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Macrophage Binding and the Uptake of Oxidized Low Density Lipoprotein Are Regulated by Intracellular Protein Phosphorylation[1]

Masatoshi Beppu, Kenji Ohishi, Masaaki Kasahara, Kengo Kizaki, Yuko Inohana, and Kiyomi Kikugawa[2]

School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392

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The involvement of intracellular protein phosphorylation in macrophages in the binding and uptake of oxidized low density lipoprotein (oxLDL) was investigated. The treatment of fibronectin-unstimulated and stimulated mouse thioglycolate-induced macrophages with inhibitors of myosin light chain kinase, protein kinase C and protein tyrosine kinase resulted in decreased macrophage binding of oxLDL, macrophage foam cell formation, and whole intracellular protein phosphorylation. The treatment of fibronectin-unstimulated and stimulated macrophages with inhibitors of protein serine/threonine and tyrosine phosphatases caused enhanced macrophage binding of oxLDL, macrophage foam cell formation, and whole intracellular protein phosphorylation. Fibronectin, which stimulates macrophage activity, enhanced macrophage intracellular protein phosphorylation. Myosine light chain phosphorylation may be involved in the fibronectin stimulation of macrophages. Treatment of fibronectin-unstimulated and stimulated macrophages with thiophosphate, which forms thiophosphate esters of intracellular proteins that are not so susceptible to protein phosphatases, enhanced macrophage binding of oxLDL. The above results indicate that intracellular protein phosphorylation maintains and enhances macrophage binding and the uptake of oxLDL.

Key words: fibronectin, macrophage, oxidized LDL, protein phosphorylation.

Lipid-laden foam cells in the arterial wall of atherosclerotic lesions are considered to originate from macrophages that have accumulated cholesterol within the cells (*1, 2*). It has been found that macrophages take up oxidized low density lipoprotein (oxLDL) *in vitro* (*2, 3*), resulting in cholesterol accumulation, and that oxLDL is present in atherosclerotic lesions (*4–6*). These findings have confirmed the role of oxLDL uptake by macrophages in atherogenesis.

Fibronectin enhances the scavenger receptor activity of mouse macrophages (*7*) by increasing the number of scavenger receptors by either increasing transport from the cytoplasmic receptor pool or by redistribution from cell attachment sites to the apical surface of the cell (*8*). Fibronectin stimulates macrophages through fibronectin receptors (*9–11*) causing increased Ca^{2+} signaling and an enhancement of scavenger receptor activity.

The aim of the present study was to examine whether the intracellular protein phosphorylation of fibronectin-unstimulated and stimulated macrophages is involved in the binding and uptake of oxLDL. Intracellular protein phosphorylation is induced by myosin light chain kinase (MLCK) (*12–15*), protein kinase C (PKC) and protein tyrosine kinase (PTK) (*17*), and controlled by protein serine/threonine (*18*) and tyrosine phosphatases (*19*). In the present study, we used known inhibitors of protein kinases and phosphatases to investigate the participation of protein phosphorylation in the binding and uptake of oxLDL by macrophages. It was found that macrophage activity is maintained and enhanced by the intracellular protein phosphorylation of macrophages by MLCK, PKC, and PTK, and that the activity is modulated by intracellular protein dephosphorylation by protein phosphatases

MATERIALS AND METHODS

Materials—Fluid thioglycollate medium, RPMI 1640 medium (phosphate free) and penicillin-streptomycin were obtained from Gibco Laboratories, Grand Island, NY. Fetal calf serum (FCS) was from Bio Whittaker, Walkersville, MD, and Hanks' balanced salt solution (HBSS) (without phenol red) was from Nissui Pharmaceuticals, Tokyo. Cytodex-1 and Ampholine (pH 3.5–9.5) were from Pharmacia Biotech, Uppsala, Sweden, and *N*-2-hyroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) was from Dojindo Laboratories, Kumamoto. Nonidet P-40 (NP-40), bovine serum albumin (BSA, γ-globulin free), phenylmethylsulfonyl fluoride (PMSF), and oil-red-O were purchased from Sigma

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[2] To whom correspondence should be addressed Tel +81-426-76-4503, Fax +81-426-76-4508, E-mail: kikugawa@ps toyaku ac jp

Abbreviations BSA, bovine serum albumin, DFP, diisopropyl fluorophosphate, DPBS, Dulbecco's phosphate buffered saline, DPBS (–), Ca^{2+}- and Mg^{2+}-free DPBS, FCS, fetal calf serum, HEPES, *N*-2-hyroxyethylpiperazine-*N'*-2-ethane sulfonic acid, HBSS, Hanks' balanced salt solution; IEF, isoelectrofocusing; LDL, low density lipoprotein; MLC, myosin light chain, MLCK, myosin light chain kinase; NP-40, Nonidet P-40, oxLDL, oxidized LDL, PAGE, polyacrylamide gel electrophoresis, PKC, protein kinase C, PMSF, phenylmethylsulfonyl fluoride; RBC, red blood cell; SDS, sodium dodecylsulfate, TCA, trichloroacetic acid.

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Chemical, St. Louis, MO. Diisopropyl fluorophosphate (DFP), leupeptin, herbimycin A, genistein, okadaic acid, sodium orthovanadate (vanadate), and Mayer's hematoxylin solution were obtained from Wako Pure Chemical Industries, Osaka. Protein kinase inhibitors, ML-9, ML-7, and H-7, were obtained from Seikagaku, Tokyo. Staurosporine was from Kyowa Hakko Kogyo, Tokyo. $H_3{}^{32}PO_4$ was from ICN Biomedicals, Costa Mesa, CA. Sodium thiophosphate was from Aldrich Chemical, Milwaukee, MI.

