

# Lipoprotein(a) stimulates the proliferation of cultured human arterial smooth muscle cells through two pathways

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**Abstract** We investigated the effect of lipoprotein(a) (Lp(a)) on proliferation of human arterial smooth muscle cells (SMCs) and its mechanisms of action. Low density lipoprotein (LDL), Lp(a) and apolipoprotein(a) (apo(a)) significantly stimulated the proliferation of SMCs. Lp(a) and apo(a) reduced the amount of active transforming growth factor- $\beta$  (TGF- $\beta$ ) with the mink lung epithelial cell bioassay, however LDL had no effect. Lp(a), but not apo(a), significantly stimulated the proliferation of SMCs even in the presence of a neutralizing antibody for TGF- $\beta$ . Our results suggest that Lp(a) stimulates the proliferation of SMCs via apo(a)-induced inhibition of TGF- $\beta$  activation and stimulation of SMCs by the LDL-particle of Lp(a).

**Key words:** Lipoprotein(a); Smooth muscle cell; Transforming growth factor- $\beta$

## 1. Introduction

Lipoprotein(a) (Lp(a)) discovered by Berg in 1963 [1] is believed to be a risk factor for coronary heart disease [2–6], vein graft stenosis after coronary artery bypass [7] and restenosis after percutaneous transluminal coronary angioplasty [8,9]. Lp(a) is an low density lipoprotein (LDL)-like particle with a characteristic polymorphic glycoprotein known as apolipoprotein(a) (apo(a)) that is disulfide linked to the apolipoprotein B moiety of LDL [10]. The complementary DNA sequence of human apo(a) has been found to be homologous to human plasminogen [11]. Because Lp(a) contains both an LDL particle and apo(a), it is believed to have both proatherogenic and prothrombotic properties. However, the precise mechanisms by which Lp(a) promotes atherosclerosis have not been clarified.

The proliferation and migration of vascular smooth muscle cells (SMCs) are major contributors to atherosclerosis and restenosis after angioplasty. Lp(a) is reported to enhance the migration [12] and proliferation [13] of cultured SMCs by reducing the level of transforming growth factor- $\beta$  (TGF- $\beta$ ), which inhibits migration and proliferation of SMCs. The striking homology between apo(a) and plasminogen suggests that Lp(a) inhibits plasmin-induced activation of TGF- $\beta$  by inhibiting plasminogen activation. A recent study suggests that activation of TGF- $\beta$  is inhibited in the aortic wall and in serum in transgenic apo(a) mice [14]. In the present study, we investigated the mechanisms of action in detail which Lp(a) stimulated the proliferation of SMCs.

## 2. Materials and methods

### 2.1. Cell culture

SMCs were explanted from the medial layer of nonatherogenic autopsy specimens obtained from adult humans. SMCs were cultured in MCDB 107 medium (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) supplemented with basic fibroblast growth factor (1 ng/ml, provided by Takeda Medical Industries, Osaka, Japan), epidermal growth factor (10 ng/ml, Becton Dickinson Labware, Bedford, MA), 5% FBS (Gibco, Grand Island, NY), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and amphotericin B (0.05  $\mu$ g/ml) (basic medium) in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. SMCs from the third to sixth passage were used for experiments. The smooth muscle cell nature of the cultures was confirmed by microscopic identification of their characteristic 'hill-and-valley' growth pattern and by immunohistochemical identification of smooth muscle actin using a specific monoclonal antibody (HHF35, Enzo Diagnostics, Syosset, NY).

### 2.2. Lipoprotein-deficient serum

Lipoprotein-deficient serum (LPDS) was prepared from FBS by the method of Chen et al. [15]. Briefly, FBS was adjusted to a density of 1.21 g/ml with solid potassium bromide (KBr) and centrifuged at 140,000  $\times$  g for 48 hours. After centrifugation, the upper fraction with a density below 1.21 g/ml was removed. The bottom fraction, referred to as LPDS, was dialyzed extensively against phosphate-buffered saline.

