Ligand Binding to Macrophage Scavenger Receptor-A Induces Urokinase-type Plasminogen Activator Expression by a Protein Kinase-dependent Signaling Pathway*

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Macrophage scavenger receptor-type A (MSR-A) has been implicated in the transmission of cell signals and the regulation of diverse cellular functions (Falcone, D. J., and Ferenc, M. J. (1988) J. Cell. Physiol. 135, 387–396; Falcone, D. J., McCaffrey, T. A., and Vergilio, J. A. (1991) J. Biol. Chem. 266, 22726–22732; Palkama, T. (1991) Immunology 74, 432–438; Krieger, M., and Herz, J. (1994) Annu. Rev. Biochem. 63, 601–637); however, the signaling mechanisms are unknown. In studies reported here, we demonstrate that binding of both lipoprotein and non-lipoprotein ligands to MSR-A induced protein tyrosine phosphorylation and increased protein kinase C (PKC) activity leading to up-regulated urokinase-type plasminogen activator (uPA) expression. Specifically, the binding of acetylated low density lipoprotein and fucoidan to MSR-A in human THP-1 macrophages triggered tyrosine phosphorylation of many proteins including phospholipase C-γ1 and phosphatidylinositol-3-OH kinase. Inhibitors of tyrosine kinase dramatically reduced MSR-induced protein tyrosine phosphorylation and PKC activity. Moreover, inhibitors of tyrosine kinase and PKC reduced uPA activity expressed by THP-1 macrophages exposed to MSR-A ligands. The intracellular signaling response for tyrosine phosphorylation following ligand binding was further demonstrated by using the stable MSR-transfected Bowes cells that express surface MSR-A. These findings establish for the first time a signaling pathway induced by ligand binding to MSR-A and suggest a molecular model for the regulation of macrophage uPA expression by specific ligands of the MSR-A.

Atherosclerosis is a chronic inflammatory disease characterized by the early and persistent presence of macrophages (5–8). Following their migration from the blood and transformation into lipid-laden cells (foam cells), monocyte-derived macrophages play a multifaceted role in lesion development (5, 9). The mechanisms by which macrophages are transformed into lipid-laden foam cells are not fully characterized. It has been demonstrated that macrophages express scavenger receptors types A and B (MSR-A, -B) that mediate the high affinity binding and internalization of modified forms of low density lipoprotein (LDL) (4, 10). These forms include acetoacetylated LDL (11), acetylated LDL (Ac-LDL) (10), malondialdehyde-modified LDL (12, 13), and oxidized LDL (14–17). The expression of MSR-A in macrophages and foam cells in atherosclerotic lesions (18), supports the proposed role for scavenger receptors in atherosclerosis (4, 19–21).

In addition to modified lipoproteins, a diverse group of polyanionic ligands are recognized by the MSR (4). These include sulfated polysaccharides (fucoidan and dextran sulfate) (1, 2), polyribonucleotides (poly(I) and poly(G)) (1, 2, 22), lipopolysaccharide (LPS), and lipopolysaccharide (LPS) (23), anionic phospholipids such as phosphatidylserine (24), crocidolite asbestos (25) and advanced glycation end products (26, 27). Owing to the broad nature of its ligand specificity, scavenger receptors have been proposed to play an important role in several macrophage functions including adhesion (27–29), clearance of pathologic substances (23, 25), and host defenses (3, 4, 21).

Several recent studies have demonstrated alterations in macrophage function following incubation with MSR-A ligands (2–4). For example, various MSR-A ligands including Ac-LDL, fucoidan, poly(I), and dextran sulfate have been shown to up-regulate the expression of urokinase-type plasminogen activator (uPA) by murine RAW264.7 macrophages (2). The induction of uPA expression by fucoidan was protein kinase C-dependent and required protein and RNA synthesis (2). Importantly, the enhanced expression of uPA by RAW264.7 macrophages following Ac-LDL challenge leads to plasmin-dependent extracellular matrix degradation and the release of matrix-bound growth factors, such as basic fibroblast growth factor and transforming growth factor-β (29). Taken together, these data suggest that the binding of specific ligands to the MSR-A may transmit a signaling response in the cell. However, direct evidence for MSR-mediated signal transduction event has not been demonstrated.

