Mitogenic Activity of Oxidized Lipoprotein (a) on Human Vascular Smooth Muscle Cells

Norio Komai, Ryuichi Morishita, Shingo Yamada, Mitsuru Oishi, Sota Iguchi, Motokuni Aoki, Minako Sasaki, Ikunosuke Sakurabayashi, Jitsuo Higaki, Toshio Ogihara

Abstract—Although oxidized lipoproteins may play an important role in the progression of atherosclerosis, no report has mentioned the significance of oxidized lipoprotein (a) (Lp[a]) in the pathogenesis of cardiovascular disease. Initially, we compared the mitogenic actions of Lp(a) and oxidized Lp(a) on human vascular smooth muscle cells (VSMC). Lp(a) significantly stimulated the growth of human VSMC in a dose-dependent manner, whereas oxidized Lp(a) showed a stronger stimulatory action on VSMC growth than native Lp(a). Interestingly, antioxidants probucol and fluvastatin inhibited the oxidation of Lp(a). Moreover, the stimulatory effect of oxidized Lp(a) on human VSMC growth was significantly inhibited by probucol. Finally, we elucidated the molecular mechanisms of how Lp(a) stimulated the growth of VSMC. Extracellular signal-regulated kinase (ERK), as those controlled by kinases, modulate critical cellular functions such as cell growth, differentiation, and apoptosis, was transiently phosphorylated by oxidized Lp(a) as well as native Lp(a) from 5 minutes, and the phosphorylation disappeared within 30 minutes. The degree of ERK phosphorylation by oxidized Lp(a) was much higher than that by native Lp(a). Administration of a specific inhibitor of MEK, PD 98059, significantly attenuated VSMC growth induced by native Lp(a) or oxidized Lp(a) in a dose-dependent manner (P<0.01). The current study demonstrated that oxidized Lp(a) is more potent than native Lp(a) in stimulating VSMC growth. Oxidized Lp(a) may play an important role in the pathogenesis of vascular disease. (Hypertension. 2002;40:902–908)

Key Words: muscle, smooth, vascular ■ atherosclerosis ■ vasculature ■ remodeling ■ lipoproteins

A high concentration of serum lipoprotein (a) (Lp[a]) is a risk factor for atherosclerosis, restenosis after angioplasty, ischemic heart disease, and cerebral stroke.1–6 Lp(a) consists of LDL with an additional protein component, apolipoprotein (a), a homologue of plasminogen.7 Lp(a) and apolipoprotein (a) have been thought to enhance proliferation of human vascular smooth muscle cells (VSMC).8–10 On the other hand, Lp(a) has been postulated to bind to endothelial cells and macrophages and to extracellular components such as fibrin and inhibit cell-associated plasminogen activation.11,12 Recently, numerous types of stress such as oxidation have been suggested to be involved in the development of atherosclerosis. For example, oxidized LDL caused by oxidative stress such as that in hypertension and diabetes but not native LDL has been postulated to be related to atherosclerosis.13 Other lipoproteins such as Lp(a) could also be modified by oxidation. Expectedly, oxidative modification enhances the inhibitory effect of Lp(a) on plasminogen binding to U937 cell surfaces.14 These findings suggest that the oxidative form of Lp(a) rather than native Lp(a) may attenuate fibrinolytic activity through reduction of plasminogen activation. We discovered a monoclonal antibody that recognizes an epitope of modified Lp(a) as a result of oxidation treatment.15 Since this epitope is hidden on the native Lp(a) molecule, we successfully developed a new ELISA system by using this antibody, which can distinguish native and oxidized Lp(a). With the use of this antibody, the presence of oxidized Lp(a) could be detected in human serum and human atherosclerotic lesions.15 However, the exact role of oxidized Lp(a) is still largely unknown. This study demonstrates the potent mitogenic activity of oxidized Lp(a) compared with native Lp(a) in the growth of human aortic VSMC.

