degrade the ECM. Recently, phagocytosis has been suggested as a possible additional mechanism by which invasive cells degrade ECM. In support of this idea, ultrastructural evidence for phagocytosis of specific ECMs has been demonstrated during turnover of soft connective tissues. The possibility that RPE cells could also show specific and preferential phagocytosis of ECM components has not been considered previously. The receptors involved in ECM phagocytosis by other cells include the αvβ3 integrin for endocytosis of vitronectin by human fibroblasts, the α5β1, αvβ3, and αvβ1 integrins for fibronectin phagocytosis by mononuclear cells, and αvβ1 integrin for phagocytosis of gelatin and Matrigel by breast cancer cells. RPE cells express a wide variety of integrins that could potentially play a role in the phagocytosis of the ECM. Ligand binding of integrins leads to the activation of a number of diverse signaling pathways that may control the phagocytic process by regulating the cytoskeleton and intracellular trafficking. Intracellular signaling is often cell-type specific and may differ depending on the activation state of the cell.

The purpose of the present study, therefore, was to determine in vitro whether RPE cells demonstrate specific and differential phagocytosis of ECM components and, if so, which receptors and signal transduction pathways are involved.

**METHODS**

**RPE Cell Culture**

Human RPE cells were isolated as previously reported. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum (Gemini Bioproducts, Calabassas, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (Omega Scientific, Tarzana, CA). Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO2. The medium was changed every 3 days. After confluence was reached, the cells were passaged by digestion with 0.05% trypsin/0.02% EDTA (Irvine Scientific). For experiments, cells between passage 3 and 5 were seeded in 6-well plates, at a density of 2 × 105 cells/well, for 24 hours, at which time the cells reached between 70% and 90% confluence. Subconfluent cultures were specifically chosen for study because they mimic the conditions found within PVR-affected membranes as opposed to the confluent conditions of the resting monolayer. The purity of cultured RPE cells was evaluated by immunocytochemical staining using antibodies against cytokeratin (Dako, Carpinteria, CA), von Willebrand factor (Dako), and CD11c (Dako).

**Analysis of Phagocytosis**

A fluorescent latex bead model was chosen to study ECM phagocytosis because this model is reproducible and quantitave. The quantal increase in phagocytosis found with increasing numbers of ingested beads simplifies comparative analysis. Fluorescent latex beads (yellow-green, emission maximum = 515 nm, 2.0 μm in diameter; Molecular Probes, Eugene, OR) were used alone or as a vehicle for ECM coating to measure phagocytosis. The beads were coated by one of the ECM components (fibronectin, Sigma Chemical, St. Louis, MO; collagen type I or IV, Becton Dickinson, Bedford, MA; thrombospondin, GibCO/BRL, Gaithersburg, MD) at three different concentrations (0.1, 1, and 10 μg/ml) at 37°C for 10 minutes. Coated beads were diluted in phosphate-buffered saline (PBS) to an appropriate concentration. Each well of subconfluent RPE cells was layered with 100 μl of DMEM containing 10% serum and 5 × 106 beads and was incubated at 37°C for 3 hours. Preliminary experiments revealed that ECM phagocytosis was poor with serum concentrations less than 1% and was maximal with 10% serum; because ROS phagocytosis experiments were also optimal with 10% serum, all experiments were performed using this serum condition. Confocal microscopy using anti-αvβ1 antibody demonstrated the presence of αvβ1 on apical and basal surfaces of the subconfluent RPE cells.

**Flow Cytometry**

After a 3-hour challenge, the cells were detached using 0.05% trypsin/0.02% EDTA, washed 3 times with PBS, and resuspended in 0.5 ml PBS for the flow cytometric assay. The external adhering beads were largely removed by this procedure.

