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Remnant Lipoproteins Induce Endothelial Plasminogen Activator Inhibitor-1

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Remnant lipoproteins (RLPs) accumulate in type III hyperlipoproteinemia, a condition associated with significant cardiovascular morbidity. The effect of RLPs on fibrinolysis is unknown. Our aim was to study the effect of RLPs on endothelial expression of plasminogen activator inhibitor-1 (PAI-1). After 24-h culture of human aortic endothelial cells with RLPs at concentrations of 0 (control), 0.038, or 0.076 mg triglyceride/mL, postculture PAI-1 antigen concentrations were: 870 ± 80, 1963 ± 183 ($P = 0.005$), and 3551 ± 177 ng/mL ($P < 0.001$), respectively. Furthermore, after 24-h incubation of endothelial cells with RLPs (0 or 0.076 mg triglyceride/mL), PAI-1 activity increased from 0.667 ± 0.144 to 1.268 ± 0.198 U/mL, respectively ($P = 0.008$) and endothelial PAI-1 mRNA increased to 2.7 ± 0.66 that of control ($P = 0.048$). In conclusion, RLPs from patients with type III hyperlipoproteinemia induce endothelial cell PAI-1 expression, which may contribute to a prothrombotic state. © 2001 Academic Press

Key Words: **remnant lipoproteins; plasminogen activator inhibitor-1; fibrinolysis; endothelial cells.**

Chylomicron remnants and very low-density lipoprotein (VLDL) remnants, collectively termed "remnant lipoproteins" (RLPs), are formed from the metabolism of chylomicrons and very low density lipoprotein after triglyceride hydrolysis by lipoprotein lipase. RLPs accumulate in type III hyperlipoproteinemia, a condition associated with premature atherosclerosis and cardiovascular morbidity (1, 2). Type III hyperlipoproteinemia or familial dysbetalipoproteinemia is most commonly caused by homozygosity for apolipoprotein E2 (apo E2) (3, 4). However, an elevation of remnant lipoproteins is also observed in conditions such as type 2 diabetes mellitus in the absence of apo E defects (5–7). Moreover, in patients without familial dysbetalipoproteinemia, elevated remnant lipoproteins have been associated with progression of coronary artery disease and cardiovascular events (8, 9). Remnant lipoproteins are believed to be highly atherogenic for several reasons, including, avid uptake by monocyte-derived macrophages, negative effect on endothelial-dependent vasorelaxation, and positive effect on platelet aggregation (10–15). The effect of RLPs on endothelium-associated fibrinolysis has not been studied.

Plasminogen activator inhibitor-1 (PAI-1) is secreted by the endothelium and negatively regulates fibrinolysis (16). PAI-1 inhibits tissue plasminogen activator (tPA) and urokinase plasminogen activator, thereby promoting a prothrombotic state (17, 18). Furthermore, PAI-1 appears to be preferentially expressed in atherosclerotic human arteries (19–24). Also, elevated plasma PAI-1 antigen concentrations or activity are associated with myocardial infarction, cardiovascular death, ischemic stroke, and peripheral vascular disease (25–35). Thus, PAI-1 is postulated to play a role in atherogenesis and precipitation of acute coronary syndrome (36, 37).

Lipoproteins such as oxidized low-density lipoproteins, lipoprotein (a), and VLDL induce endothelial secretion of PAI-1, whereas high density lipoproteins may attenuate PAI-1 secretion (38–42). The effects of RLPs on PAI-1 expression has not been previously studied. Our objective was to examine the effects of RLPs from patients with type III hyperlipoproteinemia on endothelial secretion of PAI-1 and tissue plasminogen activator.

MATERIALS AND METHODS

Isolation and purification of RLPs. The diagnosis of type III hyperlipoproteinemia was confirmed in 30 subjects by ultracentrifugation (UC), lipoprotein electrophoresis, and enzymatic determina-

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tion of cholesterol and triglycerides in various UC fractions. Inclusion criteria included presence of a broad beta-band on lipoprotein electrophoresis, a VLDL cholesterol/VLDL triglyceride ratio of >0.35 and beta VLDL cholesterol concentration greater than 100 mg/dL. EDTA-fasting plasma (1 mg/mL EDTA) was pooled in three separate preparations (plasma from 10 different subjects per preparation) and applied to a column packed with an immunoaffinity gel mixture of anti-apo-A-I and anti-apo B-100 monoclonal antibodies (Japan Immunoresearch, Takasaki, Japan) (43). The eluted unbound fraction was ultracentrifuged (150,000*g*, 20 h, d < 1.006) and dialyzed against phosphate-buffered saline containing EDTA (50 μM) and butylated hydroxytoluene (BHT) (20 μM) for 24 h at 4°C. The presence of RLPs was then confirmed by lipoprotein electrophoresis. Other lipoproteins such as low-density lipoprotein, VLDL, and lipoprotein (a) were undetectable. Triglyceride content of RLPs was measured enzymatically on a Hitachi 912. The purified RLPs were not substantially oxidized as determined by measurement of lipid hydroperoxides (Kamiya Biomedical Company, WA). RLPs were sterilized using a 0.22 mμ filter (Millipore, Inc., Bedford, MA) and confirmed to be endotoxin-free by a limulus amebocyte lysate assay (Biowhittaker, Inc., Walkersville, MD). The Mayo Foundation Institutional Review Board approved the study.