### 2.3. Isolation of Lp(a), apo(a), LDL and HDL

Plasma was obtained from donors with elevated Lp(a) concentrations after 12 hours of fasting. Butylated hydroxytoluene (10  $\mu$ M) was added during all procedures to avoid the oxidation of lipoproteins.

Lp(a) was isolated according to the modified method of Albers et al. [16]. Briefly, the plasma was adjusted to a density of 1.060 g/ml with solid KBr and then centrifuged at 150,000  $\times$  g at 10°C for 24 h. The bottom fraction was readjusted to a density of 1.090 g/ml with solid KBr and centrifuged at 150,000  $\times$  g for 26 hours. The resulting lipoproteins with densities of 1.060 to 1.090 g/ml were subfractionated by ascending gel chromatography on 6% agarose gel (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA) at 18°C using a 2.6  $\times$  90 cm column at a flow rate of 12 ml/hour. The sample volume was 4 to 6 ml and the eluting buffer contained 0.1 M Tris-HCl, 0.15 M NaCl and 0.001 M EDTA (pH 8.2). Fractions were collected at 4 ml/tube. The absorbance of the eluates was measured with an ultraviolet spectrophotometer at 280 nm. Appropriate fractions were pooled, concentrated with MINICON-B (Amicon, Beverly, MA) and then dialyzed with MCDB 107 medium. Plasma and peak fractions of Lp(a), LDL and high density lipoprotein (HDL) were analyzed by Western blotting for apo(a) using an antibody against apo(a) (provided by Daiichi Pure Chemicals, Tokyo, Japan).

Apo(a) was isolated by the method of Armstrong et al. [17]. Briefly, 10  $\mu$ M of dithiothreitol/mg of protein was added to purified Lp(a), and the solution was incubated at 37°C for 3 hours and then adjusted to a density of 1.063 g/ml with solid KBr. After centrifugation at 150,000  $\times$  g for 20 hours at 10°C, pellets sedimented at the base of the tube were collected and dialyzed with MCDB107 medium.

HDL (*d* 1.085 to 1.210 g/ml) and LDL (*d* 1.010 to 1.063 g/ml) were isolated from plasma by the method of Havel et al. using KBr and ultracentrifugation [18].

The protein content was determined by BCA protein assay reagent (Pierce, Rockford, IL).

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#### 2.4. Proliferation assay

We determined SMC proliferation by counting cells with a Coulter counter (Coulter Electronics, Luton, England) and measuring [ $^3\text{H}$ ]thymidine incorporation in SMCs as described previously [19].

#### 2.5. TGF- $\beta$ bioassay

To prepare conditioned medium, human arterial SMCs were first cultured in the control medium (1% LPDS) for 24 hours. The control medium was then removed and attached SMCs were rinsed and incubated with DME (Gibco, Grand Island, NY) alone for 24 hours. To determine the total amount of latent and active TGF- $\beta$ , the conditioned medium was acidified to pH 2.0 with 1N HCl and after 1 hour at 4°C was neutralised to pH 7.5 with 1 N NaOH. TGF- $\beta$  activity in conditioned medium of human arterial SMCs was confirmed by the method of Gangrade et al. [20] using mink lung epithelial cells (kindly provided by Dr. Y. Sato, Oita Medical University, Oita, Japan). Mink lung epithelial cells (500,000 cells/cm<sup>2</sup>) were cultured in 24-well dishes in DME containing 5% FBS in triplicate in the presence or absence of various concentrations of conditioned medium or TGF- $\beta_1$  (King Brewing, Kanagawa, Japan) and incubated at 37°C in a humidified gas (95% air, 5% CO<sub>2</sub>) chamber. The conditioned medium was removed after 24 hours and [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{ml}$ ) was added to cultures for 4 hours. Incorporation of [ $^3\text{H}$ ]thymidine in the DNA of mink lung epithelial cells was determined with a liquid scintillation counter (TRI-CARB 4430, Packard Instrument, Downers Grove, IL). Standard curves for TGF- $\beta$  were established by logit-log transformation of [ $^3\text{H}$ ]thymidine incorporation as a function of the TGF- $\beta$  dose (10 to 1000 pg/ml). This produced a linear plot from which the content of TGF- $\beta$  in experimental samples was calculated.

