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Ascochlorin Suppresses oxLDL-Induced MMP-9 Expression by Inhibiting the MEK/ERK Signaling Pathway in Human THP-1 Macrophages

Jeong Han Kang,[1,2] June-Ki Kim,[2] Won-Hwan Park,[2] Kwan-Kyu Park,[1] Tae-Sung Lee,[3] Junji Magae,[4] Hiroo Nakajima,[5] Cheorl-Ho Kim,[6] and Young-Chae Chang[1]*

[1]Department of Pathology, Catholic University of Daegu School of Medicine, Daegu 705-718, Korea
[2]Cardiovascular Medical Research Center, Dongguk University, Kyungpook 780-714, Korea
[3]Department of Obstetrics and Gynecology, Catholic University of Daegu School of Medicine, Daegu 705-718, Korea
[4]Institute of Research and Innovation, Takada, Kashiwa 277-0861, Japan
[5]Department of Endocrine, Breast Surgery, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan
[6]Department of Biological Science, Sungkyunkwan University, Kyunggi-Do 440-746, Korea

Abstract The critical initiating event in atherogenesis involves the invasion of monocytes through the endothelial walls of arteries and the transformation of monocytes from macrophages into foam cells. Human THP-1 monocytic cells can be induced to differentiate into macrophages by phorbol myristate acetate (PMA) and can then be converted into foam cells by exposure to oxidized low-density lipoprotein (oxLDL). Also, during a chronic inflammatory response, monocytes/macrophages produce the 92-kDa matrix metalloproteinase-9 (MMP-9) that may contribute to the extravasation, migration, and tissue remolding capacities of the phagocytic cells. Here, we investigate the effect of ascochlorin (ASC), a prenylphenol antiviral compound from the fungus *Ascochyta viciae*, on oxLDL-induced MMP-9 expression and activity in human THP-1 macrophages. ASC reduced oxLDL-induced MMP-9 expression and activity in a time-dependent and dose-dependent manner. Also, an analysis of MMP-9 activity using pharmacologic inhibitors showed that ASC inhibits MMP-9 activity via the extracellular signal-regulated kinase 1 and kinase 2 pathways. Our results suggest that ASC may be useful as a potent clinical antiatherogenic agent, a topic of considerable interest in the biological chemistry of chemotherapeutic agents. J. Cell. Biochem. 102: 506–514, 2007.

Key words: ascochlorin; atherosclerosis; oxidized low-density lipoprotein; matrix metalloproteinase-9; activator protein-1; THP-1

Ascochlorin (ASC) is a prenylphenol antifungal antibiotic isolated from an incomplete fungus, *Ascochyta viciae*. Although it was originally reported to be an antiviral antibiotic [Tamura et al., 1968], ASC and derivatives exhibit a large variety of physiological activities including hypolipidemic activity [Hosokawa et al., 1981], suppression of hypertension [Hosokawa et al., 1981], amelioration of type I and type II diabetes [Hosokawa et al., 1985], immunomodulation [Magae et al., 1986], and antitumor activity [Hong et al., 2005]. ASC and one of its derivatives inhibit oxidative phosphorylation by inhibiting ubiquinone-

Abbreviations used: ASC, ascochlorin; AP-1, activator protein-1ECM, extracellular matrix; MMP, matrix metalloproteinase; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; oxLDL, oxidized low-density lipoprotein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PMA, phorbol myristate acetate; TIMPs, tissue inhibitors of metalloproteinases; TNF-α, tumor necrosis factor-α.

*Correspondence to: Young-Chae Chang, PhD, Department of Pathology, Catholic University of Daegu School of Medicine, 3056-6, Daemyung-4-Dong, Nam-gu, Daegu 705-718, Korea E-mail: ycchang@cu.ac.kr

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dependent electron transport in isolated mitochondria [Magae et al., 1993], and it has been suggested that this is the basis of the antiviral activity of ASC. ASC also modulates the activity of nuclear hormone receptors and activates the transcription of the human estrogen receptor [Togashi et al., 2003], suggesting that mechanisms other than those involving the respiratory chain contribute to the physiological activities of ASC. ASC-related compounds show profound antitumor activity against a variety of transplantable tumors and suppress the metastasis of melanomas and lung carcinomas in murine experimental models [Magae et al., 1988].

