Lipoprotein(a) Enhances the Expression of Intercellular Adhesion Molecule-1 in Cultured Human Umbilical Vein Endothelial Cells

Shigeki Takami, MD; Shizuya Yamashita, MD, PhD; Shinji Kihara, MD, PhD; Masato Ishigami, MD, PhD; Kaoru Takemura, MD, PhD; Noriaki Kume, MD, PhD; Toru Kita, MD, PhD; Yuji Matsuzawa, MD, PhD

Background—We reported an increase in serum lipoprotein(a) [Lp(a)] levels in patients with thromboangiitis obliterans, suggesting that Lp(a) could also contribute to the pathogenesis of cardiovascular diseases by a mechanism different from atherosclerosis. Adhesion molecules were shown to contribute to the development of not only atherosclerotic but also inflammatory vascular diseases.

Methods and Results—We evaluated the effect of Lp(a) on the expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin in human umbilical vein endothelial cells by a cell ELISA. Lp(a) dramatically enhanced the levels of ICAM-1 in a dose-dependent manner. A discernible increase in ICAM-1 expression was observed at a physiological concentration of 0.26 mmol cholesterol/L Lp(a) after 48-hour incubation. A 1.8-fold increase in ICAM-1 expression was observed 48 hours after the addition of Lp(a) (1.04 mmol cholesterol/L). Northern blot analysis demonstrated that the amount of ICAM-1 mRNA was increased after treatment with Lp(a). In contrast to ICAM-1, the expression of VCAM-1 and E-selectin was not significantly affected by Lp(a). Lp(a<sup>−/−</sup>) [apolipoprotein(a)-removed Lp(a) by reduction with dithiothreitol] and LDL had no significant effect on the expression of ICAM-1. In contrast, recombinant apolipoprotein(a) protein alone significantly enhanced ICAM-1 expression. Lp(a) decreased the level of active transforming growth factor (TGF)-β in the conditioned medium. Furthermore, recombinant TGF-β significantly decreased the Lp(a)-induced ICAM-1 expression. These findings suggested that Lp(a) may enhance the ICAM-1 expression by decreasing active TGF-β level.

Conclusions—Lp(a) could contribute to the development of cardiovascular diseases by enhancing the expression of ICAM-1 in endothelial cells. (Circulation. 1998;97:721-728.)

Key Words: cardiovascular diseases ■ cells ■ growth substances ■ leukocytes ■ lipoproteins

Serum Lp(a) was first identified in 1963 by Berg. A high concentration of Lp(a) has been demonstrated to be one of the major risk factors for premature development of atherosclerosis. Lp(a) is a particle with an unusual structure consisting of apo(a), which is linked to apo B-100 of an LDL-like particle through disulfide bonds. cDNA nucleotide sequence analyses have shown that apo(a) has a high degree of homology to plasminogen, one of the important factors in fibrinolysis system. Therefore it is suggested that the pathophysiological effects of Lp(a), including those on fibrinolysis, may be attributable to apo(a). Lp(a) was shown to bind to vascular endothelial cells and macrophages and to extracellular components such as fibrin and inhibits cell-associated plasminogen activation in vitro.

One of the earliest events in atherogenesis in cholesterol-fed animals is an increased binding of monocytes to endothelial cells and their entry into vessel walls. It is hypothesized that these monocytes contribute in several ways to plaque formation. Although the molecular mechanism is not completely understood, in vitro studies have identified three molecules, ICAM-1, E-selectin (endothelial-leukocyte adhesion molecule-1 [ELAM-1]), and VCAM-1. These adhesion molecules are inducible on the endothelial cell surface and can support the adhesion of various leukocytes, including monocytes. Of these, ICAM-1 was shown to be expressed in human atherosclerotic plaques by an immunohistochemical method and may be a candidate that plays an important role in mediating the localization of monocytes in the intima of arteries. ICAM-1 (CD54) is a markedly glycosylated adhesion molecule belonging to the immunoglobulin gene superfamily. Its expression is restricted on resting cells but is highly inducible by activation such as exposure to IL-1β or TNF-α.
binds to its counterreceptor—leukocyte function-associated-1 molecule (LEA-1, or CD11a/CD18)—as well as to Mac-1 (CD11b/CD18).