Neutralizing mouse monoclonal antibody against TGF- $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  was purchased from Genzyme (Cambridge, MA).

#### 2.6. Statistical analysis

Data are presented as the mean  $\pm$  S.D. Differences of mean values between groups were analyzed by the unpaired Student's *t*-test. Probability values of less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Isolation of Lp(a)

The elution profile of lipoprotein fractions with densities of *d* 1.060 to 1.090 g/ml showed three peaks (Fig. 1). Western blot analysis of serum and the three peak fractions identified apo(a) bands in serum and the Lp(a) fraction (Fig. 1).

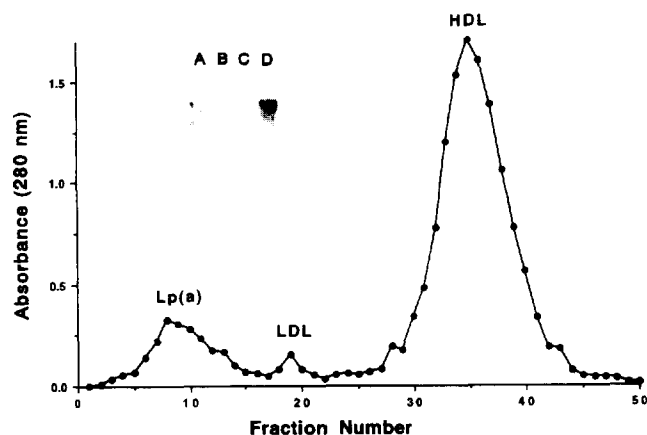


Fig. 1. Elution profile of a representative 1.060 to 1.090 g/ml lipoprotein fraction separated on Bio-Gel A-5m. Three elution peaks were observed: Lp(a), LDL and HDL. Insert: Western blot analysis. Serum and the Lp(a) fraction showed apo(a) bands. Lane A: serum, lane B: peak fraction of HDL, lane C: peak fraction of LDL, lane D: peak fraction of Lp(a).