In studies reported here, we demonstrate for the first time that MSR ligands can trigger signal transduction pathways involving protein kinases in the macrophage. In addition, we demonstrate that macrophage uPA secretion following incubation with lipoprotein and non-lipoprotein ligands of the MSR-A is indeed dependent on MSR-mediated signaling. Finally, using transfection approaches, we confirm that the observed signal transduction is dependent on expression of the MSR-A.

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1 The abbreviations used are: MSR-A or MSR, macrophage scavenger receptor type-A; LDL, low density lipoprotein; Ac-LDL, acetylated-LDL; uPA, urokinase-type plasminogen activator; PLC-γ1, phospholipase C-γ1; PI 3-kinase, phosphatidylinositol-3-OH kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
EXPERIMENTAL PROCEDURES

Materials—Disposable tissue culture materials were purchased from Corning Glass Works (Corning, NY). Medium RPMI 1640, macrophage serum-free medium, l-glutamine, penicillin, streptomycin, and fetal calf serum were purchased from Life Technologies, Inc. Leupeptin and aprotinin were obtained from Boehringer Mannheim (Germany). Calphostin C and herbinicin A were purchased from Calbiochem (La Jolla, CA). Wortmannin was purchased from Calbiochem Research Laboratories, Inc. (Plymouth Meeting, PA). Sodium orthovanadate was obtained from Aldrich. HEPES, NaCl, glycerol, Triton X-100, MgCl₂, phenylmethylsulfonyl fluoride, phosphor 12-myristate 13-acetate (PMA), and bovine serum albumin (fraction V) were purchased from Sigma. Immobilon® membrane was purchased from Millipore. DuPont Western blot chemiluminescence Reagent, Renaissance®, a non-radioactive light-emitting system for chemiluminescence, was purchased from NEN Life Science Products. Primary antibody 5-Vale-Lys-aminomethylcoumarin was obtained from Enzyme Systems Products (Dublin, CA). Bovine plasminogen and high molecular weight uPA were obtained from American Diagnostica (Greenwich, CT). The Pfu DNA polymerase and PCR-script® SK+ cloning kit were obtained from Stratagene (La Jolla, CA). The vector pcDNA3, a selectable mammalian expression vector with a neomycin resistance marker, and calcium phosphate transfection kit for introduction of DNA into mammalian cells were obtained from Invitrogen (San Diego, CA). Human recombinant macrophage colony stimulating factor were obtained from R&D Systems, Minneapolis, MN. Anti-phosphotyrosine monoclonal antibody 4G10, anti-PLC-γ1 monoclonal antibody, and anti-PI 3-kinase polyclonal antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal anti-human uPA IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-human macrophage scavenger receptor antibody (hSRII-2 anti-macrophage scavenger receptor-A peptide antibody) was a gift from Dr. T. Kodama, University of Tokyo, Japan (18). Rabbit IgG, purified immunoglobulin from serum, was purchased from Sigma, and goat F(ab')₂ rabbit immunoglobulin (gamma and light chains) fluorescein conjugates for Flow Cytometric analysis, was purchased from BioSource International (Camarillo, CA). Cell Culture—Human monocyte-like THP-1 cells were cultured as described previously (30). In this paper, suspension cultures of human monocytic THP-1 cells were referred to as THP-1 monocytes; PMA-differentiated macrophage-like THP-1 cells are referred to as PMA-differentiated THP-1 macrophages or THP-1 macrophage as described (30). Bowes human melanoma cells, obtained from Dr. D. B. Rifkin, New York University Medical Center, New York, were grown in Dulbecco’s modified Eagle’s medium with high glucose (Life Technologies, Inc.) plus 10% fetal calf serum. Transfected Bowes cells were grown in the same medium containing the neomycin analog Geneticin, G418 sulfate (Life Technologies, Inc.) at 500 μg/ml.