Regulated expression of matrix metalloproteinase-9 (MMP-9) has been implicated in renal development, macrophage differentiation, atherosclerosis, inflammation, rheumatoid arthritis, and tumor invasion [Nabeshima et al., 2002]. The MMP enzymes are known to play a critical role in migration of monocytes into intima during inflammatory reactions and plaque disruption by macrophages in the late stage of atherosclerosis. The MMP enzymes degrade collagen and elastin, the major components of the extracellular matrix (ECM) in the basement membrane or fibrous cap. In situ zymography has demonstrated a net increase in matrix-degrading activity in human atherosclerotic plaques at sites of in vivo MMP expression [Galis et al., 1995b]. Taken together, these findings suggest that macrophage-derived MMPs may increase matrix breakdown in plaques, thereby predisposing the plaques to disruption. MMP-9, a major MMP, is a 92-kDa gelatinase that is produced by human monocytes/macrophages [Nagase et al., 1992] MMP-9 cleaves basement membrane collagen IV and collagen V.

The factors that trigger expression of MMPs in the artery wall are largely unknown. One such factor may be oxidized low-density lipoprotein (oxLDL). This material has been shown to upregulate expression of MMP-9 by macrophages in vitro [Xu et al., 1999]. Among its atherogenic properties, oxLDL is immunogenic, and autoantibodies against oxLDL have been used as its marker in vivo [Palinski et al., 1989]. The present study was conducted to examine the effect of ASC on oxLDL-induced MMP-9 activity and expression in human THP-1 macrophages. We found that ASC inhibited MMP-9 activity and expression and inhibited expression of the ECM components fibronectin and laminin.

MATERIALS AND METHODS

Cytotoxicity Assay

An assay involving the reduction of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Roche Applied Science) was performed as described in the supplier's protocol to evaluate the cytotoxicity of oxLDL and ASC.

Oxidation of LDL

LDL (density = 1.020–1.063 μg/ml) was isolated by sequential flotation ultracentrifugation from human plasma as described previously [Burdon and Kinppenberg, 1984]. Before oxidative modification, LDL was dialyzed against PBS, filtered through a 0.2 μm Millipore membrane (Millipore, Bedford, MA), and stored in PBS containing 1 mM EDTA at 4°C. OxLDL was prepared as described previously [Williams and Tabas, 1995]. Briefly, LDL concentration was adjusted to 2 mg/ml of protein by dilution in PBS, and the solution was dialyzed at 4°C against PBS to eliminate EDTA. Cu^{2+}-mediated oxidation was conducted at 37°C for 24 h by dialysis against 5 μM $CuSO_4$/PBS. The degree of oxidation was measured with a thiobarbituric-acid-reactive substances assay [Steinberg, 1997] and by examination of relative electrophoretic mobilities [Heinecke et al., 1984]. The concentrations of LDL preparations were measured using the BCA protein assay reagent (Pierce, Rockford, IL). Contamination by endotoxins of all reagents and culture media used in this study was monitored using a chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Cell Culture and Treatment

Human THP-1 cells were grown in RPMI 1640 medium containing 10% (v/v) fetal serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.15% (w/v) sodium bicarbonate, 0.45% (w/v) glucose, 1×10^{-5} M β-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in an atmosphere of 5% (v/v) CO_2. The cell medium was replaced every 2 days during culture prior to initiation of cell treatment. Cells were seeded at a density of 5×10^5 cells /mL in medium containing phorbol

myristate acetate (PMA) (Sigma Chemical Company, St. Louis, MO) at 1×10^{-8} M and grown for 48 h. The medium was then replaced by culture medium with LDL (50 μg/ml) or oxLDL (50 μg/ml). Also, preincubation with the pharmacologic agents PD98059, U0126, wortmannin, AG1478 (concentration optimized in orienting experiments) and variously antiviral prenyl-phenol compounds were for 10 min prior to oxLDL addition.

