Lipoprotein (a) Inhibits the Generation of Transforming Growth Factor β: An Endogenous Inhibitor of Smooth Muscle Cell Migration

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Abstract. Conditioned medium (CM) derived from cocultures of bovine aortic endothelial cells (BAECs) and bovine smooth muscle cells (BSMCs) contains transforming growth factor-β (TGF-β) formed via a plasmin-dependent activation of latent TGF-β (LTGFβ), which occurs in heterotypic but not in homotypic cultures (Sato, Y., and D. B. Rifkin. 1989. *J. Cell Biol.* 107: 1199–1205). The TGF-β formed is able to block the migration of BSMCs or BAECs. We have found that the simultaneous addition to heterotypic culture medium of plasminogen and the atherogenic lipoprotein, lipoprotein (a) (Lp(a)), which contains plasminogen-like kringles, inhibits the activation of LTGF-β in a dose-dependent manner. The inclusion of LDL in the culture medium did not show such an effect. Control experiments indicated that Lp(a) does not interfere with the basal level of cell migration, the activity of exogenous added TGF-β, the release of LTGF-β from cells, the activation of LTGF-β either by plasmin or by transient acidification, or the activity of plasminogen activator. The addition of Lp(a) to the culture medium decreased the amount of plasmin found in BAECs/BSMCs cultures. Similar results were obtained using CM derived from cocultures of human umbilical vein endothelial cells and human foreskin fibroblasts. These results suggest that Lp(a) can inhibit the activation of LTGF-β by competing with the binding of plasminogen to cell or matrix surfaces. Therefore, high plasma levels of Lp(a) might enhance smooth muscle cell migration by decreasing the levels of the migration inhibitor TGF-β thus contributing to generation of the atheromatous lesions.

LIPOPROTEIN (a) (Lp(a)) is an LDL-like lipoprotein whose increased concentration in plasma is related to increased risk of atherosclerosis (Scanu and Fless, 1990). In addition to apolipoprotein B-100, Lp(a) contains a unique apolipoprotein called apolipoprotein (a) which is disulfide linked to the apolipoprotein B-100 (Fless et al., 1986). Recent studies have revealed that apolipoprotein (a) shares a remarkable structural homology with plasminogen, the zymogen form of the protease plasmin (McLean et al., 1987). Apolipoprotein (a) resembles plasminogen as it contains one copy of the plasminogen kringle-5 domain, a variable number of copies of the kringle-4 domain and a serine protease domain; however little enzymatic activity has been detected associated with the purified molecule (McLean et al., 1987; Utermann, 1989). Because of the presence of the large number of kringle domains that are responsible for the binding of plasminogen to fibrin, matrix components, and cell surfaces, it has been suggested that Lp(a) competitively inhibits plasminogen binding to the endothelial cell surface and, therefore, modulates fibrinolysis (Miles et al., 1989; Hajjar et al., 1989). Lp(a) also attenuates fibrin-dependent tissue-type plasminogen activator (tPA) activity through competitive inhibition of the binding of plasminogen and tPA to fibrin (Harpel et al., 1989; Loscalzo et al., 1990). However, the precise mechanism of Lp(a) atherogenicity remains to be elucidated.

The transforming growth factor-β (TGF-β) family consists of a number of related, but functionally distinct, proteins (Barnard et al., 1990; Roberts and Sporn, 1990). The best characterized member of this family is TGF-β1. Although TGF-β1 and the related molecules TGF-β2 and TGF-β3 are produced and secreted by many normal and neoplastic cells in a latent high molecular weight form (LTGF-β) that does not bind to the specific high affinity TGF-β receptors (Derynck et al., 1985; Barnard et al., 1990; Roberts and Sporn, 1990). Although TGF-β1 was originally described as a molecule that induced anchorage independent growth of cells in soft agar, a hallmark of in vitro transformation (De Larco and Todaro, 1978), TGF-β1 is now recognized to be a multifunctional cytokine with both growth promoting and growth inhibiting activities. Some of the cell types whose growth is inhibited by TGF-β are epithelial cells, lymphocytes, endothelial cells (ECs), and smooth muscle cells (SMCs) (Moses et al., 1985; Kehrl et al., 1986*a*,*b*; Heimark et al., 1986; Owens et al., 1988).