Extracellular matrix-coated bead uptake was measured using a fluorescence-activated cell sorter (FACStar plus; Becton Dickinson, Mountain View, CA). Cells were analyzed with 488-nm excitation (laser power = 15 mW) and a 530 ± 15 nm band-pass filter in the emission path. Forward and side light scatter was used to gate the desired scattered events (RPE cells) from dead cells, debris, and free beads. A negative control consisting of untreated RPE cells was used to set the gate in each experiment. Each flow cytometry run consisted of 5000 scattering events. The fluorescent beads were calibrated using fluorescence reference standards (Quantum 24 fluorescein-5-isothiocyanate [FITC]-labeled microbeads; Flow Cytometry Standards, San Juan, PR). The phagocytic index was determined in each experiment by multiplying the percentage of gated positive (bead-containing) cells by their mean fluorescence. These data were expressed as a histogram. The assay was validated by comparison of data obtained by flow cytometry with direct observations obtained by fluorescence microscopy. In each experiment, the phagocytic index for fibronectin (10 μg/ml) phagocytosis was normalized to 100; the phagocytic index for phagocytosis of other substrates or manipulations was then presented in relation to this value.

**Electron Microscopy**

Electron microscopy was used to verify that the phagocytic beads were ingested within the RPE cells. The collected cells were fixed in half-strength Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer; pH 7.2-7.4) at 4°C for 48 hours. The cells were rinsed for 15 minutes in cacodylate buffer and postfixed in 2% osmium tetroxide (Polyscience, Warrington, PA) for 2 hours. After dehydration and infiltration, the cells were embedded in Epon.
and thin sections were cut on an ultramicrotome using a diamond knife. The sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM10 (Carl Zeiss Inc., Thornwood, NY) electron microscope.

**Direct Phagocytosis of ECM**

To verify whether RPE cells could directly phagocytose fibronectin without using beads as vehicles, fibronectin was labeled with FITC using the method of Pallis et al.\textsuperscript{28} The labeled fibronectin was added to the cell culture medium at a concentration of 4 µg/ml and incubated with subconfluent RPE cells at 37°C for 3 hours. To quench external FITC fluorescence, samples were incubated with 0.2% trypsin blue for 10 minutes before trypsinization.\textsuperscript{27} The RPE cells were trypsinized, and phagocytosis was quantified by flow cytometry.

**Phagocytosis Inhibition**

**Antibody Blocking Assay.** Phagocytosis experiments were performed using fibronectin-coated beads in the presence of monoclonal antibodies known to be inhibitory and to block adhesion of cell surface receptors to their respective ligands.\textsuperscript{29–34} All anti-integrin antibodies (α1, MAB 1973; α3, MAB 2056; α5, MAB1986; β1, MAB2253Z; α5β1, MAB1969; αvβ3, MAB1976; αvβ5, MAB1961) were obtained from Chemicon International (Temecula, CA). An adhesion-inhibiting CD36 monoclonal antibody (clone FA6-152) was obtained from Immunotech (Westbrook, ME). RPE cells were preincubated with these monoclonal antibodies for 1 hour and then fed with the fibronectin-coated beads. Quantification of internalized beads was determined by flow cytometry as described above. The dose response of anti-α5 antibody inhibiting the phagocytosis of fibronectin-coated beads was obtained by using four different concentrations of anti-α5 antibody (0.1, 0.4, 1.0, and 2.0 µg/ml). All other antibodies were used at 5 µg/ml.

Because the flow cytometric assay measured only internalized beads, we performed an in situ assay to determine bead binding. RPE cells were challenged with fibronectin-coated beads for different times (5, 15, 30, 45, and 60 minutes), washed three times with PBS, and fixed with 4% paraformaldehyde. The number of beads on the RPE cells was determined under an inverted microscope. Five randomly chosen high-power fields of similar cell density were counted, and the result was expressed as the number of beads per 500 cells. To determine internalization in these samples, the cells were detached using 0.05% trypsin/0.02% EDTA, washed three times with PBS, and cytocentrifuged onto slides. The number of beads for different times (5, 15, 30, 45, and 60 minutes), was measured by flow cytometry after 3 hours.