We previously reported an increase in Lp(a) levels in patients with thrombogenicity obliterator, suggesting that Lp(a) could contribute to the pathogenesis of cardiovascular disorders by a mechanism different from atherosclerosis. Adhesion molecules including ICAM-1 were shown to contribute to the development of not only atherosclerotic but also inflammatory vascular disorders by regulating cell adhesion between leukocytes and endothelial cells. To date, there has been no report of atherogenesis not only atherosclerotic but also inflammatory vascular disorders by regulating cell adhesion between leukocytes and endothelial cells. To date, there has been no report of atherogenesis.

In the current study, to investigate the effects of Lp(a) on the expression of adhesion molecules in endothelial cells, cultured HUVEC were subjected to human plasma-derived Lp(a). We evaluated the effects of Lp(a) on the expression of ICAM-1, VCAM-1, and E-selectin in HUVEC by cell ELISA and Northern blotting. We demonstrated an enhanced expression of ICAM-1 but not VCAM-1 or E-selectin by addition of Lp(a) to culture medium. Moreover, to clarify the mechanism of Lp(a)-induced ICAM-1 enhancement, we investigated the effects of TGF-β and anti–TGF-β antibody on the enhanced expression of ICAM-1 by Lp(a).

Methods

Reagents

Medium 199, HBSS, and FCS were purchased from GIBCO Laboratories; lysine–Sepharose was purchased from Pharmacia–LKB; mouse anti-human Lp(a) monoclonal antibodies from Chemicon International; mouse anti-human ICAM-1 monoclonal antibodies, BBA3, from British Bio–technology; mouse anti-human VCAM-1 monoclonal antibodies, mouse anti-human E-selectin monoclonal antibodies, recombinant human TGF-β, and mouse anti-human TGF-β monoclonal antibodies from Genzyme; peroxidase-conjugated goat anti–mouse IgG from Orgonon Teknika–Cappel; and endotoxin-specific limulus amoebocyte lysate, LAL-ES from Wako Pure Chemical Industries. Recombinant apo(a) protein was a generous gift from Prof Gert M. Kostner (Kaar-Franzens-University of Graz, Graz, Austria). All other chemicals were of reagent grade.

Isolation of Lp(a), Lp(a−), and LDL

Lp(a) was prepared from the plasma of a male donor whose apo(a) had a high affinity for lysine–Sepharose as reported by Fless and Sydney. The donor had a plasma Lp(a) concentration of ~1000 mg/mL and an apo(a) phenotype B (single band) by the typing system of Utermann et al Briefly, total lipoproteins (d<1.210 g/mL) were isolated by ultracentrifugation and extensively dialyzed against 0.1 mol/L phosphate buffer (pH 7.4) containing 0.1% Na2-EDTA, 0.1% NaN3, and 1 mmol/L benzamidine. These lipoproteins were passed through a column containing lysine-Sepharose. The column was washed with 0.5 mol/L NaCl, 0.1 mol/L NaHCO3, 1 mmol/L benzamidine, pH 8.3. Lp(a) was eluted with 20 mol/L 6-aminohexanoic acid dissolved in 0.1 mol/L phosphate buffer (pH 7.4), 1 mmol/L benzamidine, and 0.01% Na2-EDTA and NaN3. The unbound lipoproteins obtained after lysine-Sepharose chromatography were dialyzed against EDTA-saline (d=1.006 g/mL), and then LDL (d=1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation according to Havel’s method. The isolated Lp(a) and LDL were extensively dialyzed against 0.15 mol/L NaCl, 0.01% Na2-EDTA, and 0.01% NaN3.

The Lp(a) particles were separated into two products: apo(a) and a floating apoB–100–containing lipoprotein, Lp(a−). To make Lp(a−), 1 mol/L DTT was added to the Lp(a) solution to give a final concentration of 0.01 mol/L. Reduction of Lp(a) by DTT was achieved by incubation at 37°C for 15 minutes. After reduction with DTT, apo(a) and Lp(a−) were separated by ultracentrifugation at 4×106 rpm for 18 hours in a Beckman 50.2 Ti rotor. The obtained Lp(a−) fraction did not contain apo(a) as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or by Western blot analysis using a monoclonal anti-human Lp(a). Lp(a−) was dialyzed against 0.15 mol/L NaCl, 0.01% Na2-EDTA, and 0.01% NaN3. Isolated samples were dialyzed finally against medium 199 and sterilized by ultrafiltration before use.