A critical step in the regulation of TGF-β action is the acti-

vation of the latent molecule. TGF-β1 is synthesized and released as a 390-amino acid precursor polypeptide, which is both glycosylated and phosphorylated (Derynck et al., 1985, 1986). Cleavage in the carboxyl-terminal region yields the 112-amino acid TGF-β1 monomer. The 25-kD mature TGF-β consists of two identical monomer units linked by disulfide bonds (Roberts and Sporn, 1990; Wakefield et al., 1989). Latent transforming growth factor-β (LTGF-β) contains one mature TGF-β molecule noncovalently associated with the amino-terminal portions of the propeptide dimer (Wakefield et al., 1989). (The propeptide dimer is often referred to as the latency associated peptide or LAP). Acidification (pH 3) disrupts these noncovalent interactions releasing TGF-β and is used as the standard method for the activation of LTGF-β (Barnard et al., 1990; Roberts and Sporn, 1990). However, this is unlikely to be the physiological activation mechanism. Lyons et al. (1988, 1990) reported that proteases, specifically, plasmin and cathepsin D, can activate LTGF-β by cleavage within the amino-terminal region of LAP. Recently two papers described the activation of LTGF-β in cocultures of bovine ECs with either pericytes or SMCs (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). Both cell types were shown to produce LTGF-β and the activation appears to require cell-cell contact. The amount of LTGF-β activated is low, perhaps, due to the self-regulation of the system (Sato et al., 1990). Sato and Rifkin (1989) demonstrated that this activation requires cell-associated plasmin, produced by the conversion of plasminogen to plasmin by plasminogen activator (PA) (Sato and Rifkin, 1989; Sato et al., 1990). The requirement for two different cell types does not appear to be related to activation of the PA-plasmin system as both cell types produce PA and both cell types contain PA receptors. The activation can be blocked by antibodies to PA and inhibitors of plasmin. Thus, plasmin may be a relevant physiological activator of LTGF-β.

The requirement for surface bound plasmin for TGF-β generation suggested that Lp(a) might inhibit the activation of LTGF-β in cocultures by competing for binding of plasminogen molecules. We tested this assumption by monitoring the effect of Lp(a) added to culture medium on the activation of LTGF-β in cocultures. Under these conditions Lp(a) blocks the production of the negative regulator TGF-β presumably by competing for plasminogen binding. Moreover, the inclusion of Lp(a) in the culture medium abrogates the migratory restraint normally observed when SMCs and ECs are in contact. Thus, under conditions of high levels of Lp(a) LTGF-β activation may be suppressed resulting in migration of SMCs from the media to the intima.

Glossary

αMEM	alpha minimal essential medium
αPAI-1	plasminogen activator-1 antibody
αTGF-β	anti-transforming growth factor β antibody
BAECs	bovine aortic endothelial cells
bFGF	basic fibroblast growth factor
BSMCs	bovine smooth muscle cells
CM	conditioned medium
CS	calf serum
EC	endothelial cells
HFFs	human foreskin fibroblasts
HUVECs	human umbilical vein endothelial cells
LAP	latency associated peptide
Lp(a)	lipoprotein (a)
LTGF-β	latent transforming growth factor β
M-6-P	mannose-6-phosphate
PA	plasminogen activator
PAI-1	plasminogen activator-1
SMCs	smooth muscle cells
TGF-β	transforming growth factor β

Materials and Methods

Materials

Lp(a) and LDL were purified from fresh human plasma obtained from donors with elevated Lp(a) concentrations according to described methods (Harpel et al., 1989). Briefly, Lp(a) and LDL purified from a single individual with an elevated Lp(a) concentration (80 mg/dl) according to described methods (Harpel et al., 1989). The purity of the preparation was documented by SDS-PAGE, followed by immunoblot analysis with monospecific polyclonal antibodies against apo(a) and apo B-100, as well as by native 2–16% gradient polyacrylamide slab gel electrophoresis (Harpel et al., 1989). The donor had a double-band Lp(a) phenotype, assessed by the method of Gaubatz (Gaubatz et al., 1990) and kindly performed by Dr. T. Parker, Cornell University Medical College, New York. The lower molecular mass band 4 (M_r = 553 kD) was more intense than the higher molecular mass band 8 (M_r = 742 kD). Purified lipoproteins were dialyzed against culture medium overnight at 4°C before being added to cells. The preparation of plasminogen-depleted calf serum (CS) and the isolation of human plasminogen from fresh plasma were performed using lysine-Sepharose affinity chromatography (Robbins and Summaria, 1976). The plasminogen prepared in this manner was composed of >95% Glu-plasminogen and <5% Lys-forms as determined by a densitometric method following SDS-PAGE in the presence of 6 M urea (Suenson and Thorsen, 1981). Human plasmin and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human TGF-β1 was a generous gift from Genentec, Inc. (South San Francisco, CA). Porcine TGF-β and antibody to it (αTGF-β) were obtained from R & D Sys., Inc. (Minneapolis, MN). This αTGF-β has previously been shown to neutralize porcine, human, and bovine TGF-β1 (Keski-Oja et al., 1987; Sato and Rifkin, 1989). The recombinant and porcine TGF-βs were found to be equivalent in all assays. Anti-bovine PA inhibitor-1 (PAI-1) IgG (αPAI-1) was prepared from rabbit anti-bovine PAI-1 serum which was a generous gift from Dr. David J. Loskutoff (Scripps Clinic and Research Foundation, La Jolla, CA) using protein A-Sepharose (Pharmacia, Piscataway, NJ).