**Effect of ROS on Fibronectin Phagocytosis.** Competition experiments were performed to determine whether the same receptors were involved in the phagocytosis of fibronectin and ROS. RPE cells were concurrently incubated with equal numbers of fibronectin-coated beads and ROS (5 × 10^{-6} M, well), at 37°C for 3 hours in DMEM containing 10% fetal bovine serum. The concentration of ROS is consistent with previous studies.\textsuperscript{8,13,25} Completely saturating concentrations of ROS cannot be used because at these high concentrations, the ROS nonspecifically inhibit the binding of uncoated beads to the RPE cells, presumably due to physical constraints.\textsuperscript{3} The phagocytic index of fibronectin-coated beads was then analyzed by flow cytometry. ROS were isolated from fresh bovine eyes using a discontinuous sucrose gradient method described by Papamater.\textsuperscript{35}

**Signal Transduction Pathway Inhibition.** To investigate the intracellular mechanisms by which RPE cell phagocytosis of fibronectin is regulated, we examined the effects of inhibiting tyrosine kinase, PI3-kinase, protein kinase C (PKC), and mitogen-activated protein kinase (MAP-K) pathways. Inhibitors of these pathways included genistein (Calbiochem, San Diego, CA) for tyrosine kinase; wortmannin (Calbiochem) for PI3-kinase; calphostin C (Calbiochem) for PKC; and PD98059 (New England Biolabs, Beverly, MA) for MAP-K. Cells were pretreated with genistein (20, 50, 100 µg/ml) for 1 hour; wortmannin (0.1, 1, and 5 µM) for 30 minutes; calphostin C (100, 200, and 400 nM) for 1 hour under light; or PD98059 (1, 3, 30, and 100 µM) for 1 hour. Fibronectin-coated beads were then added to the cells and incubated for another 3 hours in the presence of the inhibitors. After treatment with inhibitors, and before flow cytometry, cell viability was evaluated by the trypan blue exclusion test. Flow cytometry for analysis of phagocytosis was performed as above. To determine whether there was an additive effect of these inhibitors, cells were also pretreated with the following combinations of inhibitors: genistein (100 µg/ml) plus wortmannin (5 µM); genistein (100 µg/ml) plus calphostin C (400 nM), and wortmannin (5 µM) plus calphostin C (400 nM).

**Statistical Analysis**

For quantitative data, each assay was repeated at least three times, and the mean and SD of the mean (mean ± SD) were calculated. Comparisons between two samples were performed using the Student’s t-test.

**RESULTS**

**Phagocytosis of ECMs by RPE Cells**

The phagocytosis of fibronectin-, collagen type I-, collagen type IV-, and thrombospondin-coated beads by human RPE cells was studied at ECM concentrations of 0.1, 1.0, and 10.0 µg/ml. Phagocytosis was most prominent when beads were coated at a concentration of 10 µg/ml. Electron microscopic examination confirmed that when studies were performed as described, essentially all beads identified in association with the RPE cells were internalized and present within the cytoplasm (Fig. 1). At 10 µg/ml, the phagocytic index for fibronectin-coated beads was almost 10 times the amount that of the uncoated controls (Figs. 2, 3). Collagen type IV (3.1 times control), collagen type I (1.8 times control), and thrombospondin (2.8 times control) coating of the beads resulted in only a moderate increase in phagocytosis when compared to that of the uncoated bead control (Fig. 2, 3). Fibronectin
stimulated significantly more phagocytosis than any of the other three ECMs ($P < 0.0005$); therefore, it was chosen as the substrate to study further the receptor utilization and signal transduction pathways involved in the RPE cell phagocytic pathway.