Gelatin Substrate Gel Zymography

The matrix-degrading activity of MMP-9 was assayed by zymography performed using a previously described procedure with minor modifications [Hong et al., 2005]. Cells were seeded at 5×10^5 cells /ml in medium containing PMA at 1×10^{-8} M for 48 h. The dishes were incubated until the cultures were 80% confluent, and the medium was then changed to fresh serum-free medium with or without ASC compounds. Supernatants were collected after incubation for 12 h. The medium was subjected to SDS–PAGE in 10% (w/v) polyacrylamide gels that had been copolymerized with 1 mg/ml gelatin. After electrophoresis, the gels were washed several times in 2.5% (w/v) Triton X-100 for 1 h at room temperature, then incubated for 24 h at 37°C in buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) for 1 h and destained. Proteolytic activity at a particular gel location yielded a clear band against the blue background of the stained gelatin. The intensity of bands from zymograms was estimated with the Quantity One 1D analysis software program (Bio-Rad). The values are expressed as means ± SE values.

Western Blot Analysis

Cell lysates were prepared by suspending 3×10^5 cells/35 mm-diameter dish in 30 μl of lysis buffer [50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% (v/v) NP 40, 100 μM phenylmethylsulfonyl fluoride, 20 μM aprotinin and 20 μM leupeptin, adjusted to pH 8.0]. The cells were disrupted and proteins extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore). Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit following the manufacturer's (Amersham Biotechnology, UK) instructions. Antibodies specific for MMP-9, p-MEK, MEK, fibronectin and laminin, β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse Transcription–Polymerase Chain Reaction

After treatment of cells with ASC, total RNA was isolated using the TRIzol reagent (Sigma) according to the manufacturer's instructions. RNA yield and purity were assessed by spectrophotometric analysis. Total RNA (10 μg) from each sample was subjected to reverse transcription with random hexamers, dNTPs, and M-MLV reverse transcriptase (Promega) in a total reaction volume of 20 μl. PCR of cDNA was performed using specific primers. PCR products were resolved electrophoretically on a 1.0% (w/v) agarose gel, and DNA bands were visualized by staining the gel with ethidium bromide. The expression of measured genes in each sample was normalized to β-actin expression. All samples were analyzed in triplicate. The primers were: MMP-9 sense: 5′-CGG AGC ACG GAG ACG GGT AT-3′, MMP-9 antisense: 5′-TGA AGG GGA AGA CGC ACA GC-3′, TIMP-1 sense: 5′-GGG GAC ACC AGA AGT CAA CCA GA-3′, TIMP-1 antisense: 5′-CTT TTC AGA GCC TTG GAG GAG CT-3′, β-actin sense: 5′-GCC ATC GTC ACC AAC TGG GAC-3′, β-actin antisense: 5′-CGA TTT CCC GCT CGG CCG TGG-3′.

Immunofluorescence Microscopy

Cells were cultured and treated on poly-L-lysine-coated coverslips before being fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. After a 5 min wash with 2 mg/mL glycine in PBS, cells were permeabilized with 0.2% (w/v) Triton X-100 in PBS for 5 min. After two washes with PBS, cells were blocked with 10% (v/v) normal goat serum in PBS for 1 h in a humidified chamber. Cells were then incubated with primary antibodies [1:100, diluted in PBS containing 2% (v/v) normal goat serum] overnight at 4°C with gentle shaking. The cells were then washed three times before being incubated with FITC, TRITC-conjugated secondary antibody for 60 min, and with DAPI for 3 min at room temperature. Finally, the cells were washed five times with PBS containing 0.05% (v/v) Tween 20 and 1% (w/v) BSA. Cover slips were mounted with 90% glycerol and sealed with nail polish. Slides were examined and scanned on a fluorescence microscope.