Cell Culture

Bovine aortic endothelial cells (BAECs) and smooth muscle cells (BSMCs) were isolated and grown in alpha minimal essential medium (αMEM) containing 10% CS or Dulbecco's modified essential medium (DMEM) containing 10% CS, respectively. Human umbilical vein endothelial cells (HUVECs), obtained from Dr. Richard I. Levin (Bellevue Hospital Center, New York, NY), and human foreskin fibroblasts (HFFs), were grown in either RPMI-1640 medium containing 20% FCS plus basic fibroblast growth factor (bFGF) (15 ng/ml) or DMEM containing 10% FCS, respectively.

Preparation of Conditioned Medium (CM)

BAECs and BSMCs were detached from culture dishes with 0.25% trypsin-0.1 mM EDTA. Cells were washed twice with serum-free αMEM and were seeded in 35-mm dishes (8 cm^2) at a density of 5×10^4 cells/cm^2 in αMEM containing 10% plasminogen-depleted CS plus the indicated amount of plasminogen. For coculture experiments, 3.2×10^5 BAECs and 0.8×10^5 BSMCs were seeded in the same 35-mm dishes in αMEM containing 10% plasminogen-depleted CS plus the indicated amount of plasminogen with and without Lp(a) or LDL. Since CS does not contain Lp(a) (Utermann, 1989), the potential effects of Lp(a) in CS or FCS did not have to be considered. After a 2-h incubation at 37°C that permitted the cells to attach, the cultures were rinsed twice with PBS and incubated in 1 ml of serum-free αMEM containing 0.1% BSA (αMEM-BSA) for an additional 6 h to produce CM. The CM was centrifuged to remove cell debris, diluted to 1:4 with αMEM-BSA, and used in wound assays to measure BAEC migration. For wound assays using BSMCs, CM was prepared using DMEM containing 0.1% BSA. CM from HUVECs and/or HFFs were prepared as above except that RPMI-1640 medium plus 20% plasminogen-depleted FCS was used for the initial 2-h incubation.

Wound Assay for Cell Migration

Wound assays for BAEC or BSMC migration were carried out as described previously (Sato et al., 1990). Briefly, a denuded area is made in a confluent monolayer using a razor blade. The cultures are incubated in the presence of the additions to be tested for 24 h, the cells fixed, and the number of cells that have migrated from the original edge counted (Sato and Rifkin, 1988). TGF-β suppresses the migration of BAECs (Heimark et al., 1986; Sato and Rifkin, 1988). The number of cells which migrate is inversely proportional to the amount of TGF-β present. This assay can be used to detect concentrations of TGF-β as low as 10–20 pg/ml (Sato et al., 1990). In the case of BSMC, the incubation period was extended to 36 h because BSMCs migrated more slowly than BAECs. The data are presented as the number of cells that have migrated >125 μm from the original edge of the wound in order to exclude those cells which moved across the origin before the TGF-β had an effect. This number represents the average obtained by counting the cells in six random fields from each of two replicate dishes.

Assay of Cellular PA Activity

Cellular PA activity was assayed as follows. After the initiation of incubations in αMEM-BSA, the CM was aspirated at 0, 1, 3, and 6 h, the cells were washed three times with PBS, and the monolayer was extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1. The cell extracts were assayed for PA activity using the ^{125}I-fibrin plate assay (Gross et al., 1982).

Assay of Cell-associated Plasmin Activity

Trypsinized cells were replated into 24-well Linbro dishes (2 cm^2) and incubated for 2 h as described in Preparation of CM. Plasmin bound to the cell surface was then recovered and assayed according to the method of Stephens et al. (1989). Briefly, after rinsing the cells with PBS three times, bound plasmin was eluted with 3 mM tranexamic acid in PBS. Plasmin activity in the elute was assayed using a chromogenic substrate, S-2251 (Lottenberg et al., 1981).

