Lysophosphatidylcholine Induces Urokinase-Type Plasminogen Activator and Its Receptor in Human Macrophages Partly Through Redox-Sensitive Pathway

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Abstract—Urokinase-type plasminogen activator (uPA) and its cell surface receptor (uPAR) have been shown to be expressed in macrophages in atherosclerotic arterial walls, but the regulatory mechanisms of their expression remain unclear. The present study was performed to examine the effects of lysophosphatidylcholine (lysoPC), an important atherogenic lipid, on the expression of uPA and uPAR in human monocyte–derived macrophages. LysoPC upregulated the mRNA expression of uPA and uPAR, and it increased the protein expression of uPA in the culture medium and bound to the cell surface and of uPAR in the particulate fraction of the cells. LysoPC significantly increased the binding of the amino-terminal fragment of uPA to the treated cells and the cell-associated plasminogen activator activity. LysoPC stimulated superoxide anion production and increased intracellular oxidant levels in the cells. The combined incubation with reduced glutathione diethyl ester or N-acetylcysteine, antioxidants, suppressed the upregulation of uPA and uPAR mRNA and the increase in plasminogen activator activity by lysoPC. uPA and uPAR mRNA expression was also induced by the incubation with xanthine and xanthine oxidase, a superoxide anion–generating system. The results suggest that lysoPC increased the expression of uPA and uPAR and their functional activities in human monocyte–derived macrophages, at least in part through a redox-sensitive mechanism. This coordinate increase in the expression of uPA and uPAR in human macrophages by lysoPC could play an important role in plaque formation and disruption, arterial remodeling, and angiogenesis in atherosclerotic arterial walls. (Arterioscler Thromb Vasc Biol. 2000;20:244-250.)

Key Words: atherosclerosis n lysophosphatidylcholine n monocyte-derived macrophages n plasminogen activators n antioxidants

The coordinate action of urokinase-type plasminogen activator (uPA) and its cell surface receptor (uPAR) plays an important role in cell migration and tissue remodeling through plasmin-mediated proteolysis. UPAR plays a role in cell adhesion and migration and participates in signal transduction responses independent of its plasmin-mediated proteolytic activity. UPA and uPAR are shown to be highly expressed in a number of cells under pathological conditions, including monocytes/macrophages in atherosclerotic arterial walls, in inflammatory sites, and in neoplasms. UPA and uPAR play a crucial role in angiogenesis, plaque formation and disruption, tissue remodeling, and the progression and metastasis of neoplasms in these tissues. However, the regulatory mechanisms of the increased expression of uPA and uPAR in these tissues are not fully understood.

Lysophosphatidylcholine (lysoPC) is generated from phosphatidylcholine by the action of phospholipase A2. The activity of secretory type II phospholipase A2, one of the enzymes responsible for lysoPC production, is increased in atherosclerotic arterial walls, inflammatory sites, and neoplasms, and lysoPC content is increased severalfold in atherosclerotic arterial walls. Furthermore, we and others have shown that lysoPC caused impairment of endothelium-dependent vasorelaxation, induction of various proatherogenic and proinflammatory molecules in endothelial cells, and mitosis of monocytes/macrophages, all of which are observed in atherosclerotic arterial walls. Thus, multiple biological activities of lysoPC on vascular cells may play an important role in the pathogenesis of atherosclerosis and inflammation. Therefore, we hypothesized that lysoPC could upregulate the expression of uPA and uPAR molecules in vascular cells. The present study examines the effects of lysoPC on the expression of these molecules in cultured human monocyte-derived macrophages. We found that lysoPC induced uPA and uPAR in the cells through a redox-sensitive pathway.
Methods

Preparation and Culture of Human Monocytes
Human peripheral blood monocytes were purified from citrate-anticoagulated dextran-sedimented venous blood samples from healthy volunteers over Ficoll–sodium diatrizoate gradient centrifugation, as described previously. The mononuclear cell fraction was suspended in medium 199 (M-199) with antibiotics and plated in plastic Petri dishes for 2 hours. The nonadherent cells were then removed by washing 3 times with PBS, and the adherent cells were detached by cold PBS (4°C) containing 0.05% EDTA. The detached cells were washed in M-199 and resuspended in M-199 supplemented with 10% human serum and antibiotics and replated in plastic Petri dishes of the indicated sizes. The cells were incubated for 7 days in a humidified atmosphere of 95% air/5% CO₂, and the culture medium was replaced every 2 days. This study examined only the monocytes cultured for 7 days, which were differentiated into macrophages on the basis of the observation of morphological characteristics with Giemsa staining and esterase positivity. The cells were composed of >98% macrophages with >98% viable cells by the trypan blue dye exclusion test.