Incubation of HUVEC With Lp(a), Lp(a−), LDL, and Recombinant Apo(a) Protein

HUVEC were purchased from Kurabo Industries, Ltd. HUVEC were seeded in plastic plates precoated with collagen (type I) (Becton & Dickinson Labware) and cultured in medium 199 supplemented with 20% FCS, 12.5 μg/mL endothelial cell growth supplement, 1 μg/mL hydrocortisone, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 37°C in humidified 5% CO2 and 95% air. HUVEC were used for experiments at passages 3 to 4.

Cell ELISA of ICAM-1, VCAM-1, and E-Selectin on HUVEC

The protocols used for cell ELISA of ICAM-1, VCAM-1, and E-selectin were modified from that of Rothlein et al Briefly, HUVEC in 96-well microplates, which had been treated with Lp(a), Lp(a−), or LDL, were washed with warm HBSS containing 0.1% BSA. The monolayers were washed and then incubated with mouse anti-human ICAM-1, VCAM-1, or E-selectin monoclonal antibodies at a final concentration of 0.5 μg/mL in HBSS containing 0.1% BSA to detect the surface expression of these adhesion molecules. After
incubation of cells at room temperature for 30 minutes, the plates were washed five times with HBSS containing 0.1% BSA and then treated with 0.1 mL/well of peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution in HBSS containing 0.1% BSA). After 1-hour incubation at room temperature, the plates were washed five times with HBSS containing 0.1% BSA and incubated at room temperature in 0.1 mL/well of the substrate solution (10 mL 0.1 mol/L phosphate-citrate buffer [pH 5] + 4 mg o-phenylenediamine+15 µL 30% H₂O₂; mixed immediately before incubation). After an incubation for 15 minutes in a dark place, 50 µL/well of 2 mol/L H₂SO₄ was added and spectrophotometric readings were made at 492 nm, using a microplate reader (model 450, Bio-Rad).

**Northern Blot Analysis**
Total cellular RNA from cultured HUVEC was extracted by acid-phenol-chloroform method²⁷ and electrophoresed through 1% agarose gels containing formaldehyde and transferred onto nitrocellulose membranes. Northern blots were hybridized with human ICAM-1 cDNA probes labeled with [α-³²P]dCTP using random hexanucleotide primers.²⁸ A 1.3-kb Xho I fragment of human ICAM-1 cDNA,²⁹ kindly provided by Dr Brian Seed (Massachusetts General Hospital, Boston, Mass), was used. The blots were rehybridized with radiolabeled human β-actin cDNA probe for comparison.

**TGF-β Assay**
The amount of active TGF-β in the medium was determined by a modification of the mink lung epithelial cell assay.²⁹ Briefly, the conditioned media and TGF-β standards were diluted 1:100 in serum-free DMEM. DNA synthesis was determined by ['H]thymidine (1 μCi/mL) incorporation during 1 hour plus 23 hours after the addition of the conditioned media with or without neutralizing TGF-β antibody. TGF-β activity was calculated as the proportion of the inhibition of DNA synthesis that was reversed in the presence of the neutralizing antibody. The TGF-β samples and conditioned media both contained 5% FCS. The amount of total (active plus latent) TGF-β in the media was determined by an ELISA, using a commercial kit (Quantikine; R&D Systems). Briefly, to activate latent TGF-β to the immunoreactive form, 1N HCl was added to the conditioned medium to give a final concentration of 0.167N HCl. After incubation for 10 minutes at room temperature, the acidified medium was neutralized with 1.2N NaOH/0.5 mol/L HEPES free acid (pH 7.2 to 7.6). Ninety-six-well microplates coated with recombiant TGF-β soluble receptor type II were incubated for 1 hour with the samples and the TGF-β standards on a horizontal orbital microplate shaker at 500 rpm, with peroxidase-conjugated antibody to TGF-β at room temperature on a horizontal orbital microplate (1 hour), and then with the chromogenic substrate tetramethylbenzidine at room temperature on a horizontal orbital microplate (1 hour), and then with the chromogenic substrate tetramethylbenzidine at room temperature for 10 minutes at room temperature, the acidified medium was neutralized with 1.2N NaOH/0.5 mol/L HEPES free acid (pH 7.2 to 7.6). Ninety-six-well microplates coated with recombinant TGF-β soluble receptor type II were incubated for 1 hour with the samples and the TGF-β standards on a horizontal orbital microplate shaker at 500 rpm, with peroxidase-conjugated antibody to TGF-β at room temperature on a horizontal orbital microplate (1 hour), and then with the chromogenic substrate tetramethylbenzidine at room temperature on the benchtop (20 minutes). Absorbances at 450 nm were converted into quantities with a standard curve.