Results

We determined whether Lp(a) would interfere with the conversion of LTGF-β to TGF-β in our coculture system because of the apparent plasmin dependence of LTGF-β activation (Sato and Rifkin, 1989; Sato et al., 1990) and the reports that Lp(a) competes with plasminogen/plasmin binding (Miles et al., 1989; Hajjar et al., 1989). This first required quantitation of the minimal amount of plasminogen necessary for activation of LTGF-β to conserve Lp(a). Therefore, BAECs and BSMCs were trypsinized and cocultured for 2 h in medium containing 10% plasminogen-depleted CS reconstituted with known amounts of purified human plasminogen. The cultures were washed with PBS and incubated in αMEM-BSA for an additional 6 h to produce CM. The CM was then tested for TGF-β activity in the BAEC wound migration assay (Fig. 1). While the number of cells that migrated >125 μm was similar when either fresh medium or CM from cocultures without plasminogen were tested, the number of migrating cells decreased as increasing amounts of plasminogen were added to the serum used for the initial 2-h incubation. Inhibition of BAEC migration was abrogated by neutralizing antibodies to TGF-β demonstrating that the inhibition was due to the activation of LTGF-β. Maximal suppression of migration was achieved at a plasminogen concentration of 55 nM (5 μg/ml). This corresponds to ~50% of the plasminogen concentration found in 10% CS (Summaria et al., 1973). Similar results were obtained when cells were incubated in a medium containing 10% mixtures of normal CS and plasminogen-depleted CS at various ratios with the maximal suppression obtained with a 1:1 mixture (data not shown).

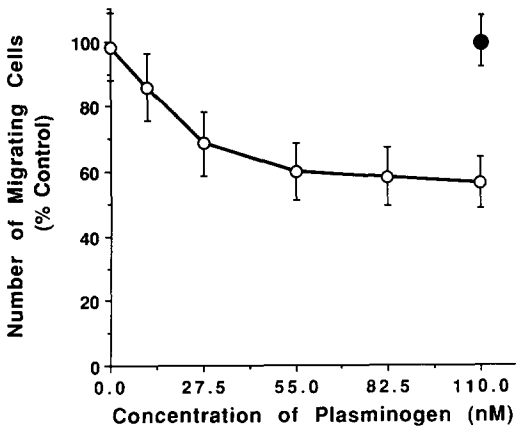

Figure 1. Plasminogen dose dependency for LTGF-β activation as measured by suppression of BAEC migration. CM was prepared from cultures of BAECs/BSMCs incubated in medium containing serum reconstituted with increasing plasminogen concentrations as described in Materials and Methods. The CM was added to wounded BAEC monolayers, and cell migration was quantitated as described. The number of cells that had migrated >125 μM was expressed as a percent of control using fresh medium. The number of cells that had moved >125 μm in the control was 42. (○) CM containing increasing amounts of plasminogen. (●) CM prepared in the presence of 110 nM plasminogen and incubated with wounded BAECs in the presence of neutralizing antibodies (10 μg/ml) to TGF-β.

To test the effect of Lp(a) on LTGF-β activation in cocultures, the cells were exposed to a fixed concentration of plasminogen (55 nM) plus increasing concentrations of Lp(a) during the initial 2-h incubation. As illustrated in Fig. 2, a fivefold molar excess of Lp(a) to plasminogen alleviated the normally observed suppression of BAEC migration with CM. The effect was dose dependent and a significant abrogation

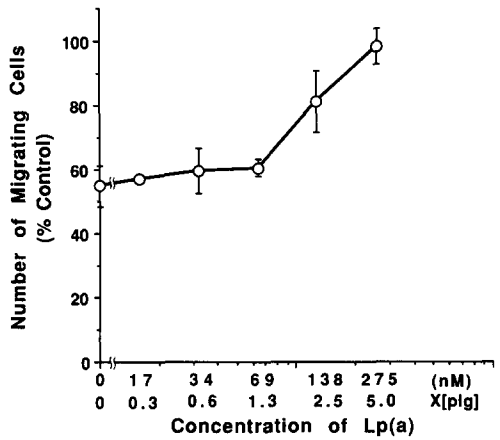

Figure 2. Inhibition of LTGF-β activation by Lp(a). Lp(a) was added to cocultures of BAECs/BSMCs at the indicated concentrations together with 55 nM plasminogen as described. CM was prepared and tested for TGF-β activity in wound assays using BAEC migration as before. Cell migration was expressed as a percent of control using fresh medium. The number of cells that had moved >125 μm in the control was 30. The molar excess of Lp(a) to plasminogen is also illustrated on the abscissa.

of inhibition, e.g., decrease in TGF-β generation, was observed at a 2.5 M excess of Lp(a) to plasminogen. Similar results to those illustrated in Figs. 1 and 2 were obtained using PA activity levels in BAEC to quantitate the generation of TGF-β rather than the BAEC migration (data not shown). This assay relies upon the observation that TGF-β suppresses PA activity in EC (Saksela et al., 1987) and detects TGF-β in the 10–60 pg/ml range (Dennis and Rifkin, 1991). The amount of TGF-β present in the coculture CM (Fig. 1) ranged from 35 ± 6 pg/ml at the highest plasminogen concentration (110 nM), 18 ± 3 pg/ml at 55 nM plasminogen, and 8 ± 1 pg/ml at 13.7 nM plasminogen.