Determination of Plasminogen Activator Activity—Plasminogen activator activity was quantitated utilizing a previously described modification of a sensitive functional assay for plasmin (29). Aliquots of serum-free conditioned media were added to microtiter wells containing 0.05% Dulbecco's phosphate-buffered saline containing Tween 20, plasmin substrate, 0.1% sodium aprotinin, and bovine plasminogen. Samples were mixed and incubated at 37 °C for 2.5 h. Cleavage of the substrate was monitored by measuring the increase in fluorescence in a Fluoroscan microplate reader. Concentrations of uPA in the conditioned media were extrapolated from a standard curve utilizing high molecular weight uPA. Plasminogen activator activity in media was completely inhibited when preincubated with a polyclonal anti-human uPA IgG, as described previously (2).

Immunoprecipitation—The methods for immunoprecipitation are described previously (30). Briefly, THP-1 macrophages or Bowes cells were grown in medium containing 0.01% fetal calf serum and maintained for 24 h prior to challenge with various reagents as indicated. Cell lysates were prepared in Triton lysis buffer (TL buffer: 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2 mM EDTA, pH 7.5, 1% Triton X-100, 0.1 mg/ml calf serum, 0.1% sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Lysates were normalized for protein concentration and incubated with anti-PLC-γ1 monoclonal antibody or anti-PI 3-kinase polyclonal antibody at 4 °C for 4 h. The next day, protein A/G plus agarose was added to the immune complexes and incubated at 4 °C for 2 h. Precipitated immunoprecipitates were washed three times in fresh TL buffer. The pellets were solubilized in SDS sample buffer with mild reducing conditions (boiling for 10 min in the presence of 2-mercapto- ethanol). Protein was loaded in each lane, separated by SDS-PAGE gel electrophoresis, and transferred to Immobilon™ membrane.

Immunoblotting—The methods for immunoblotting are described previously (30). Briefly, lysates were separated by SDS-PAGE and transferred to Immobilon™ membrane. The membranes were blocked with blocking buffer (PBS + 0.1% Tween 20 with 1% BSA) at room temperature for 1 h with slowly shaking. Then the membranes were immunoblotted with anti-phosphotyrosine monoclonal antibody 4G10 in a new blocking buffer for 2 h at room temperature with slowly shaking. After blotting, the membranes were washed in washing buffer (PBS + 0.1% Tween 20 with 1% BSA) once for 15 min followed by two washes for 5 min. The membranes were blocked again in a new blocking buffer for 1 h at room temperature with slowly shaking and immunoblotted with appropriated secondary antibodies conjugated with horseradish peroxidase in the same blocking buffer for 1 h at room temperature with slowly shaking. Afterward, membranes were further washed in washing buffer once for 15 min followed by two washes for 5 min. Protein visualization on each immunoblot was developed and performed with Renaissance®, DuPont Western blot chemiluminescence reagent (NEN Life Science Products) as described previously (30).

Assay of Protein Kinase C Activity in THP-1 Macrophages—THP-1 macrophages were treated with several agonists as described in Fig. 4. At the desired time, cells were washed twice with ice-cold PBS (without Ca²⁺, Mg²⁺), harvested, and pelleted at 325 × g, 4 °C. The cell pellet was resuspended in TE (20 mM Tris, pH 7.4, 20 mM EDTA), containing 2 mM EGTA, 10 μg/ml leupeptin, 0.3 mM phenylmethylsulfonyl fluoride, and 10% glycerol. The assay for total PKC activity was performed as described previously (31) or with the use of the Amersham Protein Kinase C enzyme assay system (Amersham Corp.).

Assay of Protein Kinase A Activity from THP-1 Macrophages—The cell pellet prepared was similar to the above PKC assay. To assay the PKA activity in the cell, we followed methods described previously (31) or used the Life Technologies, Inc., protein kinase A enzyme assay system (Life Technologies, Inc.).

Isolation of LDL and Preparation of Modified LDL—Human LDL (d 1.019–1.063 g/ml) was prepared as described (32). LDL preparations were occasionally screened for peroxides (33).