Fibronectin was purified from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B columns according to the method of Engvall and Ruoslahti (*20*) with minor modifications as described previously (*21*). Phosphate-free fibronectin was prepared by dialysis against 10 mM HEPES (pH 7.4)/0.02% NaN_3/saline, and stored in the presence of 1 mM DFP. The fibronectin concentration was determined spectrophotometrically using E (1%, 1 cm) at 280 nm = 12 8 (*22*). Mouse macrophage myosin was purified according to the procedures described elsewhere (*23, 24*).

Coating of Culture Substrates with Fibronectin—Round coverslips, 18 mm or 15 mm diameter, in microplate wells were loaded with 200 or 140 μl of a solution of fibronectin (100 μg/ml), respectively, incubated at room temperature for 12–24 h, rinsed several times with Dulbecco's phosphate-buffered saline (DPBS), and used immediately.

Cytodex-1 beads, microcarrier beads for cell culture, stored in 70% ethanol at 6 mg/ml, were washed four times with buffer A, comprising 140 mM NaCl, 5 mM KCl, and 10 mM HEPES (pH 7.4), and twice with RPMI 1650 medium (phosphate free) containing 20 mM HEPES (pH 7.2) [RPMI-HEPES medium (phosphate-free)] by centrifugation at 1,500 ×*g* for 5 min. For fibronectin-coating, a mixture of the phosphate-free fibronectin solution in buffer A at 100 μg/ml and Cytodex-1 beads in buffer A was incubated at room temperature for more than 6 h with gentle shaking to ensure coating of the beads with fibronectin. The beads were washed with buffer A and successively with RPMI-HEPES medium (phosphate-free), and resuspended in RPMI-HEPES medium (phosphate-free) at 90 mg/ml. Cytodex-1 beads not coated with fibronectin were similarly prepared without the addition of fibronectin.

Macrophages—Macrophages were obtained from the peritoneal cavity of 7–12-week-old male ddY mice 4 d after intraperitoneal injection of 2–3 ml of 3% thioglycollate medium. The peritoneal exudate cells obtained were washed twice with HBSS by centrifugation at 80 ×*g* at 4°C for 10 min, and resuspended in RPMI-HEPES medium for use. For ^{32}P-radiolabeling, the cells were washed twice with RPMI-HEPES medium (phosphate-free) by centrifugation at 80 ×*g* at 4°C for 10 min and resuspended in RPMI-HEPES medium (phosphate-free) for use.

Isolation and Oxidation of LDL—LDL were isolated from fresh human plasma as described previously (*25*). The LDL fraction obtained was dialyzed against Ca^{2+}- and Mg^{2+}-free DPBS [DPBS (–)], and subjected to Cu^{2+}-catalyzed oxidation (*26*) as described previously (*7*). The protein content in the LDL was determined by the Lowry method (*27*) using BSA as a standard.

Red Blood Cells Coated with oxLDL—Red blood cells (RBC) coated with native LDL (native LDL-coated RBC) or oxLDL (oxLDL-coated RBC) were prepared by incubating mouse RBC with 1 μg/ml of native LDL or oxLDL as described previously (*28*). The coated RBC were suspended in RPMI-HEPES medium at 2% cell suspension, and used immediately. LDL protein (2–3 ng) was adsorbed to 10^6 RBC (*28*).

Binding of oxLDL-Coated RBC to Macrophages—In the binding of oxLDL to macrophages, a simple method developed by us (*28*) was employed. A 200 μl suspension of mouse macrophages in RPMI-HEPES medium (8.5 × 10^5 cells/ml) was loaded onto a fibronectin-uncoated (plain) or a fibronectin-coated coverslip 18-mm diameter, and incubated at 37°C for 1 h in a 5% CO_2 incubator. Nonadherent cells were removed by washing three times with DPBS (–) To investigate the effect of inhibitors of protein kinases and protein phosphatases, the macrophage monolayer was preincubated with 200 μl of RPMI HEPES medium containing an inhibitor at the indicated concentration at 37°C for 2 h. The monolayer was washed with DPBS (–), and 200 μl of a 2% cell suspension of native LDL-coated RBC or oxLDL-coated RBC was loaded onto the monolayer. After incubation at 37°C for 1 h, nonadherent coated RBC were removed by gentle washing with DPBS (–) and the cells were fixed with 1.25% glutaraldehyde in DPBS (–). The number of macrophages binding two or more coated RBC on their surface was scored for random fields of the coverslip under phase-contrast microscopy. At least 200 macrophages were examined for binding of coated RBC, and the percentage of macrophages binding two or more coated RBC was determined.

Uptake of oxLDL by Macrophages and the Staining of Lipid Droplets within Cells—A 140 μl suspension of mouse macrophages in RPMI-HEPES medium (8.5 × 10^5 cells/ml) was loaded onto a fibronectin-uncoated (plain) or a fibronectin-coated coverslip, 15-mm diameter, and incubated at 37°C for 1 h in a 5% CO_2 incubator. Nonadherent cells were removed by washing three times with DPBS (–), and the coverslips with macrophage monolayers were placed in the wells, 16-mm in diameter, of a culture plate. To the wells was added 0.5 ml of RPMI 1640 medium containing oxLDL (100 μg protein/ml), 5% heat-inactivated FCS, and an inhibitor of protein kinase or phosphatase, and the cells were incubated at 37°C for 24 h. The macrophage monolayer was washed with DPBS (–), fixed with formalin, and neutral lipid droplets within the cells were stained with oil red O, after which the nuclei were stained with Mayer's hematoxylin solution. The number of macrophages containing lipid droplets stained red was scored for random fields of the coverslip. At least 200 cells were examined, and the percentage of positively stained cells was determined.