After they were plated on plastic Petri dishes of the indicated sizes, the 7-day cultures of the macrophages were washed 3 times with serum-free M-199 and then serum-starved for 12 hours. The medium was replaced with serum-free M-199, and the cells were then incubated with various concentrations of lysoPC, the same volume of PBS (as a time control), or other additions for the indicated times. The treated cells were assayed for mRNA expression, antigen levels of uPA and uPAR, binding activity of the amino-terminal fragment (ATF) of uPA to the cell surface, and plasminogen activator activity.

Northern Blotting
Total RNA was extracted from the treated cells by the guanidine thiocyanate method. Northern blot analysis was then performed by loading 10 μg of RNA in each lane of 1% agarose-formaldehyde gels, separating electrophoretically, transferring to nylon membranes (Schleicher & Schuell), and ultraviolet cross-linking. Equivalent loading of RNA in each lane was confirmed by examination of ethidium bromide–stained gels. Complementary cDNA probes were 32P-labeled by the random primer method to a specific activity of ~5×10⁶ cpm/μg DNA. The cDNA probes in the present study included the following: (1) a 600-bp cDNA probe for uPAR; (2) a 369-bp fragment corresponding to bases 19 to 387 of human uPA cDNA, which was prepared by polymerase chain reaction amplification and cloned into the vector PCR II (Invitrogen) (fidelity of the amplification was confirmed by sequencing); and (3) a 1-kb cDNA probe for GAPDH. Membranes were hybridized with either the uPA or the uPAR probes. The same blot was rehybridized with the GAPDH probe to normalize the amount of uPA and uPAR mRNA.

Measurements of Antigen Levels of uPA and uPAR
The cultures of the cells (1×10⁶ cells per well) in 12-well plates (Corning) were washed 3 times with serum-free M-199 and were serum-starved for 12 hours. After incubation of the cells with various concentrations of lysoPC or the same volume of PBS (as a time control) for the indicated times, the conditioned medium was collected. A portion of the treated cells was then washed 3 times with PBS and scraped into cold PBS with protease inhibitors. The remainder of the treated cells was further incubated with acid buffer (50 mmol/L glycine-HCl and 0.1 mol/L NaCl, pH 3.0) for 5 minutes at 23°C to elute cell-bound uPA. The eluates were neutralized by the addition of 1/4 vol of 0.5 mol/L Tris-HCl (pH 7.8). The conditioned medium and the eluates were centrifuged at 800 g for 10 minutes to remove cell debris and made in each buffer with 0.01% Tween 80, and frozen at −80°C until use. The conditioned medium was concentrated 4- to 5-fold by using a pressure-assisted stirred cell fitted with a YM-10 membrane (Amicon Corp). The scraped cells were disrupted by sonication (Sonifier 250, Branson) at 4°C. The suspension was then centrifuged at 800 g for 10 minutes at 4°C to remove unbroken cells and nuclear material. The postnuclear supernatant was centrifuged at 100 000g for 60 minutes to separate the cytosol and particulate fractions. The pellet (particulate fraction) was resuspended in cold Hanks’ balanced salt solution (HBSS) with protease inhibitors and 0.01% Triton X-100 and again sonicated at 4°C. Protein levels were measured by the Lowry protein assay with BSA used as a standard.

Antigen levels of uPA in the medium and in the eluates and the levels of uPAR in the particulate fraction of the cells were determined by the double-antibody sandwich methods that used ELISA with monoclonal antibodies against human uPA and polyclonal antibodies against human uPAR (TintElize uPA, Biopool, and IMUBIND Total uPAR ELISA, American Diagnostica Inc, respectively). The assay for uPA detects single-chain uPA and high molecular weight uPA with the same efficiency. The assay for uPAR detects uPAR, uPAR/uPA, and uPAR/uPA/plasminogen activator inhibitor type 1 with the same efficiency. Standard curves obtained from the assay kits showed that the lower limits of detection were 0.1 ng uPA/mL and 0.1 ng uPAR/mL.