Methods

Cell Culture

Human aortic VSMC (passage 5) were obtained from Clonetics Corp (San Diego, Calif) and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 ng/mL epidermal growth factor, 2 ng/mL basic fibroblast growth factor, and 1 μmol/L dexamethasone in the standard fashion.16 Cells were incubated at 37°C in a humidified atmosphere of 95% air–5% CO₂ with changes of medium every 2

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days. These cells showed the specific characteristics of VSMC by immunohistochemical examination and morphological observation. Briefly, human aortic VSMC also tested positive for α-actin and negative for expression of factor VIII antigen. All the cells were used within passages 5 to 6.

**Cell Counting Assay**

In this study, we measured cell numbers by using a WST cell counting kit (Wako). Tetrazolium salt has been used to develop a quantitative colorimetric assay for cell growth. In this study, we used sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) because this compound produces a highly water-soluble formazan dye, which makes the assay procedure easier to perform. We confirmed that a serum-stimulated increase in cell number is associated with increased absorbance at 450 nm.

**Effect of Native Lp(a) or Oxidized Lp(a) on VSMC Growth**

Human aortic VSMC were seeded onto uncoated 96-well tissue culture plates (Corning). In the preparation of experiments for determination of cell count, the cells were grown to 70% confluency in culture dishes. After 70% confluence was achieved, the medium was changed to fresh DSF (defined serum-free medium). DSF medium was supplemented with insulin (5 × 10^{-7} mol/L), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L), as previously described. The cells were then incubated for 48 hours to make them quiescent. Lp(a) used as a positive control for Western blotting was purified from plasma of donors with elevated Lp(a) concentration after 12 hours of fasting. Butyryl hydroxylsulphone (10 μmol/L) was added during all procedures to avoid the oxidation of lipoprotein. On the other hand, oxidized Lp(a) was produced with the use of copper ions as an oxidizing agent. Lp(a) was oxidized by incubation with CuCl₂ (0.3, 0.4, 1 or 10.0 μg/mL, Wako Chemical) or lipoxygenase (100 μg/mL, Wako Chemical) for 12 hours at 37°C. The degree of oxidation was quantified by 2 methods: (1) the increase in relative mobility on agarose gel (Helena Laboratory, Tokyo, Japan) and (2) the formation of t-thiobarbituric acid–reactive substances (Wako Chemical kit). During this preparation, we confirmed that Lp(a) was intact and not degraded. There was no significant difference in the preparation of oxidized Lp(a) between the two different methods. Thus, we used oxidized Lp(a) modified by copper ions to examine the mitogenic activity. On day 2, the medium was again changed to fresh DSF containing Lp(a) or oxidized Lp(a). After 4 days, an index of cell proliferation was determined to study the effects of Lp(a) and oxidized Lp(a) on VSMC growth. In addition, antioxidants (probucol and flavastatin; 150 μmol/L) were added to the solution before oxidation by CuCl₂ (100 μg/mL) or lipoxygenase (100 to examine the effect of oxidized Lp(a) on VSMC growth). Other drugs (pravastatin and simvastatin; 150 μmol/L) were used as negative control.

**Western Blotting**

Western blotting was performed for analysis of extracellular signal-regulated protein kinase (ERK) with the use of a phosphospecific antibody. VSMC were seeded onto 15-cm dishes (Corning), grown to 70% confluence, and made quiescent by incubation in DSF medium before treatment. After treatment, the cells were extracted with RIPA buffer (50 mmol/L Tris-Cl, 0.15 mol/L NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton-X, 10 mmol/L EGTA, and 10 mmol/L NaF). Samples containing 20 μg protein were run on 12.5% sodium dodecylsulfate polyacrylamide gels. Proteins were separated by SDS/PAGE, transferred to a nitrocellulose membrane (Hybond ECL, Amersham) and incubated with a polyclonal antibody to ERK (anti-human rabbit IgG, 1:1000, New England BioLabs) or phosphospecific ERK (anti-human rabbit IgG, 1:1000, New England BioLabs) at 4°C overnight. Antibodies were diluted in 4% skimmed milk and 0.1% Tween 20 in PBS. The membranes were then washed and incubated with a 1:2000 dilution of rabbit Ig horseradish peroxidase–conjugated antibody (Amersham). To quantify and compare levels of proteins, the density of each band was measured by densitometry (Shimazu). Amounts of loaded proteins were confirmed to be equal by staining with Coomassie brilliant blue R (Sigma). Staining with Coomassie brilliant blue revealed identical amounts of protein in all samples for Western blotting (data not shown).