Viability of Macrophages—For the test of cell viability during incubation with inhibitors for 2 h, a mixture of 200 μl of the suspension of mouse macrophages in RPMI-HEPES medium (8 × 10^5 cells/ml) and 200 μl of RPMI-HEPES containing the inhibitors at a concentration 2-fold of that used in the binding assay in a siliconized glass tube was incubated at 37°C for 2 h in a 5% CO_2-incubator. For the test of cell viability during incubation with inhibitors for 24 h, 140 μl of the cell suspension in a siliconized tube was centrifuged at 80 ×*g* at 4°C for 10 min, and washed with RPMI medium. To the pellet, 500 μl of RPMI-medium containing inhibitors at the indicated concentration was added and the mixture was incubated at 37°C for 24 h. Cell pellet was obtained by centrifugation at 80 ×*g* and 4°C for 10 min, resuspended in 50 μl of RPMI-HEPES, and an

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equal volume of a solution of 0.16% Trypan blue in saline was added. The percentage of viable cells among more than 200 cells was determined.

^{32}P-Phosphate Radiolabeling of Macrophage Soluble Proteins and Their Analysis—A 20 ml suspension of mouse macrophages in RPMI-HEPES medium (phosphate free) (1 × 10^7 cells/ml) was mixed with 20 ml of plain Cytodex-1 or phosphate-free fibronectin-coated Cytodex-1 suspension in a siliconized glass tube, and incubated at 37°C for 1 h with gentle swirling. The mixture was incubated with RPMI-HEPES medium (phosphate-free) containing each of the protein kinase inhibitors at the indicated concentration at 37°C for 1 h, and subsequently with RPMI-HEPES medium (phosphate-free) containing 500 μl of $H_3{}^{32}PO_4$ solution (1 mCi/250 μl) in 0.9% NaCl at 37°C for 2 h, or the mixture was incubated with the $H_3{}^{32}PO_4$ solution at 37°C for 1 h and subsequently with RPMI-HEPES (phosphate-free) containing each of the protein phosphatase inhibitors at 37°C for 2 h The mixture was allowed to stand under cooling to precipitate the Cytodex-1 beads. After removal of the supernatant, the Cytodex-1 beads were washed with buffer B, composed of 50 mM NaF, 5 mM $Na_4P_2O_7$, 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), several times by sedimentation, and finally by centrifugation at 2,000 ×*g* at 4°C for 10 min.

The cells in the pellet were solubilized in an equal amount of NP-40 buffer composed of 2% NP-40, 100 mM NaF, 50 mM $Na_4P_2O_7$, 500 mM NaCl, 10 mM EGTA, 5 mM EDTA, 50 μg/ml leupeptin, 4 mM DFP, 2 mM PMSF, and 25 mM Tris-HCl buffer (pH 7.9) by freeze-thawing twice between dry ice/acetone and 37°C. The suspension was centrifuged at 2,000 ×*g* at 4°C for 10 min to precipitate the Cytodex-1 beads, and the supernatant was centrifuged at 18,000 ×*g* at 4°C for 10 min to obtain solubilized macrophage proteins. The protein content in the supernatant was determined by the Lowry method (0.2–0.6 mg protein/ml).

Trichloroacetic acid (TCA)–precipitable proteins were obtained as follows. To the solubilized 1% NP-40 fraction, whose protein amount was adjusted at 0 1 mg, TCA solution was added to a final concentration of 10%. The mixture was kept under cooling for 10 min, and then centrifuged at 18,000 ×*g* and 0°C for 10 min to obtain a pellet. The pellet was washed with 90% acetone below 0°C by centrifugation at 18,000 ×*g* for 10 min. The pellet was solubilized in 2% sodium dodecylsulfate (SDS), and the radioactivity was counted.

For SDS–polyacrylamide gel electrophoresis (PAGE) of the macrophage solubilized proteins in 1% NP-40, 20 μl of a sample solution containing 1 mg protein/ml was subjected to SDS-PAGE with Laemmli's discontinuous buffer system (*29*) using a 5–20% polyacrylamide gradient gel (PAGEL, ATTO, Tokyo) and a 4.5% stacking gel For two-dimensional electrophoresis (*30*) of TCA-precipitable proteins, 10 μl of sample solution dissolved in 0.5% SDS at 1 mg protein/ml was mixed with 130 μl of 30% TCA, and the mixture was kept at 0°C for 10 min. The protein precipitate obtained by centrifugation at 15,000 ×*g* for 10 min was washed with 90% acetone, and dissolved in 10 μl of 1% SDS. The solution was diluted 2-fold with water to obtain 20 μl of sample solution in 0.5% SDS. To the sample solution, 20 mg of urea and 5 μl of a mixture of 16% NP-40, 44% Ampholine (pH 3.5–9.5), and 38% 2-mercaptoethanol were added for electrophoresis. For electrophoresis in the first dimension, isoelectrofocusing using a 10% polyacrylamide gel with Ampholine (pH 3.5–9.5) was performed. In the 2nd dimension, the protein bands obtained in the 1st dimension electrophoresis were transferred to a gel for SDS-PAGE, and SDS-PAGE was performed. Protein bands were stained with Coomassie Brilliant Blue R-250. For fluorography, the gel was rinsed in 10% acetic acid for 1–2 h to fix the proteins in the gel. After removal of the acetic acid, the gel was shaken in EN^{3}HANCE for 1 h. After removal of the EN^{3}HANCE, the gel was shaken in ice-cold water for 1 h, and dried. The radioactivity in the gel was visualized using Kodak XAR-5 X ray film (Tokyo) with the aid of lightning plus (Dupont de Nemours, Wilmington, DE) at –80°C. Molecular weight markers for the protein bands in SDS-PAGE, human RBC ghost, bovine serum albumin (66,300), soybean trypsin inhibitor (21,500), lysozyme (14,300), and mouse macrophage MLC (15,000 and 20,000), were used.

Effect of Thiophosphorylation of Intracellular Proteins on Macrophage Binding to oxLDL-Coated RBC—A 200 μl macrophage suspension in RPMI-HEPES medium (8 5 × 10^5 cells/ml) was loaded on a plain or fibronectin-coated coverslips, 18 mm in diameter, and incubated at 37°C in a 5% CO_2 incubator. Nonadherent cells were removed by washing with saline. To the monolayers, 200 μl of normal RPMI-HEPES medium or RPMI-HEPES medium (phosphate-free) containing sodium thiophosphate was added, and the monolayers were incubated at 37°C for 1 or 2 h After removal of the reagents, the ability of the macrophages to bind Native LDL- or oxLDL-coated RBC was assessed.