**Chemical Analyses**
Protein content was determined by the method of Lowry et al, using BSA as a standard.³⁰ Total cholesterol was measured enzymatically using a commercial kit (Kyowa Medex). Lp(a) was measured by an ELISA kit (Biopool, Umeå). The content of lipid peroxides in the conditioned medium from the control and treated HUVEC was determined as TBARS by the modified method of Yagi,³¹ using a commercial kit (Wako Pure Chemical Industries).

**Statistical Analysis**
The statistical significance of the differences between the means of groups was determined by Student’s paired t test or ANOVA.

**Results**

**Expression of ICAM-1 in HUVEC Treated With Lp(a)**
Cultured HUVEC monolayers were treated with varying concentrations of Lp(a) in medium 199 containing 5% FCS, and cell surface expression of ICAM-1 was measured by the cell ELISA method. We found that cultured HUVEC without Lp(a) treatment constitutively expressed low levels of ICAM-1. This expression was essentially constant over the time course of all experiments (data not shown). As shown in Fig 1, a physiological level of Lp(a) consistently and dramatically upregulated the levels of ICAM-1 expression in a dose-dependent manner. Lp(a) at a concentration of as low as 0.26 mmol cholesterol/L suspended in culture medium caused a significant increase in ICAM-1 expression detected at 48 hours of treatment. After 48-hour treatment with 1.56 mmol cholesterol/L Lp(a), cell surface expression of ICAM-1 was increased about twofold of the basal expression. The concentrations of Lp(a) used in the current experiment were similar to those commonly observed in vivo. This dose-response experiment in Fig 1 was a representative of two independent experiments.

Fig 2 shows the time course of Lp(a)-induced ICAM-1 upregulation. A significant increase in ICAM-1 expression was observed 24 hours after the addition of Lp(a) (1.04 mmol cholesterol/L); the expression of ICAM-1 reached a plateau by 72 hours and remained stable up to at least 96 hours. In contrast, cell surface expression of ICAM-1 in HUVEC was
not significantly affected by LDL treatment (1.04 mmol cholesterol/L) within 96 hours. Comparable results were obtained in two additional experiments under the same conditions. These results suggest that Lp(a) but not LDL enhances the expression of cell surface ICAM-1.

Northern Blot Analysis
To determine whether the enhancement of ICAM-1 expression occurred at the transcriptional level, the amount of ICAM-1 mRNA was evaluated by Northern blot analysis. Fig 3 shows the Northern blot for ICAM-1 mRNA abundance from HUVEC maintained for 24 hours in medium containing 1.04 mmol cholesterol/L lipoproteins. The addition of Lp(a) caused an apparent increase in the ICAM-1 mRNA level, whereas LDL had no obvious effect. These results were confirmed by an additional experiment.

Expression of VCAM-1 and E-Selectin in HUVEC Treated With Lp(a)
In addition, the effect of Lp(a) on the expression of VCAM-1 and E-selectin in HUVEC was measured. Cultured HUVEC monolayers were treated with 1.04 mmol cholesterol/L Lp(a) in medium 199 containing 5% FCS for 72 hours, and cell surface expression of VCAM-1 and E-selectin was measured.

Effects of LDL, Lp(a), and Recombinant Apo(a) Protein on the Induction of ICAM-1 in HUVEC
Studies were performed to elucidate the mechanism of the Lp(a)-induced enhancement of ICAM-1 expression. To determine which structural part of Lp(a) is responsible for the overexpression of ICAM-1 in HUVEC, the effects of LDL, Lp(a), [apo(a)-free Lp(a) by reduction with DTT] and recombinant apo(a) protein on the expression of ICAM-1 were examined (Fig 5). Cultured HUVEC monolayers were treated for 48 hours with LDL, Lp(a), or Lp(a) (1.04 mmol cholesterol/L, respectively) in medium 199 supplemented with 5% FCS, and the cell surface expression of ICAM-1 was measured by a cell ELISA, as described in “Methods.” Data are expressed as mean±SD of quadruplicate determinations. *P<.01 vs control.