RNA Isolation and Northern Analysis—Total RNA was isolated by the guanidinium isothiocyanate method (34). Northern blot analyses were performed as described (31, 35). The cDNA for the human uPA (36) was purchased from ATCC (Rockville, MD). The cDNA for human 28S ribosomal RNA was a gift from Dr. Iris L. Gonzalez, Hahnemann University. UPA RNA from Northern blots was quantified using a PhosImage™ (Molecular Dynamics, CA) and normalized by comparison to mRNA of 28S ribosomal RNA, a constitutively expressed gene.

Construction of N-MSR Expression Vectors, MSR cDNA Stable Transfection, and Transfectant Cell Culture—The expression vectors pcDNA3-MSR representing human normal macrophage scavenger receptor cDNA (N-MSR) was derived from the human MSR type A cDNA (15). The orientation and sequence of this construct were verified and confirmed by both restriction enzyme-digestion mapping and DNA sequencing using Perkin-Elmer/Applied Biosystems model 373 DNA Stretch Sequencer. Bowes cells were sub-passaged the day before transfection by using calcium phosphate transfection method as described (31), or the protocol provided in calcium phosphate transfection kit (Invitrogen). The pcDNA3-MSR expression vector was transfected into 60% confluent Bowes. The next day, fresh medium was added, and selection began 24 h later with the addition of Geneticin, G418 sulfate (potency 500 μg/ml) to the medium. Transfected cells were then maintained continuously in Geneticin-containing media. On days 14–16, the surviving 60 colonies were picked and subsequently grown into mass transfectant cultures and for other experiments.

Flow Cytometric Analysis of Macrophage Scavenger Receptor A Expression in MSR-transfected Bowes Cells—Rabbit polyclonal anti-human MSR-A antibody (hSRII-2 anti-macrophage scavenger receptor peptide antibody) was used in the flow cytometric analysis. Bowes cells (wild type or transfected) were removed from culture dishes with a rubber policeman, washed three times with phosphate-buffered saline (PBS), and resuspended in PBS containing 1% bovine serum albumin. Cells were then aliquoted in 50 ml volumes containing 10⁶ cells and incubated with the hSRII-2 antibody (150 μg/ml) or control rabbit IgG, purified immunoglobulin from serum, for 30 min rotated at 4 °C. The cells were then pelleted by centrifugation and washed two times with PBS, 1% BSA. Cells were then resuspended in PBS, 1% BSA with a 1:50 dilution of fluorescein-conjugated goat F(ab')₂ anti-rabbit immunoglobulin and rotated for 30 min at 4 °C. The cells were then
Ac-LDL and Fucoidan Up-regulate THP-1 Macrophage uPA Expression—The effect of lipoprotein and nonlipoprotein ligands of the MSR-A on uPA expression by macrophages was determined. For these studies, we utilized phorbol myristate acetate (PMA)-treated human THP-1 monocytes. Following PMA-induced differentiation of THP-1 monocytes into macrophage-like cells, MSR-A expression was up-regulated, and the cells became adherent (30, 39). Following a 12-h incubation with Ac-LDL or fucoidan, the steady state levels of uPA mRNA in THP-1 macrophages were dramatically increased compared with control cells or cells incubated with LDL. When examined at 24 h, Ac-LDL and fucoidan-induced increase in uPA mRNA levels remained elevated relative to control. In contrast, neither Ac-LDL nor fucoidan altered uPA mRNA levels in THP-1 monocytes, which are devoid of MSR-A activity (30) (data not shown).

We next determined whether the observed increase in uPA mRNA levels (Fig. 1) was reflected by an increase in the expression of uPA activity by THP-1 macrophages. Cells were incubated overnight in media alone or media supplemented with LDL, Ac-LDL, or fucoidan. As seen in Table I, there was a small increase (2-fold) in uPA activity expressed by THP-1 macrophages incubated with Ac-LDL. In contrast, incubation with the MSR-A ligands Ac-LDL and fucoidan stimulated uPA activity 5- and 10-fold, respectively. Taken together, these data corroborate and extend our earlier observations utilizing murine RAW264.7 macrophages (2).