We assumed that the inhibitory effect of Lp(a) on the activation of LTBF-β in cocultures could be ascribed to competition between Lp(a) and plasminogen for binding. Therefore, under conditions of Lp(a) excess, plasminogen no longer bound to BAECs/BSMCs thereby blocking the plasmin formation required for activation of LTGF-β. As a test of this hypothesis, we measured the effect of LDL in cocultures since it is similar to Lp(a) but lacks the plasminogen homologue apolipoprotein (a) (Scanu and Fless, 1990). Therefore, LDL would be expected not to affect LTGF-β activation. As shown in Fig. 3 *A*, addition of LDL and plasminogen to cocultures failed to abolish the activation of LTGF-β, while inclusion of Lp(a) did (compare samples *3* and *4* with *2*). This result suggests that, indeed, it is the apolipoprotein (a) component of Lp(a) that is important for the inhibitory effect. LDL had little effect when added during the 6-h incubation (Fig. 3 *A*, sample *6*). Interestingly, the addition of Lp(a) during the 6-h incubation period had little effect as compared with its simultaneous addition with plasminogen during the initial 2-h incubation period (Fig. 3 *A*, compare samples *3* and *5*). We presumed the reason for this difference was that Lp(a) is ineffective in removing membrane- or matrix-bound plasminogen. This was supported by the experiment illustrated in Fig. 3 *B* in which plasminogen was added during the second incubation period rather than the first. This resulted in the same suppression of BAEC migration as plasminogen addition during the 2-h incubation period (Fig. 3 *B*, samples *2* and *4*). CM collected 45 min after initiating the incubation with plasminogen, however, showed only ~28% of the TGF-β activity produced after 6-h incubation (Fig. 3 *B*, compare samples *3* and *4*). Although the simultaneous addition and coincubation of Lp(a) and plasminogen inhibited the activation of TGF-β (Fig. 3 *B*, sample *6*), the addition of Lp(a) after a 45-min incubation of cells with plasminogen did not (sample *5*). These results suggest that a maximal amount of plasminogen bound to cells within 45 min but only a fraction of the final amount of TGF-β was generated during this period. Once plasminogen bound to the cells, however, Lp(a) could not replace it under our experimental conditions and did not affct LTGF-β activation during the subsequent 5 h. Therefore, Lp(a) probably blocks TGF-β activation at the initial step of plasminogen binding.

Several control experiments were performed to insure that the effect of Lp(a) was not indirect. The addition of Lp(a) directly to wounded BAECs did not alter their migration (Fig. 4, sample *2*), thereby ruling out that the observed effect of Lp(a) (sample *5*) was simply the result of a stimulation of BAEC movement. The addition of 0.6 μM (50 μg/ml) human plasmin to Lp(a)-treated and untreated cultures during the 6-h