TBARS in Media Before and After the Incubation With HUVEC
It has been suggested that oxidatively modified lipoproteins induce the expression of adhesion molecules such as ICAM-1 and VCAM-1 in HUVEC. Therefore it is possible that the...
TABLE 1. Generation of TBARS During Treatment of HUVEC With Lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Before</th>
<th>After 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>1.24±0.57</td>
<td>44.90±9.17</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.40±0.28</td>
<td>42.80±10.47</td>
</tr>
<tr>
<td>Lp(a−)</td>
<td>1.53±0.47</td>
<td>41.11±8.47</td>
</tr>
</tbody>
</table>

HUVEC were treated with medium containing LDL, Lp(a), and Lp(a−) at the concentration of 1.04 mmol cholesterol/L, respectively. The concentration of TBARS in each medium was measured before and after 48-hour incubation, as described in “Methods.” All of these lipoproteins induced TBARS formation to a similar extent, and there was no significant difference between the media containing LDL, Lp(a), and Lp(a−). Data are expressed as mean±SD (n=4).

TGF-β in Media Containing LDL or Lp(a) After Incubation With HUVEC

We next wanted to clarify the mechanism of Lp(a)-induced ICAM-1 enhancement. It has been reported that Lp(a) stimulated the growth of human vascular smooth muscle cells in culture and this effect resulted from the inhibition of plasminogen activation and consequently the activation by plasmin of latent TGF-β, which is an inhibitor of smooth muscle cell growth. Therefore, we hypothesized the effect of Lp(a) on the expression of ICAM-1 was due to the diminishing of active TGF-β by Lp(a). To evaluate this hypothesis, the amounts of active and total (active plus latent) TGF-β in media containing LDL or Lp(a) were measured after the incubation with HUVEC.

TABLE 2. Concentration of Active and Total (Active Plus Latent) TGF-β in Media After Incubation With HUVEC

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Active</th>
<th>Total (Active Plus Latent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.2±1.8</td>
<td>7.4±1.6</td>
</tr>
<tr>
<td>LDL</td>
<td>4.0±2.6</td>
<td>7.2±0.8</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>&lt;0.1</td>
<td>6.9±2.7</td>
</tr>
</tbody>
</table>

HUVEC were treated with medium containing LDL and Lp(a) at the concentration of 1.04 mmol cholesterol/L, respectively. The concentration of TGF-β in each medium was measured after 48-hour incubation, as described in “Methods.” Lp(a) reduced the amount of active TGF-β more than 40-fold as compared with control or LDL-treated cultures. However, the amount of total (active plus latent) TGF-β was unaffected by the presence of Lp(a). Data are expressed as mean±SD (n=4).

Effect of Neutralizing TGF-β Antibodies on Upregulation of ICAM-1 by Lp(a)

To test the hypothesis further, we examined the effect of neutralizing TGF-β antibodies on the upregulation of ICAM-1 by Lp(a). Neutralizing antibodies against TGF-β (0.1, 1, 10, and 30 μg/mL at the final concentration) were added into the treatment medium and the expression of ICAM-1 in HUVEC was measured after incubation with and without Lp(a) (1.04 mmol cholesterol/L) (Fig 6). In the absence of neutralizing TGF-β antibodies, Lp(a) significantly increased ICAM-1 expression, as shown in Fig 1 (P<.01). A significant enhancement of ICAM-1 expression by Lp(a) was also observed in the presence of neutralizing TGF-β antibodies at the concentration of 0.1 μg/mL and 1 μg/mL (P<.01 and P<.05, respectively). At higher concentrations of neutralizing TGF-β antibodies >10 μg/mL, however, an additional induction of ICAM-1 expression by Lp(a) could not be observed and there was no significant difference between the expression of ICAM-1 with or without treatment of Lp(a). These data suggest that the enhancement of ICAM-1 expression by Lp(a) may be attributed to the reduction of active TGF-β by Lp(a).

Effect of TGF-β on the Expression of ICAM-1 in HUVEC Treated With Lp(a) and TNF-α

To elucidate the TGF-β-related mechanism of enhanced ICAM-1 expression in HUVEC, the effect of recombinant active TGF-β on the expression of ICAM-1 was examined in HUVEC treated with Lp(a) and TNF-α. Dose-response experiments were performed with both resting and TNF-α-treated HUVEC as well as Lp(a)-treated HUVEC. Recombinant active TGF-β was added into the treatment medium at the final concentration of 1 ng/mL and 10 ng/mL. Fig 7A shows the results with Lp(a)-treated HUVEC. The expression of ICAM-1 was significantly upregulated by Lp(a) compared with control or LDL. However, the amount of total (active plus latent) TGF-β was unaffected by the presence of Lp(a).