Statistical Analysis—Data were analyzed by Student's *t*-test.

RESULTS

Effect of Inhibitors of Protein Kinases and Phosphatases on Fibronectin-Unstimulated and Stimulated Macrophage Binding and Uptake of oxLDL—In order to examine whether intracellular protein phosphorylation and dephosphorylation are involved in fibronectin-unstimulated macrophage activity, the effects of different types of inhibitors of protein kinases and phosphatases on macrophage binding and uptake of oxLDL were investigated. Effects of inhibitors of MLCK, ML-9 and ML-7 (*31, 32*), inhibitors of PKC, H-7 (*31, 33*), and staurosporine (*34*), and inhibitors of PTK, herbimycin A (*35*) and genistein (*36*), on macrophage binding to oxLDL were examined. Similarly, the effects of a protein serine/threonine phosphatase inhibitor, okadaic acid (*37*), and a protein tyrosine phosphatase inhibitor, vanadate (*38*), on macrophage binding were examined. In the oxLDL binding assay, mouse macrophages obtained from a thioglycollate-induced peritoneal exudate were preincubated with these inhibitors, and the simple assay of macrophage binding to oxLDL (*7, 28*), in which human oxLDL are coated on mouse RBC (2–3 mg LDL protein/10^6 RBC), and macrophages bound oxLDL-coated RBC are counted under phase-contrast microscopy, was employed. The binding of macrophages to oxLDL-coated RBC was inhibited by pretreatment of the macrophages with ML-9, H-7, staurosporine, herbimycin A, and genistein in a dose-dependent fashion (Fig. 1A). Binding was enhanced by pretreatment of macrophages with vanadate in a dose-dependent fashion, whereas the binding was not enhanced by pretreatment

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with okadaic acid (Fig. 1B). Foam cell formation was inhibited when macrophages were incubated with oxLDL in the presence of ML-9, ML-7, H-7, staurosporine, herbimycin A, or genistein in a dose-dependent fashion (Fig. 2A), and en-

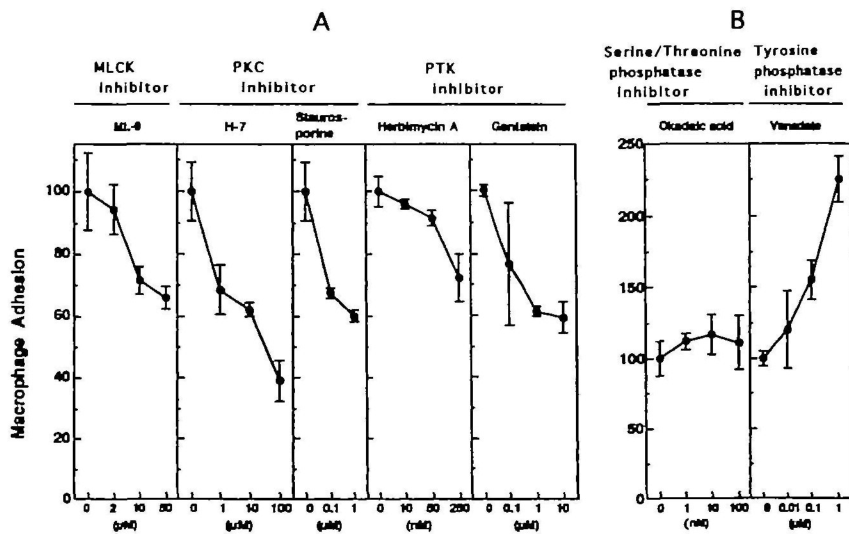

Fig 1 **Effect of inhibitors of protein kinases and phosphatases on macrophage binding to oxLDL-coated RBC.** Macrophage monolayers on plain coverslips were preincubated with inhibitors at the indicated concentrations at 37°C for 2 h After removal of the reagents, the ability of the macrophages to bind to oxLDL-RBC was assessed Results are expressed taking the ability of untreated macrophages as 100 Percent adhesion of control macrophages to native LDL-coated RBC was 23 9% (for ML-9), 26 3% (for H-7 and staurosporine), 5 44% (for herbimycin A), 3 27% (for genistein), 22.0% (for okadaic acid), and 14 7% (for vanadate), and that of control macrophages for oxLDL-coated RBC was 63 5% (for ML-9), 77 3% (for H-7 and staurosporine), 96 0% (for genistein), 46 3% (for okadaic acid), and 24 2% (for vanadate) Each point represents the mean ± SD of triplicate coverslips The decrease in the viability of macrophages treated with the concentrations employed here was less than 10%