Cell culture and stimulation. Human aortic endothelial cells (HAECs, Biowhittaker, Inc.) between 3rd and 6th passages, were cultured to confluence in T75 flasks containing Eagle basal medium (EBM) supplemented with bovine brain extract (3 mg/mL), human epidermal growth factor (10 μg/mL), hydrocortisone (1 mg/mL), 2% fetal bovine serum (FBS), gentamicin (50 mg/mL), amphotericin B (50 μg/mL) (all from Biowhittaker, Inc.) and endothelial cell growth factor (20 μg/mL, Boehringer Manheim, Indianapolis, IN), and equilibrated with 95% air/5% CO_2 at 37°C. Confluent HAECs were harvested using 0.05% trypsin/0.53 mmol/L EDTA, counted with a hemocytometer, and plated on 6-well plates (for measurement of PAI-1 and tPA antigens as well as PAI-1 activity) or in T75 flasks (for RNA extraction). Plated HAECs were incubated in EBM with additives (listed above) for 24 h. Culture medium was then replaced with "serum-enriched" medium (EBM with 2% FBS and additives listed above) with or without remnant lipoproteins for 24 h.

In experiments performed under "serum-deprived" conditions, cells were quiesced for 12 h in EBM with 0.1% FBS, gentamicin and amphotericin B (in the above concentrations), before a 24-h incubation with or without RLPs and fresh 0.1% FBS containing serum-deprived medium. When appropriate, cells were preincubated for 2 h with actinomycin D (0.2 μg/mL) before addition of RLPs.

Measurement of PAI-1 antigen, tPA antigen, and PAI-1 activity in postculture media of HAECs. Total PAI-1 and tPA antigens were measured by enzyme-linked immunosorbent assay kits, and PAI-1 activity was measured using an indirect enzymatic assay kit (American Diagnostica, Inc., CT).

Northern blot analysis. Total cellular RNA was extracted from cells using an RNeasy Mini RNA extraction kit (Qiagen, Inc., CA). RNA (6–10 μg) was denatured, fractionated on a 1.0% formaldehyde agarose gel, and transferred to a BrightStar-Plus nylon membrane (Ambion, Inc., TX). Plasmid containing the cDNA fragment encoding human PAI-1 was kindly provided by Dr. G. Shen (University of Manitoba, Canada). PAI-1 cDNA was randomly labeled with (^{32}P) d-CTP using a DECAprime II labeling kit (Ambion, Inc.). Prehybridization and hybridization were performed using NorthernMax buffer (Ambion, Inc.). The hybridized membrane was washed serially at 42°C with 2 × SSC/0.1% SDS, 1 × SSC/0.1% SDS, and 0.5 × SSC/0.1% SDS and then exposed to Kodak BMR film (Eastman Kodak, Inc., CT) with intensifying screens at −80°C. The stripped membrane was reprobed with randomly labeled mouse β-actin cDNA (Ambion, Inc.). Hybridized bands were scanned and densitometry was normalized for RNA loading to the β-actin levels using Optiquant image analysis software (Packard Instrument Co., CT).

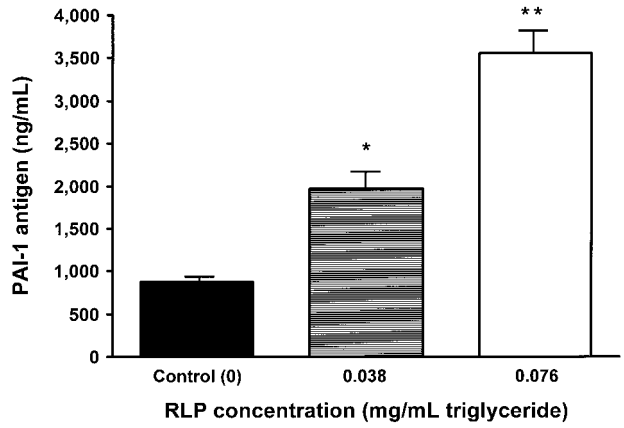

FIG. 1. Incubation of human aortic endothelial cells with remnant lipoproteins (RLPs) (0, 0.038, or 0.076 mg/mL triglyceride) was associated with increased PAI-1 antigen concentrations of conditioned medium (*P = 0.005 control vs 0.038 mg/mL remnant lipoproteins, **P < 0.001 control vs 0.076 mg/mL remnant lipoproteins).