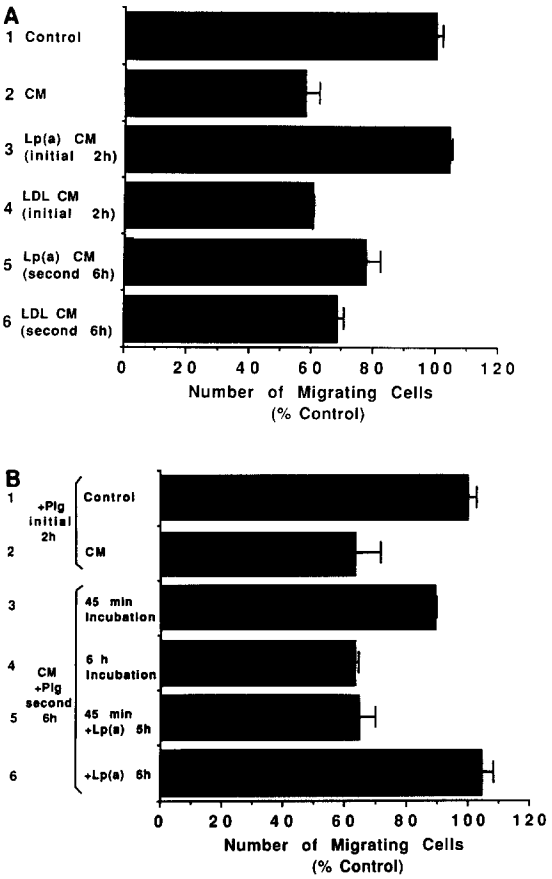

Figure 3. Characterization of the effect of Lp(a) on activation of LTGF-β. (*A*) Comparison of the effect of Lp(a) and the effect of LDL: CM was prepared from untreated cocultures (sample *2*) and cocultures treated with Lp(a) (275 nM) (samples *3* and *5*) or LDL (455 nM) (samples *4* and *6*) during the 2-h incubation period (samples *3* and *4*) or during the 6-h incubation period (samples *5* and *6*). TGF-β generated in each sample was measured by BAEC wound assays using fresh medium as a control (sample *1*). (*B*) Comparison of the effect of addition of Lp(a) at the same time or after plasminogen: Plasminogen (55 nM) was added to cocultures at the time when cells were seeded (sample *2*) or when the serum-containing medium was changed to αMEM-BSA (samples *3–6*). CM was collected after 6 h, as usual, except the CM of sample *3* was collected after 45 min. Lp(a) (275 nM) was added with plasminogen either simultaneously (sample *6*) or after a 45-min incubation of cells with plasminogen and washing the cell surface twice with PBS followed by a 5-h incubation (sample *5*). Each CM was tested for its TGF-β activity in BAEC wound assays using 4:1 mixture of BAEC CM and BSMC CM as a control (sample *1*). The number of cells that had moved >125 μm in the controls of *A* and *B* were 50 and 59, respectively.

incubation period resulted in the production of CM with equivalent ability to suppress BAEC migration (Fig. 4, samples *4* and *6*). Thus the Lp(a)-mediated block of TGF-β formation observed (Fig. 4, sample *5*) was eliminated by this treatment. This result suggested that Lp(a) did not inhibit plasmin activation of soluble LTGF-β. Acid-treated CM obtained from Lp(a)-treated cells contained migration inhibitory (TGF-β) activity (Fig. 4, sample *7*) indicating that Lp(a) did not block LTGF-β secretion. The induction of PAI-1 in cocultures has been previously shown to result in the inhibi-

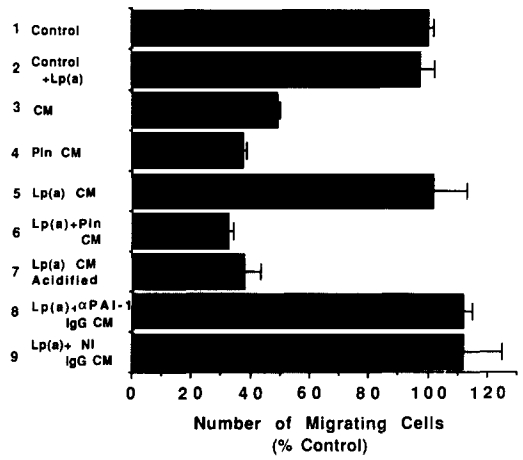

Figure 4. Specificity of Lp(a) inhibition of LTGF-β activation. Wound assays of BAEC migration were performed using medium prepared under the conditions indicated. Sample *1*, control medium which was a mixture of BAEC and BSMC CM (4:1). Sample *2*, Lp(a) (69 nM) added to control CM. Sample *3*, CM prepared from cocultures of BAECs and BSMCs exposed to serum containing plasminogen (55 nM) during the 2-h incubation period. Sample *4*, CM prepared from cocultures of BAECs and BSMCs as in sample *3* except that plasmin (0.6 μM) was added during the 6-h incubation. Sample *5*, CM prepared as in sample *3* except that Lp(a) (275 nM) was also present during the initial 2-h incubation. Sample *6*, CM prepared as described for sample *5* except that plasmin (0.6 μM) was included during the 6-h incubation. Sample *7*, CM prepared as described for sample *5* was acidified (pH 2.0, 1 h) before being tested in the migration assay. Sample *8*, CM prepared as described for sample *5* except that α PAI-1 IgG (400 μg/ml) was included during the 6-h incubation. Sample *9*, CM prepared as described for sample *5* except that nonimmune (*NI*) IgG (400 μg/ml) was included during the 6-h incubation. The total number of cells that had moved >125 μm in the control sample was 45.