**Statistical Analysis**

All values are expressed as mean±SEM. ANOVA with subsequent Bonferroni’s test was used to determine the significance of differences in multiple comparisons. Values of P<0.05 were considered statistically significant.

**Results**

**Effects of Lp(a) and Oxidized Lp(a) on Growth of Human VSMC**

First, we examined the mitogenic activity of native Lp(a) and oxidized Lp(a) in human aortic VSMC. As shown in Figure 1a, native Lp(a) significantly stimulated the growth of human VSMC in a dose-dependent manner (P<0.01). The mitogenic activity of native Lp(a) at 10 μg/mL was equivalent to that of the growth medium. In addition, addition of oxidized Lp(a) also resulted in a significant increase in the number of VSMC in a dose-dependent manner (P<0.01, Figure 1a). Unexpectedly, the mitogenic activity of oxidized Lp(a) on the growth of VSMC was significantly enhanced as compared with native Lp(a) (P<0.01, Figure 1a). The potent stimulatory effect of oxidized Lp(a) on the growth of VSMC was also confirmed by the observation that the oxidation by copper ions altered the mitogenic activity of Lp(a), as shown in Figure 1b. In addition, we confirmed the mitogenic activity of oxidized Lp(a) on VSMC growth by using antioxidants. Both flavastatin and probucol inhibited the conversion from native Lp(a) to oxidized Lp(a) by CuCl₂, as assessed by gel mobility shift assay, whereas nonantioxidants such as pravastatin and simvastatin did not inhibit the oxidation of Lp(a) (data not shown). Similarly, the treatment with flavastatin but not simvastatin and pravastatin significantly inhibited the conversion from native Lp(a) to oxidized Lp(a) by lipoxygenase as assessed by gel mobility shift assay (Figure 1c, P<0.01). In addition, the treatment with probucol also significantly inhibited the conversion from native Lp(a) to oxidized Lp(a) by lipoxygenase (Figure 1d, P<0.01). The decrease in production of oxidized Lp(a) by probucol resulted in less mitogenic activity on VSMC growth as compared with vehicle in a dose-dependent manner (Figure 1e, P<0.01).

Finally, we elucidated the molecular mechanisms of how Lp(a) stimulated the growth of VSMC. We focused on the signal transduction system, known as mitogen-activated protein (MAP) kinase modules, as those controlled by kinases modulate critical cellular functions such as cell growth, differentiation and apoptosis. The ERK/MAP kinase pathway is activated by many growth factors and hormones and is involved in mediating cellular proliferation, transformation, and differentiation. ERK was marked-edly phosphorylated by treatment with native Lp(a) (10 μg/mL), as assessed by Western blotting with a specific antibody for phosphorylated ERK. ERK was transiently phosphorylated from 5 minutes and disappeared within 30
minutes ($P<0.01$, Figure 2a). ERK was also phosphorylated from 5 minutes and disappeared within 30 minutes after stimulation with oxidized Lp(a) ($P<0.01$), as shown in Figure 2b. The specificity of phosphorylation of ERK was confirmed by the observation that no apparent change in total ERK by native Lp(a) or oxidized Lp(a) was observed (Figures 2a and 2b). Furthermore, the degree of phosphorylation of ERK was much greater in VSMC treated with oxidized Lp(a) than in those treated with native Lp(a) (Figure 2c, $P<0.01$). Importantly, administration of a specific inhibitor of MEK (the mitogen-activated protein kinase/ERK kinase), PD 98059, significantly attenuated VSMC cell growth induced by native Lp(a) or oxidized Lp(a) in a dose-dependent manner (Figure 2d, $P<0.01$). Vehicle alone (DMSO) did not affect the growth of VSMC. These findings clearly reveal the importance of the ERK pathway in the growth of VSMC stimulated by both native Lp(a) and oxidized Lp(a).
Discussion