Figure 6. Effect of neutralizing TGF-β antibodies on the induction of ICAM-1 by Lp(a) in HUVEC: Expression of ICAM-1 in untreated (open bars) and Lp(a)-treated (1.04 mmol cholesterol/L; solid bars) HUVEC in medium 199 supplemented with 5% FCS with the addition of neutralizing TGF-β antibodies (0–30 μg/mL). After 48-hour incubation, cell surface expression of ICAM-1 was measured by a cell ELISA, as described in “Methods.” Data are expressed as mean±SD of quadruplicate determinations. *P<.05, **P<.01.
ICAM-1 induction between Lp(a) and TNF-
are presented in Fig 7B. Recombinant TNF-
induced ICAM-1 expression in HUVEC suggests that there may be different mechanisms of
the exact pathogenetic mechanism for the development of
atherosclerotic cardiovascular diseases such as coronary heart
disease,2,3 stroke,37 and arteriosclerosis obliterans.22 However, the exact pathogenetic mechanism for the development of atherosclerosis in these vascular diseases has not been fully elucidated. We reported an increase in Lp(a) levels in patients with thromboangiitis obliterans, whose pathological feature is an intense inflammation leading to later arterial occlusions.22 Therefore Lp(a) could also contribute to the pathogenesis of cardiovascular diseases by a mechanism different from that of atherosclerosis. On the other hand, an increased leukocyte (especially monocyte) adhesion to the vascular endothelium is considered to be an early event in atherosclerosis and certain inflammatory disorders. It has been speculated that adhesion molecules contribute to the development of not only atherosclerotic but inflammatory vascular diseases. Therefore, it is reasonable to hypothesize that Lp(a), or one of its specific components, apo(a), might induce the expression of adhesion molecules in endothelial cells on the basis of these diseases.

In the current study, we have demonstrated that Lp(a) increases the expression of mRNA and cell surface expression of ICAM-1 but not that of VCAM-1 or E-selectin in HUVEC. ICAM-1 has been considered to play an important role in the mechanism of inflammation. Furthermore, ICAM-1 was demonstrated to be immunohistochemically expressed in human atherosclerotic plaques38 and is a candidate that may play an important role in mediating the localization of leukocytes in the intima of arteries, one of the earliest events in the process of atherogenesis. The present results support our clinical studies showing that Lp(a) could contribute to the development of both atherosclerotic and inflammatory vascular diseases.22 Once present in the developing atherosclerotic lesions, lymphocytes have the capacity to produce various cytokines, which, in turn, can activate endothelial cells locally to express adhesion molecules. Such potential paracrine mechanisms for amplifying leukocyte recruitment are becoming better defined.38

To determine which part of Lp(a) is responsible for the increased ICAM-1 expression, we compared the effect of LDL, Lp(a), and Lp(a−) at the same concentration of cholesterol (1.04 mmol/L). In contrast to Lp(a), LDL and Lp(a−) had no significant effect on the expression of ICAM-1 in HUVEC. Therefore, apo(a) itself seemed to be responsible for the overexpression of ICAM-1 induced by Lp(a). Furthermore, we demonstrated that recombinant apo(a) protein itself enhanced significantly the expression of ICAM-1 in HUVEC, while the apo(a) obtained by the reduction of Lp(a) by DTT had no significant effect on the expression of ICAM-1 (data not shown). The “kringle” structure has disulfide linkages and the reduction by DTT breaks the structure. From these findings, it is speculated that the preservation of “kringle” structure in apo(a) is indispensable for the induction of ICAM-1 by Lp(a) in HUVEC.