RESULTS

Effects of oxLDL and ASC on Cell Viability

The structure of ASC used in this study is shown (Fig. 1A). As oxLDL and ASC have antibiotic activities, we first examined possible cytotoxic effects of these compounds on human THP-1 macrophages in serum-free medium using an MTT assay. Treatment of cells with oxLDL ranging in concentration from 5 to 100 μg showed a 5–10% decrease in cell viability in serum-free medium. Also, cells with ASC ranging in concentration from 0.1 to 30 μM showed a 10–12% decrease in cell viability. We also measured the effects of various antiviral prenyl-phenol compounds on the viability of human THP-1 macrophages. Cells growing in the presence of AF, AS-6, or MAC, at concentrations of 30 μM, showed only a 12% decrease in cell viability when compared with the control (data not shown).

OxLDL Regulates MMP-9 Activity in a Dose-Dependent Manner

The effect of oxLDL on MMP-9 expression was investigated in the human monocyte cell line THP-1. Quiescent THP-1 cells secreted MMP-9 at very low levels, and stimulation with PMA markedly augmented MMP-9 protein levels in the supernatant. Human THP-1 macrophages in FBS-free medium were treated with various doses of oxLDL for 12 h, and supernatants were then collected for measurement of MMP-9 activity by zymography. oxLDL at 5–50 μg/ml slightly increased MMP-9 activity (Fig. 2A). When THP-1 cells were treated with native LDL, MMP-9 protein expression and gelatinolytic activity were not affected.

Effect of Antiviral Prenyl-Phenol Compounds on MMP-9 Activity

In a previous study, we isolated an antiviral compound from the phytopathogenic fungus *Ascochyta viciae* [Tamura et al., 1968]. We also synthesized a derivative of this compound. In the present study, we measured the effects of various prenyl-phenol compounds on oxLDL-induced MMP-9 activity in human THP-1 macrophages.

Human THP-1 macrophages were treated with ASC (10 μM), AF (10 μM), AS-6 (10 μM), or MAC (10 μM) for 12 h, and supernatants were

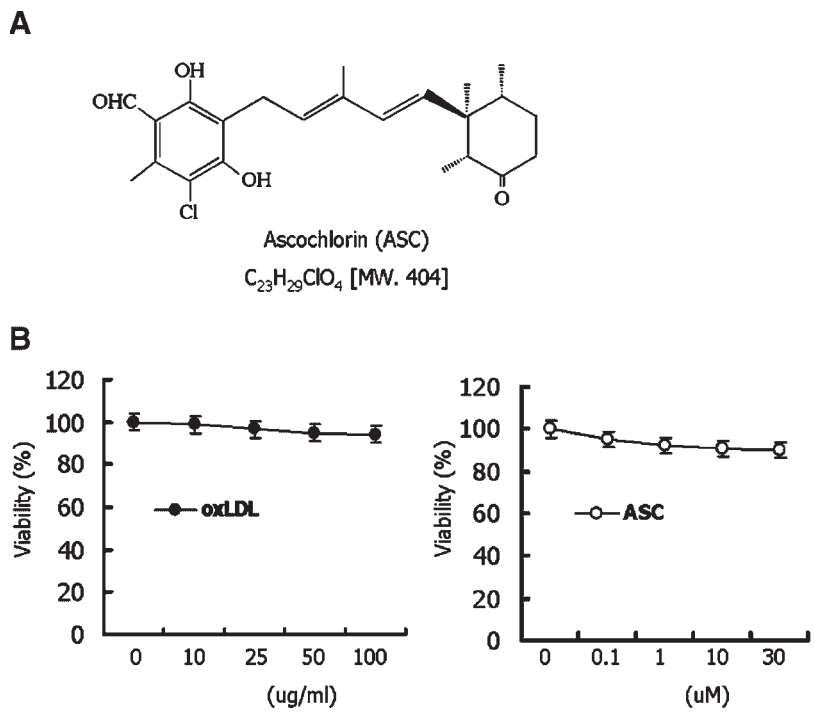

Fig. 1. Chemical structure of ASC and effects of ASC and oxLDL on viability of human THP-1 macrophages. **A**: Chemical structure and molecular weight of ASC. **B**: Effects of ASC and oxLDL on cell viability. Human THP-1 macrophages were treated with various concentrations of ASC (0.1–30 μM) and oxLDL (5–50 μg/ml), and cell viability was assessed after 12 h using the MTT assay.