Ac-LDL and Fucoidan Induce Protein Tyrosine Kinase and Protein Kinase C—To determine whether MSR-A ligands trigger specific signal transduction pathways in THP-1 macrophages, tyrosine phosphorylation was determined following incubation with Ac-LDL and fucoidan. Cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with monoclonal anti-phosphotyrosine IgG. As seen in Fig. 2A, incubation of THP-1 macrophages with Ac-LDL and fucoidan induced the appearance of many phosphotyrosyl proteins when compared with cells incubated with media alone or native LDL. MSR-A ligands did not alter the expression of phosphotyrosyl proteins in THP-1 monocytes (data not shown).

To investigate further the induction of phosphotyrosyl proteins by ligand binding to the MSR-A, we used the specific protein tyrosine kinase inhibitor, herbimycin A. As shown in Fig. 2B, preincubation of THP-1 macrophages with herbimycin A blocked the protein tyrosine phosphorylation observed in control cells following the addition of Ac-LDL or fucoidan.

Cell lysates derived from THP-1 macrophages incubated with MSR-A ligands contained 150-kDa (pp150) and 85-kDa (pp85) proteins that were prominently identified with anti-phosphotyrosine IgG (Fig. 2A). To explore further the identities of these two phosphotyrosyl proteins, cell lysates were incubated with monoclonal anti-phospholipase C-gamma specific signal transduction pathways in THP-1 macrophages was determined. For these studies, we utilized the selective and potent inhibitors herbimycin A (40–49), wortmannin (44–46), and calphostin C (47–49) for tyrosine kinase, PI 3-kinase, and PKC, respectively. As seen in Fig. 5, THP-1 macrophage uPA expression was increased approximately 3-fold when incubated with fucoidan, whereas herbimycin A or calphostin C inhibited fucoidan-induced uPA activity without affecting constitutive expression.

Inhibitors of Tyrosine Kinase and PKC Inhibit uPA Expression Induced by Ac-LDL and Fucoidan—Because MSR-A ligands induce phosphotyrosyl proteins in THP-1 macrophages, including PLC-γ1 and PI 3-kinase, and the activation of PKC, we next determined the role of tyrosine kinase, PI 3-kinase, and PKC in MSR-A ligand-induced macrophage uPA secretion. For this purpose, we utilized the selective and potent inhibitors herbimycin A (40–49), wortmannin (44–46), and calphostin C (47–49) for tyrosine kinase, PI 3-kinase, and PKC, respectively. As seen in Fig. 5, THP-1 macrophage uPA expression was increased approximately 3-fold when incubated with fucoidan, whereas herbimycin A or calphostin C inhibited fucoidan-induced uPA activity without affecting constitutive expression.
Ac-LDL and Fucoidan Stimulate Protein Tyrosine Phosphorylation in MSR-transfected Human Bowes Melanoma Cells—To support further our hypothesis that the MSR-A is a signaling receptor, we generated stable normal MSR transfectants from human Bowes melanoma cells. The expression of scavenger receptor in MSR transfectants was verified by flow cytometric analysis (Fig. 6). Moreover, we next determined whether expression of MSR-A by Bowes cells can lead to protein tyrosine phosphorylation following exposure to MSR-A ligands. As shown in Fig. 7, base-line levels of protein tyrosine phosphorylation were similar in normal MSR transfectants (N-MSR) and wild type cells. A 10-min exposure of cells to Ac-LDL resulted in increased levels of tyrosine phosphorylation in cells expressing MSR-A, whereas incubation of wild type cells with Ac-LDL did not appear to alter protein tyrosine phosphorylation. Following 60 min exposure to Ac-LDL, levels of tyrosine phosphorylation in wild type cells were increased but markedly less than that observed in MSR-A transfectants. Tyrosine phosphorylation induced by ligands for the MSR type A, fucoidan, was different than that observed with Ac-LDL in several ways. First, tyrosine phosphorylation in cells expressing MSR-A was dramatically increased following addition of fucoidan for 10 min. Second, fucoidan induced the phosphorylation of several low molecular weight proteins, which were not observed when cells were incubated with Ac-LDL. Third, wild type cells exposed to fucoidan exhibited a relatively small increase in protein tyrosine phosphorylation. Fourth, levels of tyrosine phosphorylation in wild type cells were increased but markedly less than that observed in MSR-A transfectants. Tyrosine phosphorylation induced by ligands for the MSR type A, fucoidan, was different than that observed with Ac-LDL in several ways. First, tyrosine phosphorylation in cells expressing MSR-A was dramatically increased following addition of fucoidan for 10 min. Second, fucoidan induced the phosphorylation of several low molecular weight proteins, which were not observed when cells were incubated with Ac-LDL. Third, wild type cells exposed to fucoidan exhibited a relatively small increase in protein tyrosine phosphorylation. Fourth, levels of tyrosine phosphorylation in wild type cells were increased but markedly less than that observed in MSR-A transfectants. Tyrosine phosphorylation induced by ligands for the MSR type A, fucoidan, was different than that observed with Ac-LDL in several ways. First, tyrosine phosphorylation in cells expressing MSR-A was dramatically increased following addition of fucoidan for 10 min. Second, fucoidan induced the phosphorylation of several low molecular weight proteins, which were not observed when cells were incubated with Ac-LDL. Third, wild type cells exposed to fucoidan exhibited a relatively small increase in protein tyrosine phosphorylation. Fourth, levels of tyrosine phosphorylation in wild type cells were increased but markedly less than that observed in MSR-A transfectants.