Recent in vitro studies have shown that cultured endothelial cells incubated with minimally oxidized LDL or β-VLDL exhibit an enhanced adhesiveness for monocytes.39,40 Lysophosphatidylcholine, a prominent phospholipid component of oxidized LDL and β-VLDL, has been shown to induce the expression of adhesion molecules such as ICAM-1 and VCAM-1 in cultured arterial endothelial cells.41 Furthermore, several investigators have indicated that oxidative modification of LDL can occur in arterial intima.33,42 Therefore, it is reasonable to speculate that oxidatively modified Lp(a) by

![Figure 7](http://circ.ahajournals.org/)

**Figure 7.** Effect of recombinant active TGF-β on the expression of ICAM-1 in HUVEC. A, Expression of ICAM-1 in untreated (open bars) and Lp(a)-treated (1.04 mmol cholesterol/L; solid bars) HUVEC in medium 199 supplemented with 5% FCS with the addition of recombinant active TGF-β (0, 1 and 10 ng/mL). After 48-hour incubation, the cell surface expression of ICAM-1 was measured by a cell ELISA, as described in “Methods.” B, Expression of ICAM-1 in untreated HUVEC (open bars) and HUVEC treated with 5 ng/mL (shaded bars) and 10 ng/mL (solid bars) of recombinant TNF-α. The expression of ICAM-1 was measured after 6-hour incubation. Data are expressed as mean±SD. *P<.05.

Discussion

Previous investigations showed that a high concentration of serum Lp(a) constitutes one of the major risk factors for atherosclerotic cardiovascular diseases such as coronary heart disease,2,3 stroke,37 and arteriosclerosis obliterans.22 However, the exact pathogenetic mechanism for the development of atherosclerosis in these vascular diseases has not been fully elucidated. We reported an increase in Lp(a) levels in patients with thromboangiitis obliterans, whose pathological feature is an intense inflammation leading to later arterial occlusions.22 Therefore Lp(a) could also contribute to the pathogenesis of cardiovascular diseases by a mechanism different from that of atherosclerosis. On the other hand, an increased leukocyte (especially monocyte) adhesion to the vascular endothelium is considered to be an early event in atherosclerosis and certain inflammatory disorders. It has been speculated that adhesion molecules contribute to the development of not only atherosclerotic but inflammatory vascular diseases. Therefore, it is reasonable to hypothesize that Lp(a), or one of its specific components, apo(a), might induce the expression of adhesion molecules in endothelial cells on the basis of these diseases.

In the current study, we have demonstrated that Lp(a) increases the expression of mRNA and cell surface expression of ICAM-1 but not that of VCAM-1 or E-selectin in HUVEC. ICAM-1 has been considered to play an important role in the mechanism of inflammation. Furthermore, ICAM-1 was demonstrated to be immunohistochemically expressed in human atherosclerotic plaques38 and is a candidate that may play an important role in mediating the localization of leukocytes in the intima of arteries, one of the earliest events in the process of atherogenesis. The present results support our clinical studies showing that Lp(a) could contribute to the development of both atherosclerotic and inflammatory vascular diseases.22 Once present in the developing atherosclerotic lesions, lymphocytes have the capacity to produce various cytokines, which, in turn, can activate endothelial cells locally to express adhesion molecules. Such potential paracrine mechanisms for amplifying leukocyte recruitment are becoming better defined.38

To determine which part of Lp(a) is responsible for the increased ICAM-1 expression, we compared the effect of LDL, Lp(a), and Lp(a−) at the same concentration of cholesterol (1.04 mmol/L). In contrast to Lp(a), LDL and Lp(a−) had no significant effect on the expression of ICAM-1 in HUVEC. Therefore, apo(a) itself seemed to be responsible for the overexpression of ICAM-1 induced by Lp(a). Furthermore, we demonstrated that recombinant apo(a) protein itself enhanced significantly the expression of ICAM-1 in HUVEC, while the apo(a) obtained by the reduction of Lp(a) by DTT had no significant effect on the expression of ICAM-1 (data not shown). The “kringle” structure has disulfide linkages and the reduction by DTT breaks the structure. From these findings, it is speculated that the preservation of “kringle” structure in apo(a) is indispensable for the induction of ICAM-1 by Lp(a) in HUVEC.

Recent in vitro studies have shown that cultured endothelial cells incubated with minimally oxidized LDL or β-VLDL exhibit an enhanced adhesiveness for monocytes.39,40 Lysophosphatidylcholine, a prominent phospholipid component of oxidized LDL and β-VLDL, has been shown to induce the expression of adhesion molecules such as ICAM-1 and VCAM-1 in cultured arterial endothelial cells.41 Furthermore, several investigators have indicated that oxidative modification of LDL can occur in arterial intima.33,42 Therefore, it is reasonable to speculate that oxidatively modified Lp(a) by
HUVEC, or one of its components, might enhance the expression of ICAM-1 in HUVEC. However, LDL, Lp(a), and Lp(a−) induced TBARS formation to a similar extent in the current study. There was no significant difference between the TBARS levels of LDL, Lp(a), and Lp(a−) before and after the incubation. Therefore the effect of Lp(a) was not based on the liability to generate lipid peroxides. This finding also supports the hypothesis that apo(a) is responsible for the induction of ICAM-1 expression by Lp(a) in HUVEC.