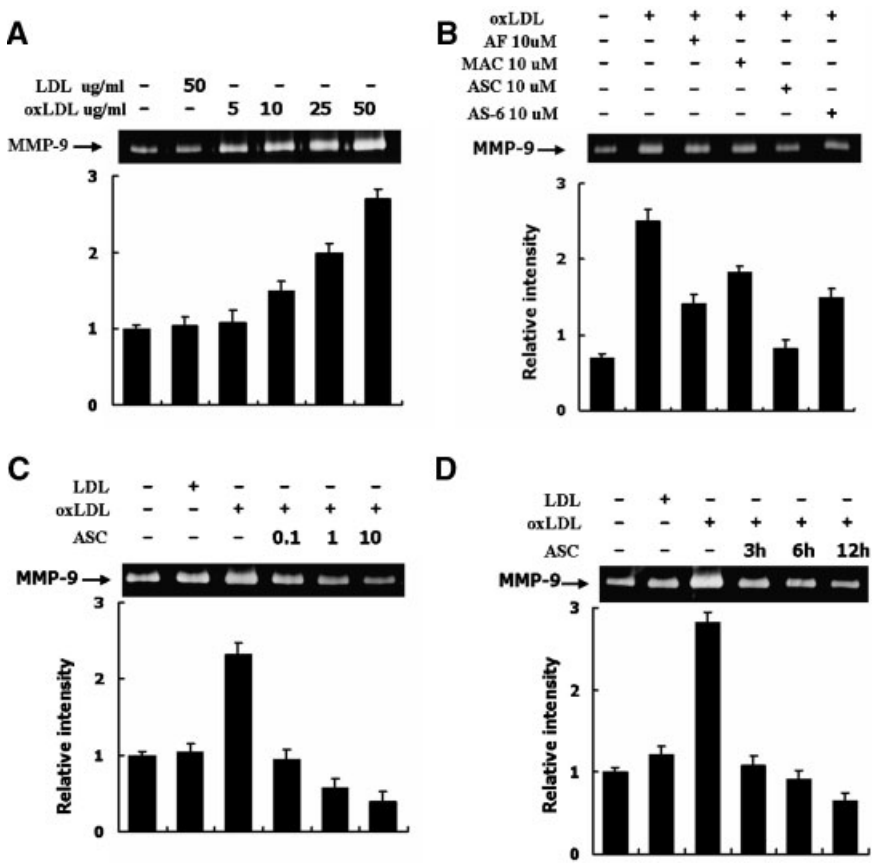

Fig. 2. Effect of ASC on oxLDL-induced MMP-9 activity. **A**: Various concentrations of oxLDL (5–50 μg/ml) were treated human THP-1 macrophages. **B**: Human THP-1 macrophages were treated with various prenylphenol anti-fungal antibiotics (10 μM). **C**: Various ASC concentrations (0.1, 1, 10 μM) were treated human THP-1 macrophages. Culture media were collected at 12 h for measurement of MMP-9 activity. **D**: Culture media were collected at various times (3 h, 6 h, 12 h) for measurement of MMP-9 activity. The bottom panels show the densities of bands measured by the Quantity One 1D analysis software program.

then collected for measurement of oxLDL-induced MMP-9 activity by zymography. Among the antiviral prenyl-phenol compounds, AS-6 and AF slightly inhibited MMP-9 activity induced by oxLDL. The inhibitory effects were small compared with that exhibited by ASC. ASC is the most potent inhibitor of oxLDL-induced MMP-9 activity in human THP-1 macrophages (Fig. 2B).

Effects of ASC on oxLDL-Induced MMP-9 Activity

Various ASC concentrations dramatically decreased oxLDL-induced MMP-9 activity in human THP-1 macrophages (Fig. 2C). Additionally, we examined the inhibitory effect of ASC on various times (Fig. 2D). These results show that ASC inhibits the enzymatic activity of MMP-9 protein secreted from human THP-1 macrophages.