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FIG. 2. Stimulation of protein tyrosine phosphorylation in THP-1 macrophages upon ligand binding to MSR-A. A, THP-1 macrophages were preincubated 24 h in 0.01% fetal calf serum. Cells were untreated (lane 1) or treated with 100 μg/ml LDL (lane 2), 100 μg/ml acetylated LDL (lane 3), 20 μg/ml fucoidan (lane 4) for 15 min at 37 °C. Cells were lysed in TL buffer and centrifuged. The supernatants were collected, and SDS sample buffer was added. The same amounts of protein were loaded onto each lane. Immunoreactive phosphorylated tyrosine proteins were identified by Western blot procedures as described under “Experimental Procedures.” B, in a similar experiment, cells were preincubated (30 min) with or without herbinycin A (5 μM) and treated with 100 μg/ml acetylated LDL (Ac-LDL, lanes 3 and 1, respectively) or with 20 μg/ml fucoidan (lanes 4 and 2, respectively). Immunoreactive phosphorylated tyrosine proteins were identified as described under “Experimental Procedures.”

FIG. 3. Identification of tyrosine-phosphorylated PLC-γ1 (A) and PI 3-kinase (B). Cells were incubated with media alone (lane 1), LDL (lane 2), Ac-LDL (lane 3), or fucoidan (lane 4) as described in Fig. 2. Immunoprecipitation of the cell lysates was then performed with anti-PLC-γ1 monoclonal antibody (A) or anti-PI 3-kinase polyclonal antibody (B). The immunoprecipitates were resolved by SDS-PAGE, and immunoblots were probed with anti-phosphotyrosine monoclonal antibody as described under “Experimental Procedures.” In addition, immunoprecipitates utilizing anti-PI 3-kinase polyclonal antibody from lysates of macrophage colony stimulating factor-treated cells were included as a positive control for phosphorylation of PI 3-kinase in B (lane 5).

DISCUSSION
The scavenger receptor type A plays an important role in several macrophage functions including adhesion (27, 28),
clearance of pathologic substances (23, 25), and host defenses (4, 21). Although several studies have demonstrated alterations in macrophage function following incubation with MSR-A ligands (2–4), there is no evidence to date that the MSRs mediate these observed alterations in macrophage function. Previously, we and others (2, 50, 51) demonstrated that exposure of Corynebacterium parvum-activated peritoneal murine macrophages and murine macrophage cell lines (RAW264.7 and J774A.1 cells) to MSR-A ligands stimulates their secretion of uPA. In studies reported here, we demonstrate for the first time that exposure of human THP-1 macrophages with MSR-A ligands can stimulate a series of signaling events, including activation of tyrosine kinase and PKC activities which lead to uPA expression. Moreover, exposure of Bowes melanoma cells transfected with MSR type A to either Ac-LDL or fucoidan can significantly up-regulate tyrosine kinase activity, whereas MSR-A ligands had no effect on mock-transfected cells. These data demonstrate that the binding of ligands to the MSR-A stimulates protein kinases leading to alterations in macrophage functions.