Ligand Binding
ATF, a receptor-binding domain of uPA consisting of amino acids 1 to 274, was radiolabeled with sodium 125I with the use of Iodo-Beads (Pierce Chemical Co) according to the manufacturer’s instructions. Unbound iodine was removed on a P-6DG column (Bio-Rad), which had been preequilibrated with PBS. ATF was labeled to a specific activity of 2×10⁶ cpm/μg.

The cultures of the cells (density of 2×10⁶ cells per well) on 6-well plates were washed 3 times with PBS and incubated with lysoPC or the same volume of PBS (as a time control) for 24 hours. Then, the cultures were rinsed twice with PBS and treated with acid buffer for 10 minutes on ice to remove endogenous uPA bound to uPAR. Subsequently, the cultures were quickly neutralized with 0.5 mol/L HEPES and 0.1 mol/L NaCl, pH 7.5, and then washed with M-199 twice. The cells were incubated at 4°C for 2 hours with binding buffer (M-199 with 3 mg/mL of BSA) containing the radiolabeled ATF in the presence or absence of a 50-fold molar excess of unlabeled ATF. After incubation, the cells were washed 3 times with phenol red-free M-199 and solubilized with 1 mol/L NaOH. The radioactivity in the lysates was determined by counting in a gamma counter. Specific binding was defined as the difference between total binding and nonspecific binding (counts bound in the presence of the unlabeled ATF). Kᵣ and Bₘ₉₉ were determined by Scatchard analysis.

Ligand Binding
The cultures of cells were detached by cold PBS (4°C) containing 0.05% EDTA. After they were washed, the suspended cells were replated into a 96-well microtiter plate at a density of 2×10⁴ cells per well and incubated for 24 hours with M-199, 10% human serum, and antibiotics in the CO₂ incubator (5% CO₂). After washing the cells with phenol red–free M-199, the culture medium was replaced with phenol red–free M-199 without serum. The cells were additionally incubated for 8 hours and then treated with lysoPC (15 μmol/L) or PBS (as a time control) in either the presence or absence of reduced glutathione diethyl ester (GSH diethyl ester, 500 μmol/L) for 24 hours. After incubation, the cells were washed twice with phenol red–free M-199 and subsequently incubated with 0.2 mmol/L of S-2251 (KabiVitrum), a chromogenic substrate for plasmin, in either the presence or absence of 50 μg/mL human Glu-plasminogen (Biopool). The absorbance at 405 nm in each well was measured periodically for time periods up to 3 hours by using a microplate reader (M-Emax, Wako) at 37°C. The cell-associated plasminogen activator activity was assessed by the difference in the values of the absorbance between wells incubated with S-2251 alone (background control) and S-2251 plus Glu-plasminogen.

Measurements of Superoxide Anion Production
Production of superoxide anion (O₂⁻) was detected by chemiluminescence of lucigenin in a luminescence reader (BLR-201, Aloka). The suspended cells were mixed with HBSS containing...
Ca\(^{2+}\) (1.3 mmol/L), Mg\(^{2+}\) (0.4 mmol/L), and lucigenin (250 \(\mu\)mol/L) at 37°C in a total volume of 1 mL (1 \(\times 10^6\) cells) in the presence or absence of superoxide dismutase (SOD), 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), or diphenyliodonium (DPI). Counts were measured at baseline and every 30 seconds until 15 minutes after addition of lysoPC into the assay mixture. NADPH-dependent \(O_2^-\) generation in the particulate fraction of the cells was also measured by the chemiluminescence in HBSS containing Ca\(^{2+}\) (1.3 mmol/L), Mg\(^{2+}\) (0.4 mmol/L), lucigenin (250 \(\mu\)mol/L), cell protein (100 \(\mu\)g), and NADPH (100 \(\mu\)mol/L) at 37°C in a total volume of 1 mL, and the counts were measured every 30 seconds until 30 minutes after the addition of cell protein with lysoPC (7.5 \(\mu\)mol/L) or PBS (control). The particulate fraction of the cells was prepared as described above. The chemiluminescence response was estimated by subtraction of the chemiluminescence in the absence of cells and cell fractions and was standardized by use of a standard curve generated from known quantities of xanthine and xanthine oxidase (X/XO). A chemiluminescence signal of 500 cpm was approximately equivalent to a rate of 1 nmol/min of \(O_2^-\) generation in our system.