ASC Inhibits Expression of MMP-9

The levels of MMP-9 protein in cells treated with ASC were determined by Western blot analysis (Fig. 3A). The expression levels of MMP-9 protein gradually decreased in a dose-dependent manner indicating that reduced MMP-9 enzyme activity was the result of decreased amounts of MMP-9 protein. To determine whether the inhibition of MMP-9 enzyme expression by ASC was due to a decreased level of transcription, we performed RT-PCR analyses. Treatment of human THP-1 macrophages with ASC decreased the level of oxLDL-induced MMP-9 mRNA expression (Fig. 3B). Because the activity of MMP-9 is tightly regulated by endogenous inhibitors, the tissue inhibitor of metalloproteinases (TIMPs) [Nabeshima et al., 2002], the levels of expres-

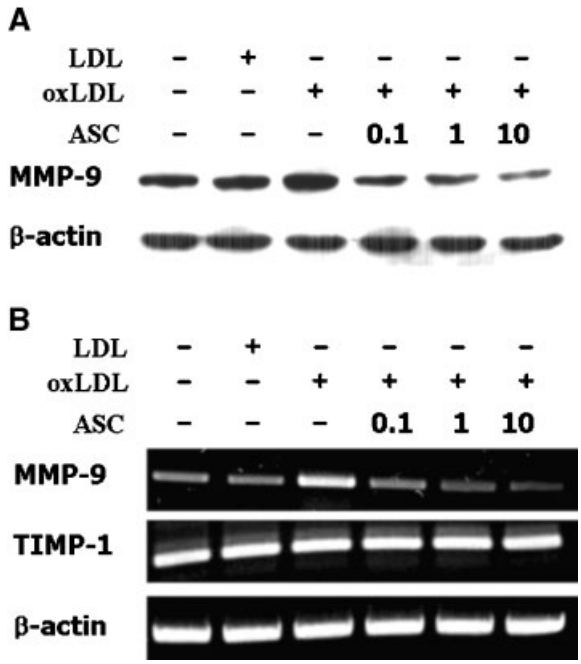

Fig. 3. ASC inhibits oxLDL-induced MMP-9 expression. **A**: Expression of MMP-9 in human THP-1 macrophages treated with ASC in the presence of oxLDL for 12 h was evaluated by Western blot analysis using anti-MMP-9 antibody. Expression of β-actin in cell lysates was used as a control. **B**: MMP-9 and TIMP-1 mRNA expression levels in cells treated as indicated were determined using RT-PCR; a representative result from three independent experiments is shown. Expression of β-actin in mRNA expression level was used as a control.

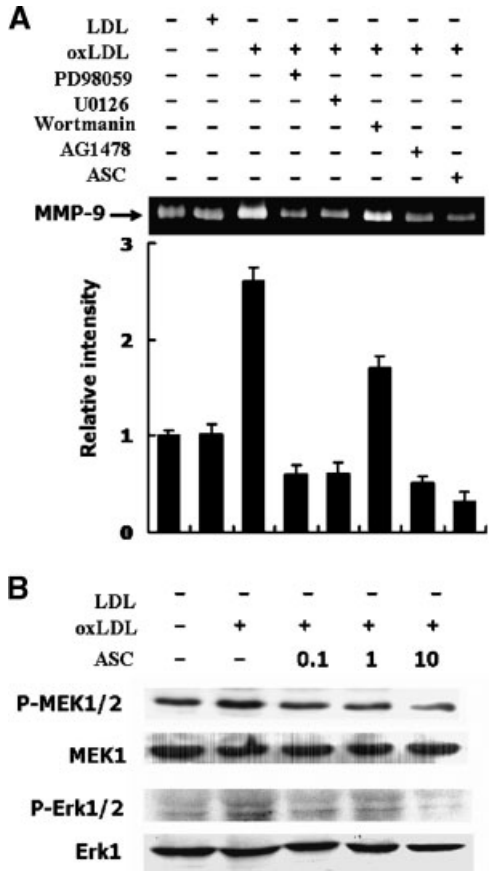

Fig. 4. oxLDL-induced MMP-9 activity in the presence of both pharmacologic inhibitors and ASC. **A**: Human THP-1 macrophages were treated with PD98059 (50 μM), U0126 (20 μM), wortmannin (50 nM), AG1478 (2.5 μM), all in the presence of 10 μM ASC. Culture media were collected at 12 h for measurement of MMP-9 activity. The bottom panels show the densities of bands measured by the Quantity One 1D analysis software program. **B**: ERK, MAPK pathway involvement in oxLDL induction of MMP-9 was confirmed in Western blot by anti-phosphorylated MEK1/2, anti-phosphorylated Erk1/2.