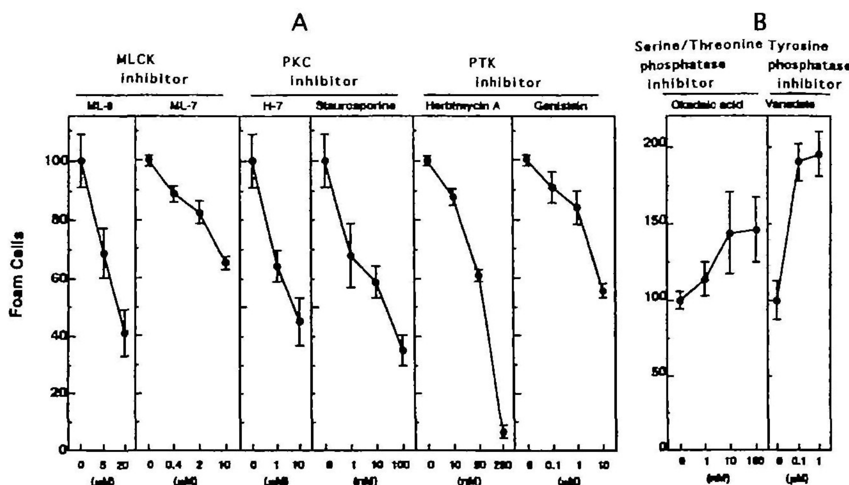

Fig 2 **Effect of inhibitors of protein kinases and phosphatases on foam cell formation.** Macrophage monolayers on plain coverslips were incubated with 100 μg/ml oxLDL in the presence of inhibitors at the indicated concentrations at 37°C for 24 h Foam cells were counted after staining with oil-red-O Results are expressed taking the ability of untreated macrophages as 100 Percent foam cell formation of control macrophages for oxLDL was 43 0% (for ML-9, H-7, and staurosporine), 94 2% (for ML-7), 90 9% (for herbimycin A and genistein), 31 9% (for okadaic acid), and 32 7% (for vanadate) Each point represents the mean ± SD of triplicate coverslips. The decrease in the viability of macrophages treated with the concentrations employed here was less than 10%

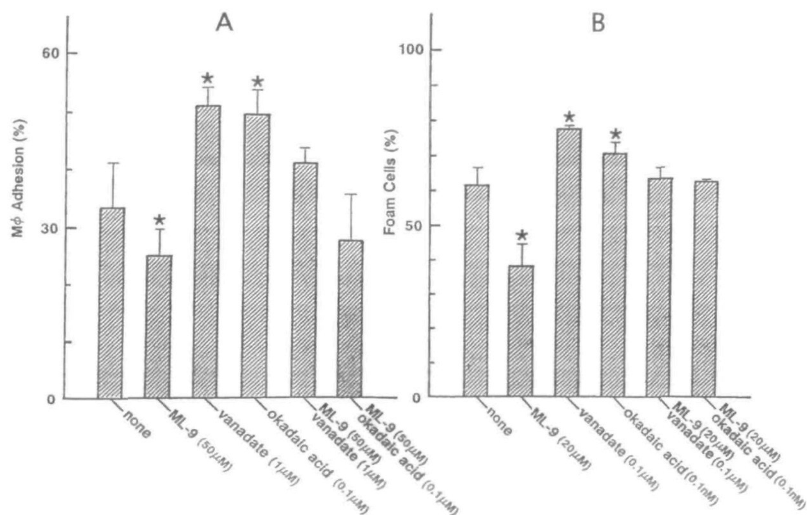

Fig 3 **Effect of a combination of a protein kinase inhibitor (ML-9) and a phosphatase inhibitor (okadaic acid or vanadate) on macrophage binding to oxLDL coated RBC (A) and foam cell formation (B).** Macrophage monolayers were treated with inhibitors as described in the legends to Fig 1 and Fig 2. Each point represents the mean ± SD of triplicate coverslips. *Significantly different from none $p < 0\ 01$

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hanced when macrophages were incubated with oxLDL in the presence of okadaic acid or vanadate (Fig. 2B). When macrophages were pretreated with a combination of ML-9 and vanadate or a combination of ML-9 and okadaic acid,

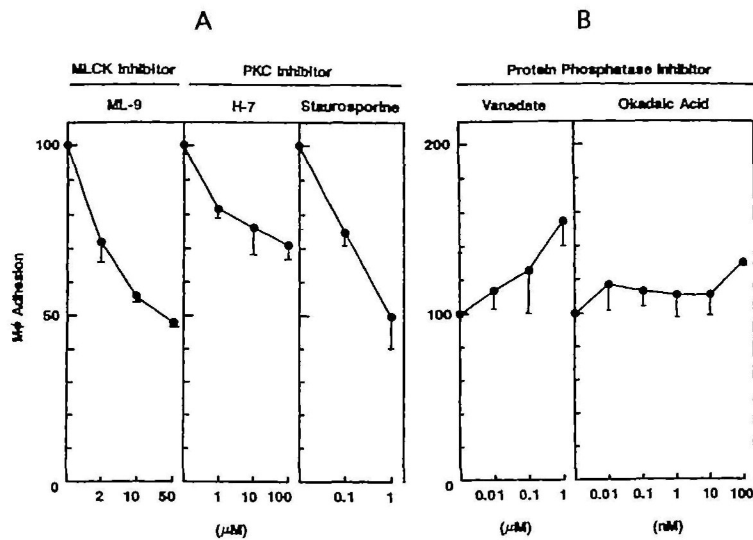

Fig. 4 **Effect of inhibitors of protein kinases and phosphatases on fibronectin-stimulated macrophage binding to oxLDL-coated RBC.** Macrophage monolayers on fibronectin-coated coverslips were preincubated with inhibitors at the indicated concentrations at 37°C for 2 h After removal of the reagents, the ability of the macrophages to bind to oxLDL-coated RBC was assessed. Results are expressed taking the ability of untreated macrophages as 100. Percent adhesion of control fibronectin-unstimulated macrophages to LDL-coated RBC was 35.7% (for ML-9, H-7, and staurosporine), 34 2% (for okadaic acid), and 24 2% (for vanadate), that of control fibronectin stimulated macrophages was 60 8% (for ML-9, H-7, and staurosporine), 54 5% (for okadaic acid), and 24 2% (for vanadate) Each point represents the mean ± SD of triplicate coverslips