Grainger et al reported that Lp(a) stimulated the growth of human smooth muscle cells in culture and that this effect resulted from the inhibition of plasminogen activation and consequently the activation by plasmin of latent TGF-β, which is an inhibitor of smooth muscle cell growth. In the current study, we confirmed that Lp(a) inhibited the activation of TGF-β, in agreement with the study of Grainger et al, and demonstrated that neutralizing TGF-β antibodies enhanced the expression of ICAM-1 in HUVEC and that there was no significant difference in ICAM-1 expression between the untreated and Lp(a)-treated HUVEC in the presence of the neutralizing TGF-β antibodies at the high concentration >10 μg/mL. These results suggest that Lp(a) may enhance the ICAM-1 expression at least in part by decreasing active TGF-β. Moreover, the enhancement of ICAM-1 expression by Lp(a) can be specifically attributed to the decrease in active TGF-β, because the addition of recombinant active TGF-β inhibited the enhancement of ICAM-1 expression in the Lp(a)-treated HUVEC but not in the TNF-α-treated HUVEC.

It is interesting to note that the enhanced expression in HUVEC by Lp(a) was identified only in ICAM-1 but not in VCAM-1 or E-selectin. These results demonstrate that Lp(a) can selectively upregulate ICAM-1 in HUVEC. This expression pattern is distinctly different from that elicited by bacterial endotoxin or inflammatory cytokines such as IL-1 and TNF-α, which characteristically induce ICAM-1, VCAM-1, and E-selectin coordinately in endothelial cells. Although it is difficult to clarify the cause of different reactions to Lp(a) between ICAM-1 and the other adhesion molecules in the present study, it is likely that the difference of intracellular second messengers and the existence of influence of TGF-β were concerned.

The sequence homology between apo(a) and plasminogen suggested a link between Lp(a) and atherosclerosis or thrombosis. Lp(a) has been shown to stimulate the growth of human vascular smooth muscle cells in culture, to inhibit the activation of plasminogen by tissue plasminogen activator, and to increase the expression of plasminogen activator inhibitor-1. Both enhancement of cell proliferation and inhibition of fibrinolysis by Lp(a) could contribute to the development of cardiovascular diseases. Furthermore, we suggest that Lp(a) may contribute to the development of cardiovascular diseases by enhancing the expression of ICAM-1 in endothelial cells and consequently increasing the attachment of leukocytes to endothelial cells.

The present study has demonstrated that Lp(a) could enhance the ICAM-1 expression by decreasing active TGF-β. Lp(a)-induced ICAM-1 may promote the adhesion and transendothelial migration of monocytes. Activated macrophages are likely to be an important source of IL-1 and TNF-α and T lymphocytes may produce IFN-γ. These cytokines could further induce the cell surface expression of adhesion molecules including ICAM-1, VCAM-1, and E-selectin on endothelial cells. Such a positive feedback system could possibly play an important role in the progression of atherosclerosis. In conclusion, the current study demonstrates that Lp(a) may play an essential role in both the earliest stage of atherosclerosis and inflammatory cardiovascular diseases by the enhancement of ICAM-1.

Acknowledgments
This work was supported by grants from the Japanese Ministry of Education, Science, and Welfare (Nos. 4404085 and 3557117). We thank Prof Gert M. Kostner for providing us with recombinant apo(a) protein.

References
Lp(a) and ICAM-1 Expression in HUVEC


Lipoprotein(a) Enhances the Expression of Intercellular Adhesion Molecule-1 in Cultured Human Umbilical Vein Endothelial Cells
Shigeki Takami, Shizuya Yamashita, Shinji Kihara, Masato Ishigami, Kaoru Takemura, Noriaki Kume, Toru Kita and Yuji Matsuzawa

Circulation. 1998;97:721-728
doi: 10.1161/01.CIR.97.8.721

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/8/721

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/