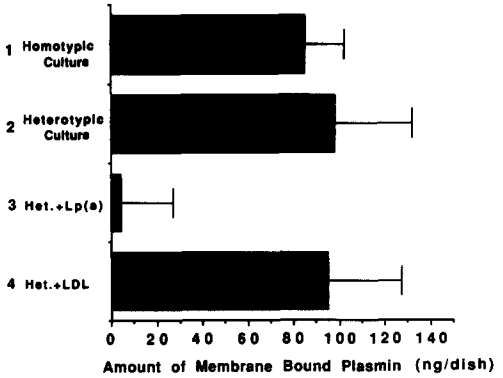

Figure 5. Recovery of membrane bound plasmin activity. After BAECs and/or BSMCs were incubated for 2 h in a medium containing 10% plasminogen-depleted CS supplemented with 55 nM plasminogen with or without Lp(a) and LDL as before, plasmin was eluted from the surface of the culture and its activity measured as described. Sample *1*, mixture of BAEC and BSMC eluates (4:1). Sample *2*, eluate from cocultures. Sample *3*, eluate from cocultures treated with 275 nM Lp(a). Sample *4*, eluate from cocultures treated with 455 nM LDL.

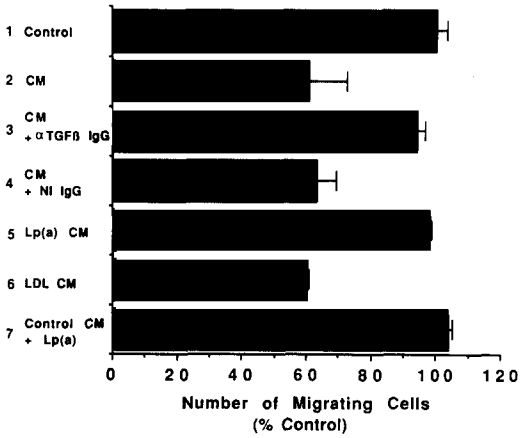

Figure 6. Effect of Lp(a) on suppression of BSMC migration by coculture CM. BSMC wound assays were performed as described in Materials and Methods testing the following additions: sample *1*, control CM defined as in Fig. 3 *B*; sample *2*, coculture CM; sample *3*, coculture CM supplemented with αTGF-β IgG (10 μg/ml); sample *4*, coculture CM supplemented with nonimmune (*NI*) IgG (10 μg/ml); sample *5*, CM from cocultures treated with Lp(a) (275 nM); sample *6*, CM from cocultures treated with LDL (455 nM); sample *7*, control CM supplemented with Lp(a) (69 nM). The number of cells that had moved >125 μm in the control was 56.

tion of LTGF-β conversion to TGF-β (Sato et al., 1990) since PA is required for the activation of plasminogen. It has recently been reported that Lp(a) stimulates HUVECs to synthesize and release PAI-1 (Etingen et al., 1991). Therefore, it was possible that exposure of cells to Lp(a) induced PAI-1 which prevented the activation of LTGF-β. However, the addition of neutralizing antibodies to PAI-1 to the cocultures during the 6-h incubation period did not diminish the effect of Lp(a) (Fig. 4, compare samples *8* and *9*). In addition to these results, other experiments (data not shown) demonstrated that Lp(a) did not block (*a*) the inhibition of BAEC migration observed upon addition of exogenous TGF-β, (*b*) the artificial activation of LTGF-β in CM by acid treatment, nor (*c*) cellular PA activity in cocultures during the preparation of CM.

These results strongly suggest that competitive inhibition of plasminogen binding to cells by Lp(a) was responsible for the observed inhibition of LTGF-β activation. Indeed, in the presence of Lp(a), the amount of membrane bound plasmin that could be recovered from cultures was reduced by 98% (Fig. 5).

Since it is SMCs that migrate within atherosclerotic regions (Ross, 1986), we determined whether BSMC migration was inhibited by coculture CM and whether this was prevented by the inclusion of Lp(a) in the medium during the initial 2-h incubation (Fig. 6). The suppression of BSMC migration by CM (Fig. 6, sample *2*) was neutralized by TGF-β antibodies (sample *3*), and was abolished by the simultaneous addition of Lp(a) and plasminogen to cocultures (sample *5*). The addition of LDL had no effect on the generation of inhibitory activity (Fig. 6, sample *6*). Lp(a) did not interfere with basal BSMC migration (Fig. 6, sample *7*). Furthermore, a similar result analyzing BAEC migration was obtained using CM obtained from cocultures of HUVECs and HFFs as a source of CM (data not shown) indicating that human as well as bovine heterotypic cocultures can generate TGF-β.