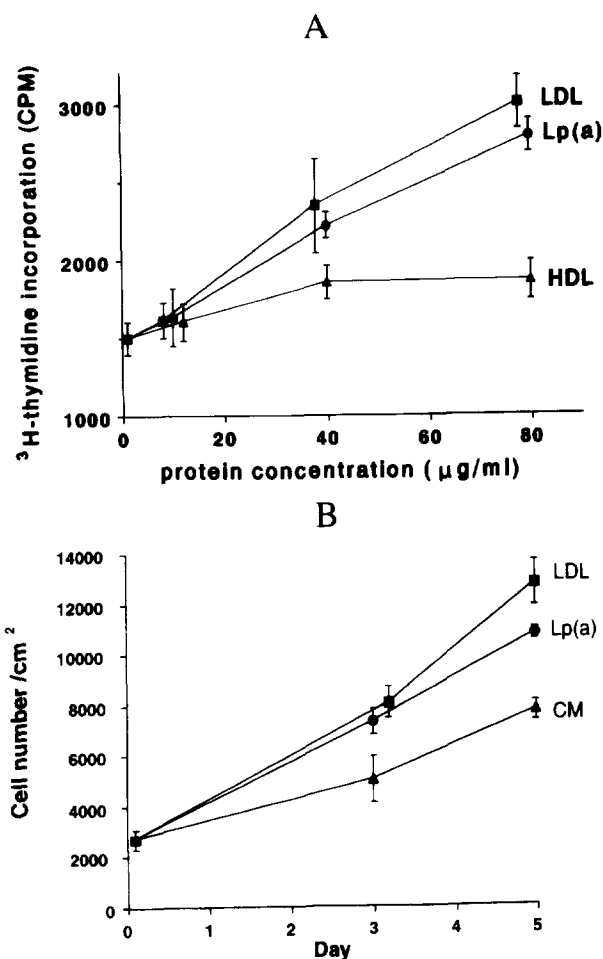


Fig. 2. Proliferation of SMCs incubated with Lp(a), LDL or HDL. A: Dose response curve of growth of SMCs incubated with Lp(a), LDL or HDL. SMCs were cultured in MCDB 107 medium supplemented with basic fibroblast growth factor (1 ng/ml), epidermal growth factor (10 ng/ml), 1% LPDS and the indicated amounts of lipoproteins. B: Time course of growth of SMCs incubated with Lp(a) or LDL. Closed triangles indicate control medium (1% LPDS), closed circles indicate control medium plus Lp(a) (80  $\mu\text{g}/\text{ml}$  protein) and closed squares indicate control medium plus LDL (80  $\mu\text{g}/\text{ml}$  protein). SMCs were counted on Day 3 and Day 5. Values are the mean  $\pm$  S.D. in a representative experiment of triplicate samples.

#### 3.2. Effect of Lp(a) on SMC proliferation

Dose-response curves showed that Lp(a) and LDL increased [ $^3\text{H}$ ]thymidine incorporation in SMCs in a dose-dependent manner (Fig. 2A). HDL caused less incorporation of [ $^3\text{H}$ ]thymidine in SMCs. The number of SMCs incubated with Lp(a) (80  $\mu\text{g}/\text{ml}$  protein) or LDL (80  $\mu\text{g}/\text{ml}$  protein) increased with time (Fig. 2B). The number of SMCs was significantly higher on Day 5 in the LDL- and Lp(a)-containing media compared with the control medium in the six experiments of triplicate samples (control:  $6905 \pm 768$ , Lp(a):  $9416 \pm 1570$ , LDL:  $11702 \pm 1303$ ; LDL vs. control:  $P < 0.01$ , Lp(a) vs. control:  $P < 0.05$ ).

#### 3.3. TGF- $\beta$ bioassay

Both Lp(a) and apo(a) reduced the concentration of active TGF- $\beta$  compared with the control medium. LDL had no effect

Table 1  
Amount of active TGF- $\beta$

	TGF- $\beta$ (ng/ml)
Control	$0.5 \pm 0.1$
Lp(a) (80 $\mu$ g/ml protein)	<0.1
apo(a) (40 $\mu$ g/ml protein)	<0.1
LDL (80 $\mu$ g/ml protein)	$0.5 \pm 0.1$
Neutralizing antibody for TGF- $\beta$	<0.1

Values represent the mean  $\pm$  S.D. of the amount of active TGF- $\beta$  of two experiments with triplicate samples.

on the TGF- $\beta$  level. The total amount of latent and active TGF- $\beta$  in the control medium was  $1.4 \pm 0.1$  ng/ml. In the medium with a neutralizing antibody for TGF- $\beta$ , active TGF- $\beta$  was less than 0.1 ng/ml (Table 1).

### 3.4. Effect of Lp(a) and apo(a) on SMC proliferation in the presence of neutralizing antibody for TGF- $\beta$

To determine whether Lp(a) stimulated SMC proliferation only by inhibiting activation of TGF- $\beta$ , we assayed SMC proliferation in the presence or absence of a neutralizing antibody for TGF- $\beta$  (Ab). We used enough amount of the neutralizing antibody for TGF- $\beta$  which can neutralize 1.0 ng/ml of active TGF- $\beta$ , because the concentration of active TGF- $\beta$  in control was  $0.5 \pm 0.1$  ng/ml. We confirmed that the antibody effectively neutralized the active TGF- $\beta$  by TGF- $\beta$  bioassay (Table 1).