**Receptors Involved in Fibronectin Phagocytosis by RPE Cells**

The utilization of specific receptors in the phagocytosis of fibronectin by RPE cells was determined by pretreating cells with adhesion-blocking antibodies against $\alpha_1$, $\alpha_3$, $\alpha_5$, $\alpha_5\beta_1$, $\beta_1$, $\alpha\beta_3$, and $\alpha\beta_5$ integrins and against CD36. Anti-$\alpha_5$, $\beta_1$, and $\alpha\beta_5$1 antibodies decreased the phagocytic index by 75.5% ($P < 0.0005$), 45.1% ($P < 0.01$), and 68.4% ($P < 0.0005$), respectively, when compared with that of untreated controls. By comparison, there was no significant inhibition of the phagocytic index demonstrated by antibodies against $\alpha_1$, $\alpha_3$, $\alpha\beta_3$, $\alpha\beta_5$, or CD36 (Fig. 4). The dose–response experiment for anti-$\alpha_5$ antibody showed that when the antibody concentration was increased to 0.4 $\mu$g/ml, the phagocytic index of fibronectin-coated beads reached a plateau that was above the level found for the uncoated control (Fig. 5). The phagocytic index of uncoated beads was not affected by anti-$\alpha_5$ antibody (Fig. 4). To determine whether internalization of fibronectin-coated beads continued in the presence of anti-$\alpha_5$ antibody, we performed the flow cytometric assay after a delay of 6 hours; anti-$\alpha_5$ antibody decreased the phagocytic index after the delay, by 36.7% compared with that of the untreated control ($P < 0.0001$; Fig. 4).

The continuing increase in bead internalization in the presence of anti-$\alpha_5$ antibody suggested that the antibody was inhibiting the binding of fibronectin-coated beads more than internalization. An in situ assay (Fig. 6A) showed that the beads were rapidly bound to the RPE cells, and by 30 minutes the internalization had already started. The binding initially occurred at a faster rate than internalization; however, after a lag of 45 minutes, both increased at a similar rate. Anti-$\alpha_5$ antibody initially inhibited both binding and internalization; however, by 60 minutes internalization had started despite the presence of antibody. The 45-minute time point was chosen for further study because it represented the latest time at which binding was almost completely inhibited. RPE cells were challenged with fibronectin-coated beads in the presence or absence of anti-$\alpha_5$ antibody for 45 minutes; unbound fibronectin-coated beads were then removed, and the incubation continued either in the absence or in the presence of inhibitory antibody. When anti-$\alpha_5$ antibody was present in the early stage (0–45 minutes), the phagocytic index was inhibited by 56.8% ($P < 0.0005$). If, instead, no antibody was used in the early stage and if unbound beads were washed away after 45 minutes, internalization of these beads could be studied by measuring internalization in the presence or absence of antibody. The possibility that the antibody caused disassociation of bound beads and prevented their rebinding must also be considered as a cause of the reduced internalization. When anti-$\alpha_5$ antibody was added after 45 minutes of incubation with beads, internalization was inhibited by only 34.4% ($P < 0.005$; Fig. 6B). This result suggests that the major effect of $\alpha_5$ integrin was on binding with fibronectin; however, it is possible, but not proven, that a significant but more modest effect on internalization of fibronectin-coated beads was also present.

To determine whether the inhibitory effect of anti-$\alpha_5$ antibody on fibronectin phagocytosis also occurred in the absence of beads, a flow cytometric phagocytic assay was performed using FITC-labeled fibronectin. After a 3-hour challenge with FITC-labeled fibronectin, the phagocytic index was 2.97-fold greater in cells pretreated with control antibody than after pretreatment with anti-$\alpha_5$ antibody ($P < 0.005$). This result indicates that RPE cells can directly phagocytose fibronectin without the use of beads as a vehicle and that both processes use the $\alpha_5$ integrin receptor.

**Competition experiments were performed to determine whether there was any overlap in receptor utilization for the phagocytosis of ROS and fibronectin by RPE cells.** As previ-
ously reported, ROS phagocytosis by RPE cells was prominent under these conditions (results not shown). Coincubation of equal numbers of ROS with fibronectin-coated beads did not affect the phagocytosis of fibronectin-coated beads by the RPE cells (Fig. 7), indicating probable utilization of distinct cell surface receptors.

**Effect of Inhibiting Signal Transduction Pathways on Fibronectin Phagocytosis**

Pretreatment of RPE cells with 100 μg/ml of the tyrosine kinase inhibitor genistein decreased the phagocytic index by 37.8% compared with that of untreated controls (Fig. 8; *P* < 0.005). In contrast, no significant decrease in the phagocytic index of uncoated beads by genistein was found. Lower concentrations of genistein were not effective in inhibiting phagocytosis of fibronectin-coated beads. Wortmannin, a specific PI3-kinase inhibitor, decreased fibronectin phagocytosis over a range of 0.1 to 5 μM. In the presence of 5 μM wortmannin, the phagocytosis of fibronectin-coated beads was decreased by 47.7% compared with that of the untreated control (*P* < 0.01), whereas no significant decrease was found for uncoated beads.