In these studies, we have used human THP-1 macrophages to determine the role of MSR-A in uPA expression. THP-1 monocyes do not normally express MSR-A (30); however, when exposed to PMA, they differentiate into macrophage-like cells (30, 39, 52) and express MSR-A (30, 39, 53). Exposure of THP-1 monocyes that are devoid of scavenger receptor activity with MSR-A ligands did not affect steady state levels of uPA mRNA or their expression of uPA activity. In contrast, exposure of THP-1 macrophages with MSR-A ligands up-regulated uPA mRNA levels and uPA activity (Fig. 1 and Table I). Therefore, the MSR-A appears to play an important role in the regulation of THP-1 macrophage uPA expression.

To understand how binding of MSR-A ligands to the MSR-A is coupled to the initiation of signaling pathways leading to uPA expression in macrophage, several signaling cascades were initially examined. We found that Ac-LDL and fucoidan induced tyrosine phosphorylation of numerous proteins in THP-1 macrophages by Western blot analysis with monoclonal anti-phosphotyrosine IgG (Fig. 2A). Preincubation of cells with herbimycin A, an inhibitor of tyrosine kinase, blocked the ability of MSR-A ligands to induce tyrosine phosphorylation in THP-1 macrophages (Fig. 2B). In preliminary studies, we determined whether MSR ligands trigger tyrosine phosphorylation and increase uPA expression in fully differentiated human macrophages. Peripheral blood monocytes were allowed to differentiate in culture for 10 days. The monocyte-derived macrophages were then exposed to LDL, Ac-LDL, or fucoidan for 15 min, following which lysates were prepared. Based on Western blots, exposure of monocyte-derived macrophages to Ac-LDL or fucoidan resulted in an increase in protein tyrosine phosphorylation. When examined by Northern blot, the steady state level of uPA mRNA was elevated in cells following an overnight exposure to fucoidan.

Of the many phosphorylated proteins observed following incubation with MSR-A ligands, we identified PLC-γ1 and PI3-kinase, two important signaling molecules, utilizing combined immunoprecipitation and Western blot analysis (Fig. 3, A and B). Tyrosine phosphorylation of PLC-γ1 and PI3-kinase is also observed following engagement of growth factor receptors and cytokine receptors (54–57). However, MSR-A ligands do
not function as growth factors and cytokines, and, unlike receptors for growth factor and cytokine, the MSR-A does not have an obvious functional tyrosine kinase domain (4, 37, 39). Therefore, these data suggest that signaling following engagement of the MSR-A is probably mediated by some associated cytosolic tyrosine kinase molecules.

It has been shown that PMA can stimulate macrophage uPA secretion (58, 59). Moreover, we demonstrated that the ability of the MSR-A ligand, fucoidan, to up-regulate murine RAW264.7 macrophage uPA expression can be blocked with inhibitors of PKC (2). In these studies preincubation of THP-1 macrophages with calphostin C, a specific PKC inhibitor, completely inhibited uPA secretion induced by the MSR-A ligands (Fig. 6), suggesting that PI 3-kinase may not involve MSR-mediated uPA activity. The role and significance of PI 3-kinase in MSR-mediated signaling in macrophages need further investigation.

Furthermore, we found that herbimycin A inhibited Ac-LDL- and fucoidan-induced tyrosine phosphorylation of PLC-γ1 as well as their ability to increase PKC activity (Figs. 2B and 4). Herbimycin A can inhibit tyrosine phosphorylation of PLC-γ1 by an IgE-induced non-receptor tyrosine kinase (63). Moreover, inhibition of PLC-γ1 activation leads to decreased phospholipid hydrolysis and decreased PKC activity (63). These data suggest that engagement of the MSR-A leads to sequential activation of tyrosine kinase, PLC-γ1, and PKC.