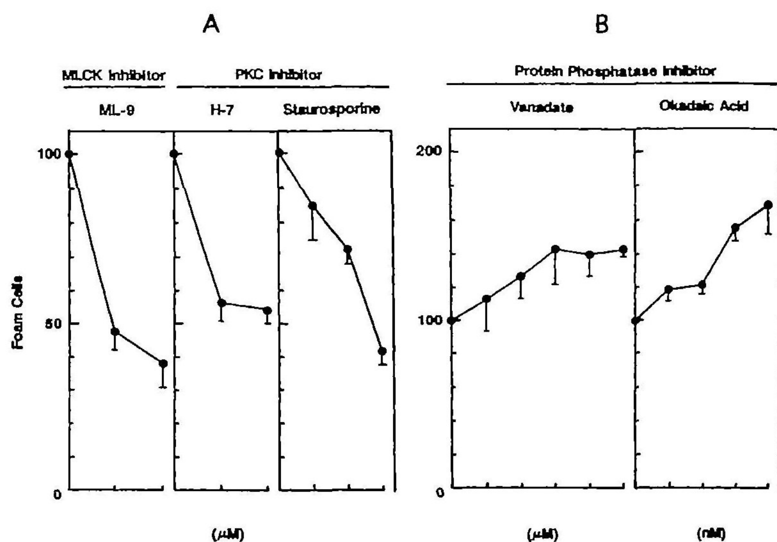

Fig 5 **Effect of the inhibitors of protein kinases and phosphatases on foam cell formation from fibronectin-stimulated macrophages.** Macrophage monolayers on fibronectin-coated coverslips were incubated with 100 mg/ml oxLDL in the presence of inhibitors at the indicated concentrations at 37°C for 24 h Foam cells were counted after staining with oil-red-O Results are expressed taking the ability of untreated macrophages as 100 The percent foam cell formation of control fibronectin-unstimulated macrophages was 26 3% (for ML-9 and H-7), 43 0% (for staurosporine), 31 9% (for okadaic acid), and 32 7% (for vanadate), that of control fibronectin-stimulated macrophages was 49 8% (for ML-9 and H-7), 61 4% (for staurosporine), 34 8% (for okadaic acid), and 52 4% (for vanadate) Each point represents the mean ± SD of triplicate coverslips

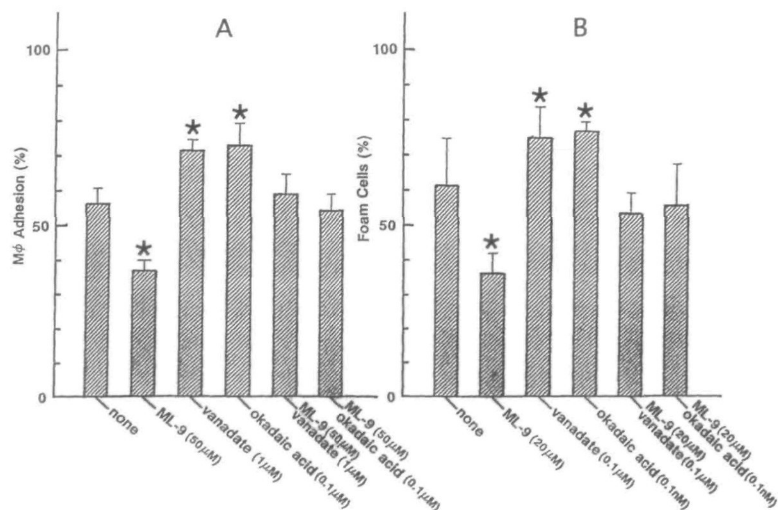

Fig 6 **Effect of a combination of a protein kinase inhibitor (ML-9) and a phosphatase inhibitor (okadaic acid or vanadate) on fibronectin-stimulated macrophage binding to oxLDL-coated RBC (A) and foam cell formation from fibronectin-stimulated macrophages (B).** Fibronectin-stimulated macrophages were treated with inhibitors as described in the legends to Fig 4 and Fig 5. Each point represents the mean ± SD of triplicate coverslips. *Significantly different from none $p < 0.01$

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the inhibitory effect or the enhancing effect of each of the inhibitors on binding (Fig. 3A) and on foam cell formation was cancelled (Fig. 3B).

The effects of the kinase and phosphatase inhibitors on fibronectin-stimulated macrophage activity (*8*) were examined. Percent adhesion of control fibronectin-stimulated macrophage binding of oxLDL was increased about 2-fold as compared with that of control fibronectin-unstimulated macrophages, and all the kinase inhibitors decreased and the phosphatase inhibitors increased fibronectin-stimulated macrophage binding (Fig. 4). Percent foam cell formation of control fibronectin-stimulated macrophages increased about 2-fold as compared to that of control fibronectin-unstimulated macrophages, and the kinase inhibitors decreased and the phosphatase inhibitors increased fibronectin-stimulated foam cell formation (Fig. 5). A combination of kinase inhibitor and phosphatase inhibitor cancelled the inhibitory and enhancing activities for binding and foam cell formation (Fig. 6).

The above results indicate that protein phosphorylation of macrophages maintains and enhances the ability of macrophages to bind to oxLDL and to form foam cells, and that the macrophage activity depends on the balance of phosphorylation and dephosphorylation of intracellular proteins.

Effects of Inhibitors of Protein Kinases and Phosphatases on Macrophage Intracellular Protein Phosphorylation—Effects of inhibitors of kinases on the incorporation of $H_3{}^{32}PO_4$ into macrophage intracellular proteins, and the effects of inhibitors of phosphatases on the release of radioactivity from proteins were examined. Thus, fibronectin-unstimulated macrophages coated on Cytodex-1 were incubated with kinase inhibitors and subsequently with $H_3{}^{32}PO_4$. In another way, macrophages were incubated with $H_3{}^{32}PO_4$ and subsequently with phosphatase inhibitors The cells were solubilized, and the radioactivity of the TCA-precipitable proteins of the solubilized cells was counted (Fig. 7). ML-9, ML-7, H-7, staurosporine and herbimycin A inhibited the incorporation of radioactivity into the TCA-precipitable proteins Although vanadate showed little effect, okadaic acid increased the radioactivity in the TCA-precipitable proteins and thus decreased the release of phosphate.