Inhibition of the PKC pathway was performed using calphostin C. Pretreatment of RPE cells with calphostin C (400 nM) increased the phagocytic index of fibronectin-coated beads by 24% (*P* < 0.05), when compared with untreated controls. In the uncoated control groups, pretreatment with calphostin C (400 nM) increased the phagocytic index to 227% compared with that of the untreated control (*P* < 0.01), whereas no significant decrease was found for uncoated beads.

Inhibition of the MAP-K pathway inhibitor was used, no significant change in fibronectin phagocytosis was found. Control experiments showed functional inhibition of these pathways as previously reported. Cell viability analyses performed by trypan blue exclusion showed no cellular toxicity of these signal transduction pathway inhibitors in the concentration range and time frame of these experiments (data not shown).
To determine the dependence of these signaling pathways on each other, we investigated the effect of combining different inhibitors on fibronectin phagocytosis. When RPE cells were pretreated with genistein (100 μg/ml) and wortmannin (5 μM), the decrease in the phagocytic index was greater than those by either of the reagents alone (*P < 0.01) and appeared to be additive. Although the effect of calphostin C (400 nM) combined with genistein (100 μg/ml) was not significantly different from that of genistein alone, wortmannin (5 μM) combined with calphostin C (400 nM) showed greater inhibition of phagocytosis than wortmannin alone (*P < 0.05). Thus, the stimulatory effect of PKC inhibition is lost when combined with a tyrosine kinase or PI3-kinase inhibitor, and the combination of tyrosine kinase and PI3-kinase inhibitors has an additive inhibitory effect.

**DISCUSSION**

This study provides conclusive evidence that RPE cells are capable of phagocytosing ECM components, either as a soluble substrate or attached on the surface of latex beads. Fibronectin is preferentially phagocytosed by the RPE cells in a dose-response manner, and although collagen type I, collagen type IV, and thrombospondin are phagocytosed at a higher level than uncoated beads, their levels of phagocytosis are significantly lower than that for fibronectin and cannot be distinguished from one another. Studies of human disease provide support for the contention that levels of fibronectin accumulation are regulated during the course of disease. A time-dependent change in fibronectin is seen in healing wounds. The glycoprotein has important adhesive roles in the early stages of wound healing; however, as the scar matures, fibronectin disappears.14,37 Similarly, when the content of ECM in human PVR-affected membranes was compared to the age of the membranes, it was found that membranes of less than 4 months’ clinical duration contained significantly more fibronectin than older membranes.14 In contrast, there was no association between membrane duration and the content of collagen subtypes I or IV or laminin. Thus, our results suggest that phagocytosis of ECM, and especially fibronectin, by RPE cells may represent a mechanism for remodeling of the provisional ECM formed during outer retinal wound healing.

The utilization of a phagocytic mechanism for ECM remodeling has only recently been proposed. Particular interest has been paid to use of this mechanism by invasive cells, including macrophages, fibroblasts, and tumor cells. In breast cancer cells, phagocytosis of the ECM is an inherent feature of the tumor cells that correlates with and may even contribute to their invasive capacity.27 Phagocytosis of gelatin by breast cancer cells has recently been used as a model to follow the ECM phagocytic pathway. Breast cancer cells invade the ECM at well-defined membrane projections termed “invadopodia.”21 The phagocytosis is a rapid actin-dependent process beginning within 30 minutes of exposure and is preceded by proteolysis of ECM components by matrix metalloproteinases (MMP).27 Most of the intracellular gelatin is routed to actively acidified vesicles, indicating intracellular degradation of the material in lysosomes.27

Phagocytosis of ECM by RPE cells is a similarly rapid process. Internalization of fibronectin beads begins within 30 minutes, whereas ROS internalization begins after 1 hour of challenge.8 In vitro, both processes appear to be dependent on the presence of serum. The nature and role of the serum components involved in phagocytosis are not well understood but may involve growth factors such as transforming growth factor-β1 that are found in the normal retinal environment as