Discussion

The present study characterizes the inhibition by Lp(a) of the activation of LTGF-β normally observed in cocultures of ECs and SMCs. Using plasminogen-depleted serum, we demonstrated that plasminogen is required for the activation of LTGF-β. Next, we showed that Lp(a) inhibits the activation of LTGF-β in BAEC/BSMC cocultures without affecting the activity of TGF-β, the plasmin-mediated activation of LTGF-β, the release of LTGF-β, the cellular PA activity, or the basal migration of BAECs and/or BSMCs. Furthermore, we demonstrated that Lp(a) inhibited plasminogen binding to BAEC/BSMC cocultures, indicating that the inhibition of LTGF-β activation by Lp(a) probably occurred at the step of plasminogen binding. The concentration of Lp(a) required for complete inhibition of LTGF-β activation is consistent with results reported by Miles et al. (1989) describing the competition by Lp(a) with plasminogen binding to HUVECs.

When the migration of BSMCs rather than BAECs was measured using different CM, a similar inhibitory effect on cell migration was observed. The inhibitor (TGF-β) produced in cocultures was absent when the CM was produced in the presence of Lp(a). We also demonstrated that cocultures of human cells activated LTGF-β and that this activation was also blocked by the inclusion of Lp(a).

Interestingly, the inhibitory effect of Lp(a) on LTGF-β activation was apparent only when Lp(a) and plasminogen were added simultaneously. Once plasminogen had bound to the cell surface, Lp(a) was ineffective under short-term conditions. We do not know the reason for this effect but it is possible that this reflects different modes of binding.

It should be noted that the amount of Lp(a) required to block LTGF-β is relatively high and exceeds the circulating levels found in normal and pathological conditions. The circulating level of plasminogen is ~1 μM, while the Lp(a) plasma concentration is 1 nM–1 μM. However, recent reports indicate that the tissue concentrations of Lp(a) in the intima of the arterial wall may be much higher than the circulating levels (Rath et al., 1989; Cushing et al., 1989; Niendorf et al., 1990). This may reflect Lp(a) binding to glycosaminoglycans, fibronectin, and fibrin fragments. Therefore, it is possible that Lp(a) concentrations do exceed plasminogen concentrations within the vessel tissue.

Numerous studies have been conducted in recent years concerning the molecular mechanisms for the regulation of cell migration. Recently it has been shown that bFGF and TGF-β may be critical in the control of migration of certain cells (Mullins and Rifkin, 1990). The regulation of the extracellular action of TGF-β is complicated because of its production in a latent form (Barnard et al., 1990; Roberts and Sporn, 1990). We previously reported that the endogenous activation of LTGF-β by plasmin occurred in cocultures of BAECs and pericytes or BSMCs (Sato and Rifkin, 1989), and that this activation may be self-regulating since generation of TGF-β stimulates PAI-1 production, which in turn decreases subsequent plasmin generation (Sato et al., 1990). Furthermore, we have proposed that LTBF-β activation proceeds on the cell surface. The evidence for this derives from the observation that the interference of the binding of mannose-6-phosphate (M6P)-containing LTGF-β to cell surface M6P/insulin-like growth factor-II receptors inhibits LTGF-β activation (Dennis and Rifkin, 1991), indicating that surface localization of LTGF-β is required for activation. It is well documented that the surface-bound proteolytic reactions offer several advantages when compared to those in the fluid phase (Hajjar et al. 1986; Plow et al., 1986). Thus, it is of interest that all of the components of LTGF-β activation, such as plasminogen, PA, plasmin, and M6P/insulin-like growth factor-II receptors, and LTGF-β exist on the cell surface. The findings in the present study stress the importance of surface plasminogen binding. It must be noted that Hajjar et al. (1986) reported that human plasminogen did not bind to BAECs or BSMCs and that the binding of plasminogen was species specific. On the other hand, Stephen et al. (1989) described that bovine plasminogen was able to bind to human fibrosarcoma cells and that there was no apparent species specificity. In our study human plasminogen did bind to bovine cells. We cannot explain this discrepancy between our results and those of Hajjar et al. (1986). It must also be noted that we have not discriminated between the binding of plasminogen to cell membranes or to cell matrix.

The current studies suggest a new role for Lp(a) in atherogenesis. We propose a molecular mechanism for the Lp(a)-induced inhibition of LTGF-β activation and suggest a relationship between the activation of LTGF-β and atherosclerosis. We suggest that under normal conditions, LTGF-β may be activated at sites where ECs and SMCs are in contact and function to maintain the tissue architecture. Thus, excess tissue levels of Lp(a) may decrease TGF-β production thereby reducing the block on SMC migration. This may account for the migration of SMCs from the media to the intima and thereby contribute to the generation of atheromas.

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