**Measurements of Intracellular Oxidant Levels by Flow Cytometry**

Intracellular levels of reactive oxygen species (ROS) were measured by flow cytometric analysis with an oxidant-sensitive fluorescence probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA, Eastman Kodak), as previously described. The cells treated with lysoPC or PBS (control) for 1 hour were incubated with phenol red–free M-199 containing 5 \(\mu\)mol/L DCFH-DA for 30 minutes at 37°C in the dark. After incubation, the cells were washed 3 times with cold PBS (4°C) and detached by adding cold PBS containing 0.05% EDTA. The suspended cells were then pelleted by centrifugation and washed 2 times with phenol red–free M-199 containing 0.1% BSA. The cells were resuspended and immediately analyzed with a fluorescence-activated cell sorter (FACScan, Becton Dickinson). For each analysis, 10,000 events were recorded.

**Materials**

All reagents for cell cultures were from Gibco-BRL. [\(\alpha\)-\(\beta\)-PdC]TP was from Amersham. Synthetic phospholipids, Tiron, SOD (S5639), lucigenin, NADPH, and other chemicals were obtained from Sigma Chemical Co. DPI was from Aldrich Chemical Co, Inc. DPI was dissolved in dimethyl sulfoxide (Sigma), and the final concentration of dimethyl sulfoxide in the assay mixture was 0.05%. This concentration of dimethyl sulfoxide had no effect on the measurement of chemiluminescence.

**Statistical Analysis**

All values were expressed as mean±SEM unless otherwise indicated. The mean values for >3 groups were compared by 1-way ANOVA. Two-way ANOVA for the repeated measurement, followed by the Bonferroni multiple comparison test, was used for comparison of curves showing the results of the experiments of plasminogen activator activity. The difference between 2 mean values was analyzed with the unpaired Student \(t\) test. A value of \(P<0.05\) was considered statistically significant.

**Results**

**Upregulation of uPA and uPAR mRNA Expression by LysoPC**

The incubation of monocyte-derived macrophages with \(\alpha\)-palmitoyl lysoPC (16:0) increased both mRNA levels of uPA and uPAR, as shown in Figure 1. uPA and uPAR mRNA expression reached a maximum 24 hours after the treatment with lysoPC (15 \(\mu\)mol/L). The maximum increase was observed at a concentration of 15 \(\mu\)mol/L lysoPC for both mRNAs. \(\alpha\)-Stearoyl lysoPC (18:0) at a concentration of 15 \(\mu\)mol/L also increased uPA and uPAR mRNA expression after incubation for 24 hours by 3.1- and 2.5-fold of the respective time control, respectively, whereas dipalmitoyl phosphatidylcholine had no effect on the mRNA expression of uPA and uPAR (data not shown).

**Increase in Antigen Levels of uPA and uPAR by LysoPC**

The incubation of monocyte-derived macrophages with lysoPC increased uPA antigen levels in the conditioned medium, as shown in Figure 2A. uPA antigen levels in the acid-treated eluates of the treated cells, which reflected levels of uPA bound to the cell surface receptor, were also increased after incubation with lysoPC, as shown in Figure 2B. The incubation of monocyte-derived macrophages with lysoPC increased uPAR content of the particulate fraction of the treated cells, as shown in Figure 2C.

**Increase in Binding of ATF by Treatment of Cells With LysoPC**

As shown in Figure 3 (inset), the binding of ATF to the treated cells was increased by the incubation of the cells with 15 \(\mu\)mol/L lysoPC for 24 hours compared with the time control. When the data in the binding isotherms (inset) were analyzed in Scatchard plots, a single class of high-affinity uPA binding sites was determined for both treated and control cells with an apparent \(K_d\) of 1.28 nmol/L and 1.75 nmol/L, respectively. In control cells, the number of uPAR molecules per cell was 1.2 \(\times 10^4\). LysoPC increased the number of uPAR molecules per cell by 1.8-fold (2.2 \(\times 10^4\) uPAR per cell).