![Figure 5. Dose–response curve of anti-α5 integrin antibody inhibiting the phagocytosis of fibronectin-coated beads by RPE cells.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933584/)
The specific role of proteolytic digestion of the ECM by RPE cells in this process is yet to be determined; however, RPE cells showed no difference in the phagocytosis of proteolyzed versus nonproteolyzed type 1 collagen-coated beads (data not shown). Studies of breast cancer cells have shown that, although cross-linked ECM requires proteolytic digestion before phagocytosis, subcellular-sized beads can be internalized without the need for prior proteolytic degradation.28 Because RPE cells are capable of increased gelatinase B (MMP-9) secretion after cytokine or phorbol ester stimulation,39,40 and because in PVR the vitreous often shows increased levels of MMP-9,41–43 the microenvironment of the RPE cell in a PVR-affected membrane may allow for proteolytic digestion of the ECM, if necessary, before phagocytosis. For this study, the choice of a subcellular-sized bead model allowed us to examine the role of receptor-mediated phagocytosis in a manner that may be independent of complicating proteolytic mechanisms.

Receptor utilization by phagocytic cells varies depending on the cell type and the substrate involved. In macrophages, phagocytosis is mediated by a variety of receptors including IgG-Fc, complement receptors, CD36, and mannose receptors.2,44 Recent experiments have proposed a critical role for integrins in the phagocytosis process in several different cell types.8,20 Integrin receptors are transmembrane heterodimeric proteins whose extracellular binding domains define substrate affinity and specificity.22 They play critical roles in attachment and migration of cells, including RPE cells, through the ECM. The preferential phagocytosis of fibronectin-coated beads in this study suggested that RPE cells express integrins with a substrate affinity for fibronectin.23,45 This correlates well with attachment studies that demonstrate that RPE cells have a clear substrate preference for fibronectin.36 Fibronectin is a glycoprotein with at least two independent cell adhesive regions with different receptor specificities. The cell adhesive region in the central portion of fibronectin is made up of a RGD and a PHSRN sequence that function in synergy, whereas the carboxyl-terminus contains LDV and REDV sequences with additive effects.46 Studies of cultured human RPE cells have shown a wide spectrum of integrin receptor expression, including $\beta_1$,
transfected with cells is the involvement of the phagocytosis of fibronectin by hematopoietic cells and RPE phagocytosis by RPE cells. An interesting difference between less significant unknown receptors involved in fibronectin nonspecific phagocytosis, suggesting that there may be other reveals that maximal inhibition did not reach the level of anti- for fibronectin phagocytosis by RPE cells. The dose–response demonstrated the clear preference for utilization of the RGDbinding sites for maximal binding. The antibody inhibition studies performed in the present study demonstrated the clear preference for utilization of the α5β1 integrin for fibronectin phagocytosis by RPE cells. The dose–response of anti-α5 antibody on fibronectin phagocytosis by RPE cells reveals that maximal inhibition did not reach the level of nonspecific phagocytosis, suggesting that there may be other less significant unknown receptors involved in fibronectin phagocytosis by RPE cells. An interesting difference between the phagocytosis of fibronectin by hematopoietic cells and RPE cells is the involvement of the αvβ3 integrin. In K562 cells transfected with αvβ3, antibodies against αvβ3 inhibit fibronectin phagocytosis by suppressing the phagocytic competence of the α5β1 integrin. This effect is not present in fibronectin phagocytosis by RPE cells and may be related to the low expression of αvβ3 in these cells.