Incorporation of [ $^3$ H]thymidine was significantly higher in SMCs incubated with the neutralizing antibody for TGF- $\beta$  (Ab) than in SMCs incubated with IgG (IgG:  $1331 \pm 183$  CPM, Ab:  $2126 \pm 248$  CPM;  $P < 0.05$ ) (Fig. 3A). Incorporation of [ $^3$ H]thymidine was also significantly higher in SMCs incubated with Ab plus Lp(a) (80  $\mu$ g/ml protein) than in SMCs incubated with Ab alone (Ab:  $2126 \pm 248$  CPM, Ab + Lp(a):  $2966 \pm 227$  CPM;  $P < 0.05$ ) (Fig. 3A). Apo(a) (40  $\mu$ g/ml protein) significantly increased [ $^3$ H]thymidine incorporation compared with IgG (IgG:  $1416 \pm 65$  CPM, IgG + apo(a):  $1900 \pm 100$  CPM;  $P < 0.01$ ) (Fig. 3B). However, in contrast to Lp(a), apo(a) did not significantly increase [ $^3$ H]thymidine incorporation in SMCs in the presence of Ab (Ab:  $2107 \pm 113$  CPM, Ab + apo(a):  $2126 \pm 128$  CPM; not significant) (Fig. 3B).

## 4. Discussion

An elevated Lp(a) level is a risk factor for coronary heart disease [2–6], stenosis of saphenous vein graft [7] and restenosis after angioplasty [8,9]. Immunohistochemical staining with a specific antibody to apo(a) has shown a substantial amount of Lp(a) in human aortic atherosclerotic plaques [21]. Lipid-staining lesions in the aorta and co-localization of apo(a) with lipid deposition in the arterial wall have been observed in transgenic mice expressing human apo(a) [22]. Possible mechanisms of Lp(a)'s involvement in atherosclerosis include inhibition of fibrinolysis [23–25], stimulation of proliferation [13] and migration [14] of SMCs, uptake of Lp(a)-glycosaminoglycan complex by macrophages [26], and the formation of an insoluble LDL-Lp(a) complex in the presence of calcium ions [27]. Studies have suggested that Lp(a) may promote thrombotic events by interfering with the fibrinolytic functions of plasminogen and/or plasmin [23–25] because the complementary DNA

sequence of human apo(a) is homologous to human plasminogen [11].

SMC migration and proliferation are believed to be key contributors to the development of spontaneous atherosclerosis and restenosis after angioplasty. Kojima et al. suggested that high concentrations of Lp(a) may enhance SMC migration by reducing the level of TGF- $\beta$ , which inhibits migration of SMCs [12]. Grainger et al. reported that Lp(a) and apo(a) stimulated the growth of human SMCs by inhibiting plasminogen activation, leading to inhibition of plasmin-induced activation of latent TGF- $\beta$ , an inhibitor of SMC growth [13]. In the present study, we also found that Lp(a) and apo(a) stimulated proliferation of cultured human arterial SMCs. Lp(a) and apo(a) reduced the amount of active TGF- $\beta$ . These results suggest that Lp(a) and apo(a) stimulated growth of SMCs by inhibiting activation of TGF- $\beta$ . To determine whether inhibition of TGF- $\beta$  activation was the sole mechanism of Lp(a)-induced stimulation of SMCs, we assessed the effect of Lp(a) on growth of SMCs in the presence or absence of a neutralizing antibody for