**Increase in \(O_2^-\) Production and Intracellular ROS Levels by LysoPC**

The addition of lysoPC to the incubation mixture stimulated \(O_2^-\) production in intact macrophages. The production of \(O_2^-\) was increased by the incubation of the cells with lysoPC.
peaked 1 minute after the addition of lysoPC and subsequently decreased to baseline within 5 minutes (Figure 4A). Combined incubation of the intact cells with Tiron (10 mmol/L) completely scavenged O$_2^-$ produced after the addition of lysoPC, whereas SOD (1000 U/mL) scavenged the induced O$_2^-$ by only 10% to 20% (chemiluminescence at peak [cpm/1 x 10^6 cells], 1672±53 after lysoPC alone, 1380±38 after lysoPC plus SOD [P<0.01 versus after lysoPC alone], and 200±11 after lysoPC plus Tiron [P<0.01 versus after lysoPC alone]; n=5 in each experiment). Thus, lysoPC-induced stimulation of O$_2^-$ production was mainly mediated by intracellular mechanisms. Combined incubation of the intact cells with DPI, an inhibitor of flavin-contained enzymes, significantly inhibited lysoPC-induced stimulation of O$_2^-$ production by 40% (n=5, P<0.01 versus after lysoPC alone), suggesting that flavin-contained enzymes such as NADH or NADPH may be partly involved in the lysoPC-induced stimulation of O$_2^-$ production in the intact cells. The addition of lysoPC into the assay mixtures containing the particulate fraction of the cells significantly augmented NADPH-dependent O$_2^-$ production in the particulate fraction (1644±87 cpm/100 μg protein for lysoPC versus 683±42 cpm/100 μg protein for PBS [control], P<0.01, n=6 for each).

Flow cytometric analyses showed that the 2',7'-dichlorofluorescein intensity was increased in the cells after 1 hour of treatment with lysoPC compared with the time-control cells (Figure 4B). This indicates that lysoPC increased intracellular levels of ROS in the cells.

Role of Oxidative Stress in LysoPC-Induced Increases in uPA and uPAR mRNA Expression and in Plasminogen Activator Activity

The combined incubation of the cells with GSH diethyl ester or N-acetylcyctisteine (NAC) significantly suppressed the lysoPC-induced increase in mRNA expression of uPA and uPAR, as shown in Figure 5. uPA and uPAR mRNA expression was significantly increased by the incubation with X/XO.
Incubation of the cells with lysoPC significantly increased their plasminogen activator activity compared with the time control, as shown in Figure 6. Absorbance at 405 nm in each well of absorbance between wells incubated with S-2251 alone (background control) and S-2251 plus Glu-plasminogen was not detectable for up to 180 minutes, indicating that the S-2251 cells treated with S-2251 without Glu-plasminogen was not a result of endogenous plasmin or other proteases that might have been bound to the cells during the period of the incubation. The addition of the antibody against human urokinase (Biopool) reduced cell-associated plasminogen activator activity by \( \approx 90\% \) compared with no addition of the antibody. These results suggest that lysoPC increased the cell-associated plasminogen activator activity through uPA. Furthermore, GSH diethyl ester significantly suppressed the lysoPC-induced increase in cell-associated plasminogen activation (Figure 6).

**Discussion**

The present study showed that lysoPC upregulated mRNA and protein expression of uPA and uPAR in human monocyte–derived macrophages. Furthermore, the upregulation of these molecules was associated with an increase in the binding activity of ATF and a concomitant increase in cell-associated plasminogen activation. Thus, lysoPC, abundantly contained in atherosclerotic arterial walls, may contribute to the increase in uPA and uPAR expression in macrophages in atherosclerotic arterial walls. The coordinate upregulation of uPA and uPAR molecules and the increase in their functional activities in macrophages by lysoPC could play an important role in cell migration, arterial remodeling, and angiogenesis in a variety of vascular diseases, including atherosclerosis. The present study further showed that lysoPC stimulated \( \mathrm{O}_2^- \) production and increased intracellular levels of ROS in human macrophages and that GSH and NAC, antioxidants, suppressed lysoPC-induced increases in uPA and uPAR mRNA expression and in plasminogen activator activity. Furthermore, X/XO, another system generating \( \mathrm{O}_2^- \), also upregulated uPA and uPAR mRNA expression. These results suggest that the induction of uPA and uPAR molecules by lysoPC was partly mediated through redox-sensitive mechanisms.