In the resting intact monolayer, one of the most critical functions of the RPE cell is its ability to phagocytose ROS. Growth of RPE cells on native ECM substrates such as Bruch’s membrane or corneal endothelial cell matrix inhibits ROS phagocytosis, possibly by altering RPE cell morphology or differentiation or intracellular metabolism. Lack of a competitive effect of ROS on fibronectin phagocytosis strongly suggests, however, that different receptors are being used in ROS and fibronectin phagocytosis. Although the ligand for ROS phagocytosis has not been established, several receptors have been proposed, including mannose receptor, CD36, and αvβ5. Our experiments show that for fibronectin phagocytosis, most of the effect is mediated through the α5β1 integrin. Although inhibiting antibody experiments showed no effect of anti-αvβ5 or anti-CD36 antibodies on pure fibronectin phagocytosis, a cooperative role for these receptors may be possible in the complex ECM environment of the PVR-affected membrane. It is likely that other integrins mediate the phagocytosis of nonfibronectin ECM components by RPE cells.

The major effect of the anti-α5 antibody was in the binding stage of fibronectin phagocytosis, although a continuing but smaller effect may also be found on internalization. This is similar to the mannose receptor and CD36, which are also thought to participate in later steps of ROS phagocytosis; however, it differs from αvβ5, which appears to affect only ROS binding and not internalization.

Although very little is known about the intracellular signaling pathways involved in ECM phagocytosis, much more is

**Figure 8.** Effects of modifying signal transduction pathways on fibronectin phagocytosis by RPE cells. Inhibitors of these pathways included genistein for tyrosine kinase; wortmannin for PI3-kinase; and calphostin C for PKC. Cells were pretreated with genistein (100 µg/ml) for 1 hour; wortmannin (5 µM) for 30 minutes; or calphostin C (400 nM) for 1 hour under light. Fibronectin-coated beads were then added to the cells and incubated for another 3 hours in the presence of the inhibitors. Fibronectin phagocytosis was quantitated using flow cytometry. To determine whether there was an additive effect of these inhibitors, cells were also pretreated with the following combinations of inhibitors: genistein (100 µg/ml) plus wortmannin (5 µM); genistein (100 µg/ml) plus calphostin C (400 nM); and wortmannin (5 µM) plus calphostin C (400 nM). Error bar represents mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001, compared with no inhibitor control or when compared with first-named inhibitor alone.
known about integrin-mediated signaling.\textsuperscript{24,49} Integrin clustering and binding leads to formation and activation of focal adhesions, wherein integrins link to cytoskeletal complexes and actin filaments. This leads to the sequential activation of a large number of molecules, including tyrosine kinases, low-molecular-weight GTPase (such as Ras and Rho), serine–threonine protein kinases (such as PKC and MAP-K), and PI3-kinase.\textsuperscript{24}

We limited our study of the effects of modulating signaling pathways to the internalization of fibronectin-coated beads because this best represents total phagocytic activity; however, we recognize that differential effects on binding and internalization may be present.

Genistein, a specific tyrosine kinase inhibitor, moderately inhibited the phagocytosis of fibronectin by RPE cells but had no effect on uncoated beads. Previous studies have shown the importance of tyrosine phosphorylation in phagocytosis. For example, phagocytosis of IgG-coated particles or enteropathogenic \textit{Escherichia coli} is accompanied by enhanced protein tyrosine phosphorylation.\textsuperscript{49,50} Miceli and Newsome\textsuperscript{51} reported insulin stimulation of ROS uptake by human RPE cells and concluded that insulin stimulates ROS phagocytosis by an as yet unknown process that may involve a specific tyrosine phosphatase. They found that genistein at 100 \textmu M (27 \textmu g/ml) had no effect on insulin stimulation of ROS phagocytosis; however, it should be noted that in the present experiment, genistein was effective only when a dose of at least 100 \textmu g/ml was used.

Our study also demonstrated that the specific PI3-kinase inhibitor wortmannin\textsuperscript{52} significantly inhibited fibronectin phagocytosis by RPE cells. A dose–response effect was observed over a range in which wortmannin remains specific for inhibition of PI3-kinase and myosin light chain kinase.\textsuperscript{52} Previous studies have found that in the presence of wortmannin, recycling receptors, such as the transferrin\textsuperscript{53} receptor, and lysosomally targeted receptors, such as the platelet-derived growth factor (PDGF) receptor,\textsuperscript{54} became arrested in early endosomal compartments and were not able to return to the cell surface or follow the endocytic pathway to lysosomes for degradation. Recently, Memmo and McKeown-Longo\textsuperscript{55} further reported that wortmannin enhanced the colocalization of vitronectin and \beta 5 integrin with endocytic vesicles but inhibited the movement of vitronectin to lysosomes, suggesting that PI3-kinase may regulate the segregation of vitronectin and \beta 5 integrin to distinct intracellular compartments.