Relationship between Fibronectin-Stimulation of Macrophages and Intracellular Protein Phosphorylation—Macrophages on fibronectin-uncoated and coated Cytodex-1 were incubated with $H_3{}^{32}PO_4$, and the cells were solubilized. The radioactivity of TCA-precipitable proteins of the solubilized cells was increased 1 4-fold by fibronectin stimulation, indicating that intracellular protein phosphorylation is enhanced by fibronectin stimulation. When the soluble proteins were subjected to SDS-PAGE and subsequent detection by fluorography (Fig. 8A), the radioactivity of all protein bands, including that of mouse macrophage MLC (20 kDa), was higher in the fibronectin-stimulated macrophages than in the unstimulated macrophages. When the TCA-precipitable proteins were subjected to 2-dimensional PAGE, isoelectrofocusing (IEF) in the first dimension and

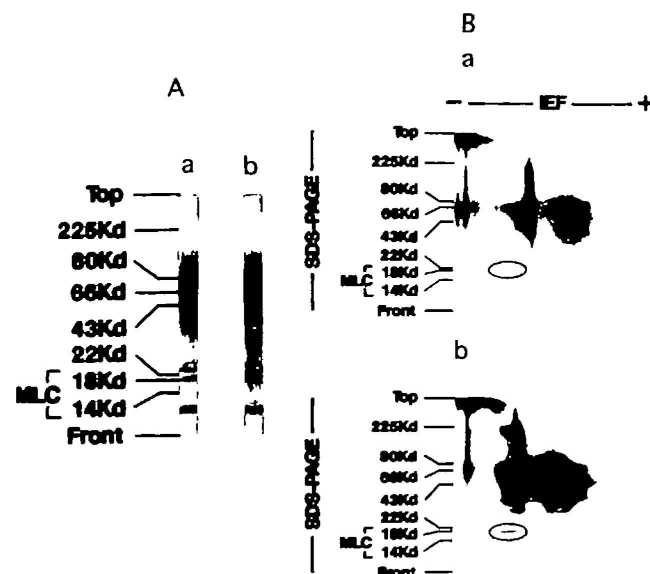

Fig 8 **Effect of fibronectin stimulation on macrophage protein phosphorylation.** Macrophage monolayers on plain (a) or fibronectin-coated Cytodexl-1 (b) were incubated with $H_3{}^{32}PO_4$ Macrophages were solubilized, and the proteins were subjected to SDS-PAGE (A) and the TCA-precipitable proteins were subjected to 2-dimensional PAGE, isoelectrofocusing (IEF) and SDS-PAGE (B) The gel was visualized by Coomassie Brilliant Blue R-250 (for molecular weight markers) and fluorography.

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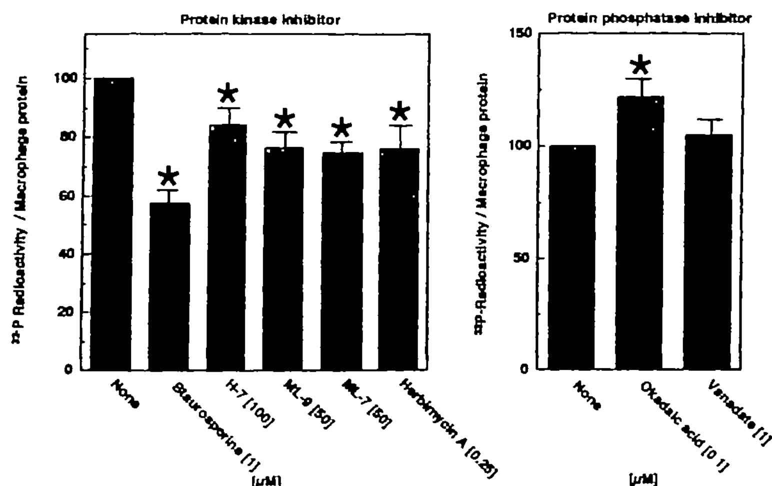

Fig 7 **Effect of inhibitors of protein kinases and phosphatases on the incorporation of ³²P-labeled phosphate into TCA-precipitable proteins of solubilized macrophages.** Macrophage monolayers on plain Cytodex-1 were incubated with each of the protein kinase inhibitors at the indicated concentration and subsequently with $H_3{}^{32}PO_4$, or $H_3{}^{32}PO_4$ and then with each of the protein phosphatase inhibitors. Macrophages were solubilized, and the proteins were precipitated with TCA The precipitate was solubilized with SDS and the radioactivity was counted The radioactivity without inhibitors was expressed as 100 Each point represents the mean ± SD of triplicate experiments. *Significantly different from none: $p < 0.05$.

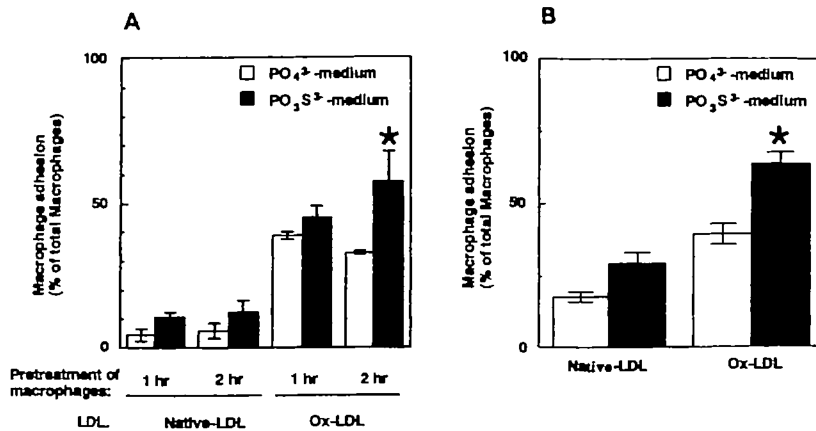

Fig 9 **Effect of thiophosphate on fibronectin-unstimulated macrophage (A) and fibronectin-stimulated macrophage (B) binding to oxLDL-coated RBC.** Macrophage monolayers on plain (A) or fibronectin-coated coverslips (B) were incubated in normal medium containing phosphate or medium containing thiophosphate at 37°C for 1 or 2 h After removal of the reagents, the ability of the macrophages to bind to native- or oxLDL-coated RBC was assessed Results are expressed taking the ability of untreated macrophages as 100. Each point represents the mean ± SD of triplicate experiments. *Significantly different from PO$_4^{3-}$ $p < 0.05$

SDS-PAGE in the second (Fig. 8B), all the proteins, including 20 kDa proteins, were phosphorylated to a greater extent in fibronectin-stimulated macrophages than in unstimulated macrophages. The results indicate that fibronectin stimulates the phosphorylation of various intracellular proteins including MLC. The increased intracellular protein phosphorylation in macrophages may be related to increased macrophage activity under fibronectin stimulation.