sion of TIMP-1 were also assessed by RT-PCR. ASC had no effect on TIMP-1 mRNA expression at the concentrations tested (Fig. 3B).

ASC Inhibits MMP-9 Expression by Suppressing the EGFR/MEK/ERK1/2 Signaling Pathway

Figure 4A shows that in cells growing in the presence of the MEK-specific inhibitors PD98059 or U0126, or the EGFR-specific inhibitor AG1478, ASC suppressed MMP-9 activity induction in response to oxLDL while the phosphatidylinositol 3-kinase blocking agent wortmannin weak protected to affect MMP-9 activity. These results suggest that MMP-9 expression is regulated by the EGFR/MEK/ERKs pathway in human THP-1 macrophages. Therefore, we confirmed that the concentration of ASC used in these studies effectively reduced the phosphorylation of MEK1/2, ERK1/2 (Fig. 4B).

Immunofluorescence Microscopy

We performed immunofluorescence microscopy to confirm the change in oxLDL-induced MMP-9 protein expression after ASC treatment of human THP-1 macrophages. As shown (Fig. 5), treatment with ASC decreased MMP-9 protein expression. Cell adhesion and spreading on the ECM are hallmarks of macrophage differentiation. Among the ECM components, fibronectin has been recognized as the key element in promoting cell adhesion and various functions of monocytes and macrophages. Also, it has been shown that laminin and entactin stimulated production of MMPs by murine peritoneal macrophages [Corcoran et al., 1995]. Therefore, we sought to show a change in oxLDL-induced fibronectin and laminin expression after ASC treatment of human THP-1 macrophages. As shown (Fig. 5), ASC

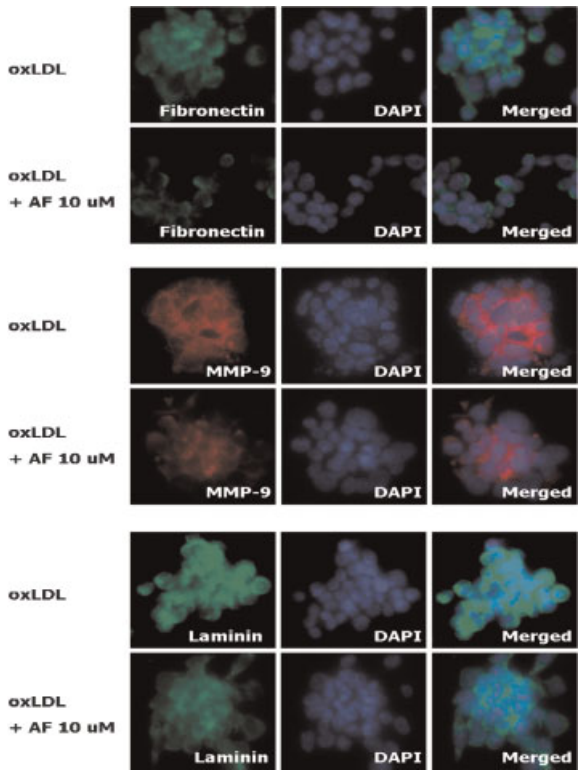

Fig. 5. ASC treatment of oxLDL-induced human THP-1 macrophages causes changes in expression of MMP-9, fibronectin, and laminin. Expression of fibronectin, MMP-9, and laminin in human THP-1 macrophages treated with ASC in the presence of oxLDL for 12 h was evaluated by immunofluorescence microscopy using anti-fibronectin, anti-MMP-9, and anti-laminin antibodies. Cells were double stained with the antibodies and DAPI and observed by fluorescence microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

decreased oxLDL-induced fibronectin and laminin expression. These results indicate that ASC may be useful as a potent clinical antiatherogenic agent.