The present study showed that a specific PKC inhibitor, calphostin C, significantly increased the phagocytic index of fibronectin-coated beads. Similarly, Hall et al.\textsuperscript{55} reported that ROS ingestion by RPE cells is inhibited by increased PKC activity. Studies on phagocytic cells other than RPE cells (monocytes, neutrophils, macrophages, and gingival fibroblasts) have shown that PKC activation stimulates, rather than suppresses, phagocytosis.\textsuperscript{26,56–59} The discrepancy between these reports may be due to differences in confluence conditions, serum conditions, receptors, second-messenger systems, phagocytic mechanisms, or distribution of PKC isoenzymes in different cell types.\textsuperscript{55} Consistent with this hypothesis, inhibition of \alpha 5\beta 1-mediated fibronectin phagocytosis in K562 cells by the PKC inhibitor H7 did not occur in cells lacking \alpha\beta 3 but was prominent in cells transfected with \alpha\beta 2.\textsuperscript{20} An interesting finding was the much greater stimulation of phagocytosis of uncoated beads, when compared with that of fibronectin-coated beads, by PKC inhibition. Although this suggests that PKC is involved in both nonspecific and receptor-mediated phagocytosis, it also implies that different regulatory mechanisms exist for these diverse pathways of phagocytosis.

In agreement with our results, Heith and Schmidt\textsuperscript{60} demonstrated that phagocytic challenge of RPE cells with either specific or nonspecific particles is linked to the activation of phosphatas and kinases and that activation of PKC may play a role in phagocytosis of ROS and polystyrene latex microspheres.

One of the best studied signaling pathways activated by integrin binding is the ERK1/ERK2 MAP-K pathway. In this study we found no effect of the MAP-K inhibitor PD98059 on fibronectin phagocytosis at a concentration known to strongly inhibit MAP-K activity in RPE cells.\textsuperscript{56} The haptotactic response of RPE cells to fibronectin can be inhibited by PD98059 at similar concentrations, suggesting that different integrin-signaling pathways mediate different cell responses.

The complexity of the signaling pathways controlling fibronectin phagocytosis in RPE is demonstrated by the experiments in which inhibitors are combined. This is further complicated by the presence of serum components, necessary to maintain adequate levels of phagocytosis. The additive effect of tyrosine kinase and PI3-kinase inhibitors suggests that integrin binding leads to stimulation of parallel signaling pathways, each of which plays a role in the fibronectin phagocytosis process. Inhibitors of PKC can either stimulate or inhibit fibronectin phagocytosis depending on the presence of other inhibitors in the environment, suggesting complex interactions among these signaling pathways. Whether the PKC, tyrosine kinase, and PI3-kinase pathways differentially act to affect changes on distinct stages of the fibronectin phagocytosis process in RPE cells is yet to be determined.

A characteristic feature of the normal RPE cell is its remarkable specificity for phagocytosis of ROS.\textsuperscript{3} We demonstrate here that nonconfluent RPE cells also show highly specific phagocytosis of ECM components and that there is differential affinity for these components with the highest affinity for fibronectin. These results suggest the possibility that phagocytosis of fibronectin by RPE cells may represent a novel mechanism for remodeling of the provisional ECM during outer retinal wound healing. Because ECM components play a critical role in RPE cell migration and survival, alteration of the composition of the ECM in PVR-affected membranes could be of therapeutic interest. If fibronectin phagocytosis by RPE cells is shown to occur during wound healing in vivo, then the \alpha 5\beta 1 integrin and the PKC, PI3-kinase, and tyrosine kinase pathways may become novel pharmacological targets for modulation of this process.

Acknowledgments

The authors thank Ernesto Barron for technical assistance and Susan Clarke for her editorial review.

References