Augmentation of Binding of Fibronectin-Unstimulated and Stimulated Macrophages to oxLDL by Thiophosphate—Hydroxyl amino acid residues in proteins are thiophosphorylated with thiophosphate by protein kinases to form thiophosphate esters, but the thiophosphate esters are hard to be released by protein phosphatases (*39, 40*). Thus, it is expected that the thiophosphorylation of macrophage proteins will augment macrophage activity. Fibronectin unstimulated (Fig. 9A) and stimulated macrophages (Fig 9B) were incubated in medium containing thiophosphate instead of phosphate for 1–2 h, and the binding of the macrophages to oxLDL-coated RBC was examined. The binding of fibronectin-unstimulated (Fig. 9A) and fibronectin-stimulated macrophages (Fig. 9B) to oxLDL-coated RBC was increased by incubation with thiophosphate. The results support the idea that intracellular protein phosphorylation is an enhancing factor for macrophages in the binding and uptake of oxLDL.

DISCUSSION

In the present study, we found that intracellular protein phosphorylation is involved in the maintenance or enhancement of the ability of mouse macrophages to bind and take up oxLDL. This was clearly demonstrated by (i) treatment of macrophages with inhibitors of MLCK, PKC, and PTK inhibited macrophage binding to oxLDL, macrophage foam cell formation, and whole intracellular phosphorylation; (ii) treatment of macrophages with inhibitors of protein serine/threonine and tyrosine phosphatases enhanced macrophage binding to oxLDL, macrophage foam cell formation, and whole intracellular phosphorylation; (iii) fibronectin, which stimulates macrophage activity, enhanced macrophage protein phosphorylation; (iv) protein kinase inhibitors inhibited and protein phosphatase inhibitors enhanced fibronectin-stimulated macrophage activity; and (v) treatment of fibronectin-unstimulated and stimulated macrophages with thiophosphate enhanced the binding of oxLDL

It is known that there are several types of scavenger receptor proteins on the macrophage surface: type I and type II class A receptors, CD36, FcγRIIB2, and macrosialin (*41, 42*). We did not discriminate among these receptors in the present study. However, previous inhibition studies (*28*) suggested that class A (type I and type II) scavenger receptors are likely to be involved in the binding of oxLDL and the thioglycollate-induced mouse peritoneal macrophages used here

Because MLCK is known to activate myosin light chain (MLC) by phosphorylation, the results obtained using MLCK inhibitors may indicate that the phosphorylation of MLC is involved in fibronectin unstimulated macrophage activity On the other hand, PKC phosphorylates various proteins including MLC (*16*), and the phosphorylation of another sites on MLC by PKC (*43*) has been shown to modulate the activity of MLC (*44, 45*). Hence, PKC-induced intracellular phosphorylation of proteins other than MLC may be also involved in the enhancement of fibronectin-unstimulated macrophage activity. Moreover, the phosphorylation of tyrosine residues in intracellular proteins by TPK has been found to be involved in the activity of fibronectin-unstimulated macrophages. The dephosphorylation of proteins with phosphoserine/threonine and phosphotyrosine residues may reduce the activity of fibronectin-unstimulated macrophages It is likely that macrophage activity is regulated by the balance of phosphorylation and dephosphorylation of various intracellular proteins.

We have previously found that fibronectin significantly enhances the scavenger receptor activity of mouse macrophages (*8*). The fibronectin-stimulated increase in macrophage activity has been found to be due to Ca^{2+} influx of extracellular Ca^{2+} and the activation of calmodulin. The stimulation of macrophages by fibronectin is inhibited by inhibitors of the Ca^{2+} signaling pathway such as Ca^{2+} channel blockers and calmodulin inhibitors, and fibronectin enhances the activity of macrophages by increasing channel-dependent Ca^{2+} influx. It was found in the present study that fibronectin increases the phosphorylation of intracellular proteins including MLC. Fibronectin may enhance macrophage activity through the Ca^{2+} signaling pathway involving Ca^{2+} influx, the activation of calmodulin, MLCK activation, the phosphorylation of MLC and finally the activation of myosin. However, PKC-induced intracellular phosphorylation of proteins other than MLC is also in-

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volved in the enhancement of fibronectin-stimulated macrophage activity. The dephosphorylation of proteins with phosphoserine/threonine residues may enhance the activity of fibronectin-stimulated macrophages.

In another series of experiments, it has been shown that cell surface lectin-like receptors of macrophages recognize the clustered sialylated poly-*N*-acetyllactosaminyl sugar chains of oxidatively damaged RBC (*46*), and that cell surface fibronectin of macrophages enhances recognition (*47*) by the Ca^{2+} signaling pathway, including Ca^{2+} influx, calmodulin activation and MLC phosphorylation. Macrophages depleted of fibronectin by trypsinization lose the ability to recognize oxidized RBC Hence, the activation of macrophage scavenger receptors and lectin-like receptors by fibronectin may be through the common mechanisms of the Ca^{2+} signaling pathway including MLC and other protein phosphorylation

In conclusion, various types of intracellular protein phosphorylation are involved in the maintenance and enhancement of the ability of fibronectin-unstimulated and stimulated macrophages to bind oxLDL and to form foam cells, and these activities depend on the balance of phosphorylation and dephosphorylation of intracellular proteins.

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