Recently, the MSR-A has been identified as an opsonin-independent phagocytosis-promoting receptor. Moreover, MSR-dependent phagocytosis was involved in the activation of tyrosine kinase and PI 3-kinase (64). In these studies, we demonstrate that MSR-A ligands trigger signaling pathways leading to tyrosine phosphorylation of PI 3-kinase (Figs. 3 and 5). In fact, we found that preincubation of THP-1 macrophages with wortmannin, a potent and selective inhibitor of PI 3-kinase (44–46), had no effect on uPA expression induced by MSR-A ligands (Fig. 6), suggesting that PI 3-kinase may not involve MSR-mediated uPA activity. The role and significance of tyrosine phosphorylation of PI 3-kinase in MSR-mediated signaling in macrophages need further investigation.

Finally, to determine whether MSR-A plays a direct role in the observed signaling events including alterations in protein phosphorylation upon ligation, we stably trans fused Bowes...
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melanoma cells with the cDNA of normal MSR-A. MSR-transfected cells (N-MSR) challenged with lipoprotein (Ac-LDL) and non-lipoprotein (fucoidan) ligands of the receptor exhibited similar protein tyrosine phosphorylation patterns as THP-1 macrophages challenged with MSR-A ligands (Figs. 2A and 7). It is unclear why there is little nonspecific induction of protein tyrosine phosphorylation by both Ac-LDL and fucoidan in wild type Bowes cells (lanes 4 and 6 in Fig. 7). Interestingly, the protein tyrosine phosphorylation induced by fucoidan in MSR-transfected cells quickly returned to the basal level. In contrast, Ac-LDL-induced tyrosine phosphorylation increased over time. These results suggest that the kinetics for protein tyrosine phosphorylation induced by fucoidan and Ac-LDL is different. The physiological significance of this particular finding is unclear.

In summary, the activation of various signaling molecules including protein kinases in both macrophages and the MSR-transfected Bowes cells shows that the macrophage scavenger receptor type A can act as a signaling receptor. Additionally, because of its role in uPA activation, it may have a significant participant in tissue remodeling during inflammation and vascular diseases.

REFERENCES

1. Falcone, D. J., and Ferenc, M. J. (1988) J. Cell. Physiol. 135, 397–396
2. Falcone, D. J., McCaffrey, T. A., and Vergilio, J. A. (1991) J. Biol. Chem. 266, 22726–22732
3. Palkama, T. (1991) Annu. Rev. Biochem. 59, 574–576
4. Krieger, M., and Herz, J. (1994) Annu. Rev. Biochem. 63, 601–637
5. Ross, R. (1993) Nature 362, 801–809
6. Hansson, G. K., Seifert, P. S., Olsson, G., and Bondjers, G. (1991) Arteriosclerosis. Thromb. 11, 745–750
7. Jonasson, L., Holm, J., Skalli, O., Bondjers, G., and Hansson, G. K. (1996) Arterioscler. Thromb. 16, 131–138
8. Gown, A. M., Tsukada, T., and Ross, R. (1986) Am. J. Pathol. 122, 203–212
9. Krieger, M. (1990) Arterioscler. Thromb. 10, 9213–9220
10. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 504–508
11. Naito, M., Suzuki, H., Mori, T., Shibayama, R., Kawabe, Y., Kodama, T., Takahashi, K., Shichiri, M., and Horiiuchi, S. (1989) Eur. J. Biochem. 200, 408–415
12. el Khoury, J., Thomas, C. A., Loike, J. D., Hickman, S. E., Cao, L., and Vassalli, J.-D. (1993) J. Cell. Physiol. 155, 585–595
13. Takahashi, K., Freeman, M., Rohrer, L., Zubarecky, J., Matsudaira, P., and Steinbrecher, U., Ishibashi, S., Maeda, N., Gordon, S., and Kodama, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 342–344