DISCUSSION

The MMPs are a family of proteases produced both by macrophages and by a wide variety of other cells. They are secreted in zymogen form requiring extracellular activation [Woessner, 1991]. MMPs are active at neutral pH, require zinc and calcium as cofactors, and are capable of degrading virtually all components of the extracellular matrix. The MMPs are tightly regulated not only at the transcriptional level but also by their requirement for extracellular activation and by specific inhibitors such as TIMP-1 and TIMP-2 that are cosecreted with the MMPs [Murphy et al., 1991]. The MMP and TIMP genes are regulated by the transcription factors NF-κB and activator protein-1 (AP-1) [Sato and Seiki, 1993]. Foam cells derived from aortas of cholesterol-fed rabbits have been shown to express MMP in cell culture [Galis et al., 1995a]. Human monocyte-derived macrophages have been shown to express MMP-1 and MMP-2 and to have the ability to induce collagen breakdown in fibrous caps of atherosclerotic plaques [Shah et al., 1995]. However, the precise factors that stimulate MMP elaboration in human lesion-associated macrophages have not been well characterized. Increased MMP activation by oxidant stress has been described by Rajagopalan et al. [1996]. A recent study has emphasized that the factors triggering expression of MMPs in the artery wall are largely unknown. One of these factors may be oxLDL, as this material has been shown to upregulate expression of MMP-9 by macrophages in vitro [Xu et al., 1999].

This study has shown that prenyl-phenol compounds affect oxLDL-induced MMP-9 activity. As described (Fig. 1), ASC and oxLDL have weak cytotoxic activity. In agreement with Xu et al. [1999], our data (Fig. 2A) clearly show that MMP-9 activity in culture medium conditioned by exposure of cells to oxLDL is significantly higher than that observed in medium from control cells or cells receiving LDL treatment only. As shown (Fig. 2B), only ASC of four prenyl-phenol compounds examined showed significant inhibition of oxLDL-induced MMP-9 activity of human THP-1 macrophages at a concentration of 10 μM. These results show that while ASC, AS-6, and AF have weak inhibitory effects on oxLDL-induced MMP-9 activity at low concentration (10 μM), they have almost no cytotoxic effect at this level. In addition, our data show that MMP-9 activities decrease significantly in oxLDL-induced THP-1 cells treated with 0.1–10 μM of ASC for 12h. The decreases were time dependent and dose dependent (Fig. 2C and D). Using Western blot and RT-PCR, we showed that ASC is an inhibitor of oxLDL-induced MMP-9 expression (Fig. 3). We also examined TIMP-1 gene expression by RT-PCR. As TIMP-1 is a major inhibitor of MMP-9, and as TIMP-1 and TIMP-2 are differentially regulated in vivo as well as in a culture system [Stetler-Stevenson et al., 1990], we ruled out possible effects of ASC on TMP-1. In a previous study by Hong et al. [Hong et al., 2005], ASC

inhibited MMP-9 expression though the ERK1/2 signaling pathway. Also, we have reported that treatment with ASC decreased EGFR protein expression in U2OS [Kang et al., 2006]. We thus showed here that oxLDL-induced MMP-9 activity was affected by pharmacologic inhibitors of the ERK1/2 pathway (Fig. 4). These results suggest that the EGFR/MEK/ERKs pathway regulates MMP-9 expression in human THP-1 macrophages. Roh et al. [1998] reported that oxLDL increased fibronectin and laminin gene expression, synthesis, and secretion, in a dose-dependent manner, in murine mesangial cells. Therefore, we used immunofluorescence microscopy to show that ASC affects ECM components. As shown (Fig. 5), ASC inhibited oxLDL induced-fibronectin and laminin expression in human THP-1 macrophages.

In conclusion, our results suggest that ASC may be useful as a potent clinical antiatherogenic agent. This is a topic of considerable interest in the biological chemistry of chemotherapeutic agents.

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