Although the present study did not define the downstream signals leading to the upregulation of uPA and uPAR molecules by oxidant stress, both genes are known to have oxidative stress–responsive elements such as activator protein-1 and/or nuclear factor-\( \kappa \mathrm{B} \) DNA binding sites in their promoter/enhancer regions. A previous report showed that ROS caused uPA activation. Our previous study showed that lysoPC was capable of stimulating the binding of activator protein-1 and nuclear factor-\( \kappa \mathrm{B} \) to the DNA binding sites. Although the present study did not examine transcriptional activity of both genes, it is possible that the activation of these transcription factors may be involved in the lysoPC-induced upregulation of both genes. However, lysoPC has been shown to modulate multiple signal transduction pathways, including stimulation of protein kinase C activity, activation of adenylyl and guanylyl cyclases, and stimulation of DNA binding activities of other transcription factors, which can possibly regulate uPA and uPAR expressions. Thus, it may be possible that synergy and/or cross talk among these diverse cellular actions of lysoPC may also play a role in the lysoPC-induced upregulation of uPA and uPAR mRNA in addition to the mechanisms involving oxidative stress.

The present study showed that the exogenous addition of lysoPC stimulated \( \mathrm{O}_2^- \) production mainly in the intracellular space of human monocyte–derived macrophages partly...
through the membrane-associated NAD(P)H-dependent system. Furthermore, intracellular levels of ROS, assessed by an oxidant-sensitive probe, DCFH-DA, were increased in the cells after treatment with lysoPC. Thus, our data suggest that lysoPC can stimulate O$_2^-$ production in macrophages, leading to exposure of these cells to oxidative stress. A growing body of evidence indicates that increase in oxidative stress plays a crucial role in the pathogenesis of various vascular diseases, including atherosclerosis.13 In this regard, lysoPC may contribute to the increase in oxidative stress in atherosclerotic arterial walls on the basis of the present and previous data, showing that lysoPC-stimulated O$_2^-$ production in smooth muscle cells25 and endothelial cells34 as well as macrophages. We cannot completely exclude the potential source of superoxide anion from the auto-oxidation of lucigenin itself.30 However, the lucigenin chemiluminescence in the present system was unlikely to be derived mainly from auto-oxidation of lucigenin because the lucigenin chemiluminescence detected in the cell suspension was only minimally abolished by extracellular addition of SOD, but it was completely abolished by Tiron, a low-molecular-weight and cell-permeable scavenger of superoxide anion, as shown in the present study. This indicates that the lucigenin chemiluminescence detected in the cell suspension was derived mainly from intracellular sources but not extracellular sources. Furthermore, the present system did not detect any significant chemiluminescence in the cell-free buffer with higher concentrations (up to 10 mmol/L) of lucigenin.

Secretory type II phospholipase A$_2$, one of the enzymes responsible for lysoPC production, has recently been shown to be highly expressed in atherosclerotic arterial walls, in inflammatory sites, and in various neoplasms.12 Lecithin:cholesterol acyltransferase can hydrolyze phosphatidylcholine to lysoPC and transfer fatty acids to cholesterol.13 Higher lecithin:cholesterol acyltransferase activity is shown to be increased in the plasma of atherosclerotic patients.13,14 In fact, lysoPC content is increased severalfold in atherosclerotic arterial walls.13,14 Thus, the enzymatic activities responsible for lysoPC production are concomitantly increased in the milieu in which the uPA/uPAR system functions. Therefore, the upregulated expression of uPA and uPAR in atherosclerotic arterial walls, in inflammatory sites, and in various neoplasms may partly be due to the action of the high levels of lysoPC in these tissues. Plasminogen activators are shown to be abundant in plasma and extravascular tissues.36 Thus, lysoPC-induced increase in uPA and uPAR expression could play an important role in cell migration, tissue remodeling, and angiogenesis through plasmin-mediated proteolysis in these tissues.

In conclusion, lysoPC increased the expression of uPA and uPAR molecules and their activities in human monocyte–derived macrophages partly through redox-sensitive mechanisms. The lysoPC-induced increase in uPA and uPAR expression in human macrophages could play an important role in their pericellular proteolysis, tissue remodeling, and angiogenesis in atherosclerotic arterial walls, in inflammatory sites, and in neoplasms.

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