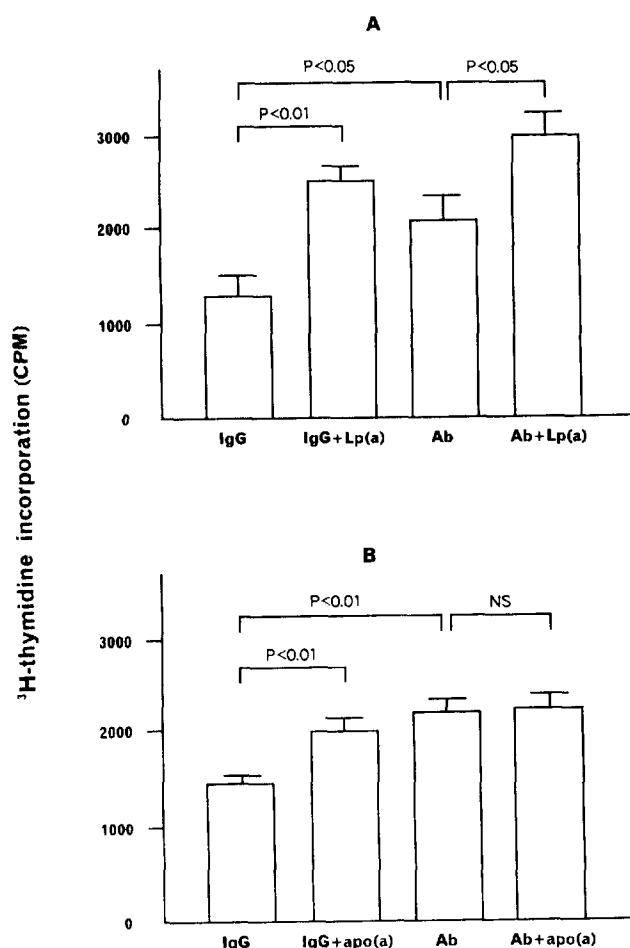


Fig. 3. Effect of Lp(a) or apo(a) on SMC proliferation in the presence or absence of a neutralizing antibody for TGF- $\beta$  (Ab). A: Incorporation of [ $^3$ H]thymidine was measured in SMCs incubated with IgG, IgG plus Lp(a) (80  $\mu$ g/ml protein), Ab alone or Ab plus Lp(a) (80  $\mu$ g/ml protein). B: Incorporation of [ $^3$ H]thymidine was measured in SMCs incubated with IgG, IgG plus apo(a) (40  $\mu$ g/ml protein), neutralizing antibody for TGF- $\beta$  (Ab) or Ab plus apo(a) (40  $\mu$ g/ml protein). Values are the mean  $\pm$  S.D. of the three experiments of triplicate samples. NS: not significant.

TGF- $\beta$ , which abolished the effect of TGF- $\beta$ . In the presence of the neutralizing antibody for TGF- $\beta$ , Lp(a), but not apo(a), stimulated SMC growth, suggesting that LDL-particle of Lp(a) may promote SMC proliferation. Our results suggest that Lp(a) may stimulate proliferation of vascular SMCs through two pathways: apo(a)-induced inhibition of activation of TGF- $\beta$  and stimulation of SMCs by the LDL-particle of Lp(a).

TGF- $\beta$  is a multifunctional peptide that controls proliferation, differentiation and other functions in many cell types [28]. TGF- $\beta$  is reported to inhibit growth of SMCs under some conditions [29–31] and to stimulate growth under other conditions [32]. In the present study, exogenous TGF- $\beta$  (0.5 to 1.0 ng/ml) inhibited SMC growth in the control medium (data not shown) and [ $^3$ H]thymidine incorporation was significantly higher in SMCs incubated with the neutralizing antibody for TGF- $\beta$  than in SMCs incubated with IgG (Fig. 3A), confirming that TGF- $\beta$  is a potent inhibitor of the growth of SMCs.

Some studies have shown that LDL stimulates SMC growth [15,33–35], but others have found no evidence of LDL-induced stimulation [13,36]. These disagreements may reflect differences either in the assay conditions or in the modification of LDL employed in various studies. In the present study, we cultured cells in a medium containing LPDS to clarify the role of lipoproteins in growth of SMCs and we obtained lipoproteins using butylated hydroxytoluene to avoid their oxidation. In our experimental system, LDL stimulated proliferation of SMCs. Lp(a), but not apo(a), stimulated SMC growth even in the presence of the neutralizing antibody for TGF- $\beta$ , suggesting that this mitogenic effect may be related to the LDL-like action of Lp(a).

In conclusion, our results suggest that Lp(a) stimulates the proliferation of SMC through two pathways: apo(a)-induced inhibition of TGF- $\beta$  activation and stimulation of SMCs by the LDL-particle of Lp(a).

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