Lp(a) has been of interest in vascular biology, since epidemiological studies indicated it to be an independent risk factor for cardiovascular disease, for example, atherosclerosis and ischemic heart disease.1–6 Recent studies demonstrated that Lp(a) stimulated the growth of VSMC by inhibition of the activation of TGF-β, an autocrine inhibitor of VSMC growth. In addition to the action of apo (a) on TGF-β activation, our previous experiments with gene transfer revealed the potential TGF-β–independent mitogenic mechanisms of Lp(a),20 consistent with a previous report.21 However, the molecular mechanism of how Lp(a) stimulated VSMC growth is unclear. In this study, we focused on the ERK pathway and how it is involved in mediating cellular proliferation, transformation, and differentiation. Expectedly, this study demonstrated that Lp(a) stimulated human VSMC growth through an ERK-dependent pathway, although further studies are necessary to elucidate the exact mechanisms of the mitogenic activity of Lp(a).

Recently, it has been reported that oxidized Lp(a) has additional specific biological properties compared with native Lp(a). The contribution of oxidized Lp(a) to the development of atherosclerosis is supported by several lines of evidence: (1) oxidative modification of Lp(a) enhanced Lp(a)-induced plasminogen activator inhibitor-1 (PAI-1) production in vascular endothelial cells,22 as native Lp(a) itself increased PAI-1 production in cultured endothelial cells23; (2) oxidized Lp(a) impaired endothelium-dependent vasodilation and was more potent than oxidized LDL24; (3) macrophages took up oxidized Lp(a) through scavenger receptors as well as oxidized LDL.25 More importantly, this study demonstrated that the biological effects of oxidized Lp(a) on the growth of human VSMC are more potent than those of native Lp(a) through the ERK pathway. This finding is extremely important because vasodilation is inhibited by oxidized Lp(a).26 Moreover, oxidized Lp(a) enhanced Lp(a)-induced PAI-1 production in vascular endothelial cells.15 Elevation of oxidized Lp(a) may explain the endothelial dysfunction observed in hypertensive patients.26–28 Interestingly, the inhibition of oxidation of Lp(a) by probucol inhibited the growth of human VSMC. Since the restenosis rates per segment were significantly reduced in the patients who were treated with probucol,29 the inhibition of the oxidation of Lp(a) by probucol might contribute to the reduction of restenosis rate.

Perspectives

The present study demonstrated that (1) oxidized Lp(a) is more potent than native Lp(a) in stimulating VSMC growth and (2) the stimulatory effects of both native and oxidized Lp(a) on the growth of human aortic VSMC are dependent on ERK. These results provide new information on the molecular mechanisms of the mitogenic action of Lp(a). With the use of a newly developed ELISA to detect modified Lp(a), especially oxidized Lp(a), the previous study demonstrated that oxidized Lp(a) was significantly increased in the hypertensive patients with vascular complication.15 Thus, elevation of oxidized Lp(a) might be related to the pathogenesis of hypertensive complications such as stroke. Interestingly, in addition to previous reports showing that plasma-derived
Lp(a) penetrates human arteries and Lp(a) accumulates in vascular lesions. Thus, plasma-derived oxidized Lp(a) present in the vascular wall may have an important role in the pathogenesis of arteriosclerosis/atherosclerosis. Oxidized Lp(a) rather than Lp(a) may play an important role in the pathogenesis of cardiovascular disease.

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