Statistics. Data are presented as mean ± standard error of the mean (SEM). The error bars on all figures correspond to SEM. Statistical comparisons between two groups were performed using unpaired Student's *t*-tests or 1-way ANOVA for multiple groups. The level of significance was defined as $P < 0.05$.

RESULTS

Culture of HAECs in serum-enriched medium with the addition of RLPs in the concentrations of 0 (control), 0.038, or 0.076 mg triglyceride/mL for 24 h (n = 3, repeated 1–2 times) resulted in the following PAI-1 antigen concentrations in postculture medium: 870 ± 80, 1963 ± 183 (P = 0.005), and 3551 ± 177 ng/mL (P < 0.001), respectively (Fig. 1). Furthermore, PAI-1 antigen concentrations were significantly higher in medium of cells exposed to 0.076 mg/mL than to 0.038 mg/mL triglyceride of RLPs, suggesting a dose-responsive effect of RLPs on PAI-1 antigen secretion (P = 0.002). Cell viability, as evaluated by trypan blue staining, was unaffected by RLP exposure. Moreover, PAI-1 mRNA content increased 2.7 ± 0.66 that of control in RLP-exposed cells versus control (PAI-1 2.4 kb mRNA band, densitometry corrected for β-actin, P = 0.049, n = 4) (Fig. 2).

Similarly, in a separate serum-deprived experiment, incubation of endothelial cells with RLPs at concentrations of 0 or 0.076 mg/mL triglyceride resulted in postculture PAI-1 antigen concentrations of 1174 ± 525 and 1890 ± 845 ng/mL, respectively (P = 0.02, n = 5, repeated 2 to 3 times). Corresponding, PAI-1 activity measurements were 0.667 ± 0.144 and 1.268 ± 0.198 U/mL, respectively (P = 0.008) (n = 3) (Fig. 3). In the same experiment, preincubation with actinomycin D (0.2 μg/mL) prior to addition of RLPs, decreased PAI-1 antigen concentration to 492 ± 284 ng/mL (P = 0.01 compared with remnant-containing medium, P = 0.05

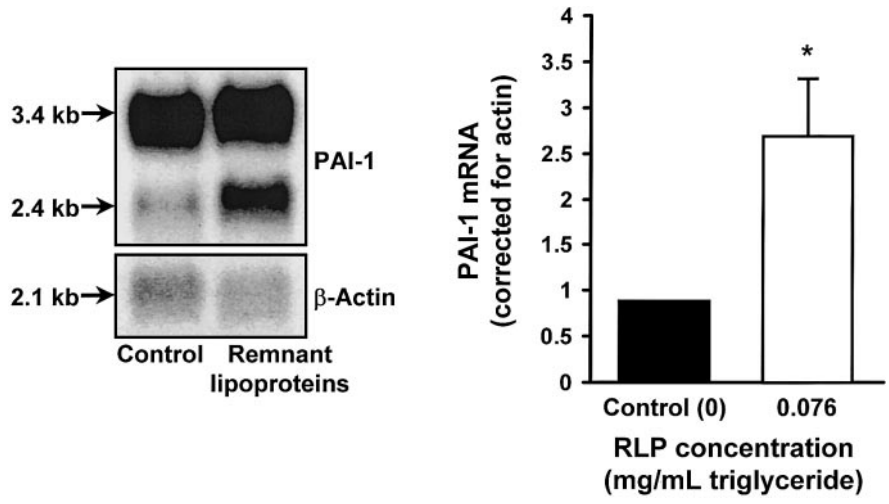

FIG. 2. Representative Northern blot showing increased expression of the 2.4-kb band of PAI-1 mRNA in endothelial cells exposed to remnant lipoproteins (0.076 mg/mL triglyceride) vs control. RNA loading was controlled by probing the blot with β-actin cDNA. Also shown, PAI-1 mRNA content of endothelial cells as measured by densitometry (2.4-kb band of PAI-1 mRNA normalized to β-actin) is increased in cells exposed to RLPs (0.076 mg/mL triglyceride) vs control ($\dagger P = 0.049$).

compared with control medium), suggesting that RLPs induced PAI-1 gene transcription. Of note, Actinomycin D treatment did not significantly effect total cellular protein concentrations.

Incubation of cells with RLPs at concentrations of 0 or 0.076 mg/mL triglyceride did not substantially affect tPA antigen concentrations (3.6 $\pm$ 1.1 and 9.6 $\pm$ 2.8 ng/mL, respectively, $P = 0.12$, $n = 3$, in duplicate).

DISCUSSION

This study is the first to examine the effects of remnant lipoproteins derived exclusively from patients with type III hyperlipoproteinemia on endothelial cell biology. We have successfully isolated RLPs using an immunoaffinity gel containing monoclonal antibodies to apo A-I and apo B-100, which do not bind apo-E-enriched RLPs. This reliable and simple technique has been developed for clinical measurement of RLPs in plasma and its use in isolation of RLPs for in vitro studies is relatively novel. This new technology should enable further in vitro studies of the effects of RLPs on vascular endothelium.

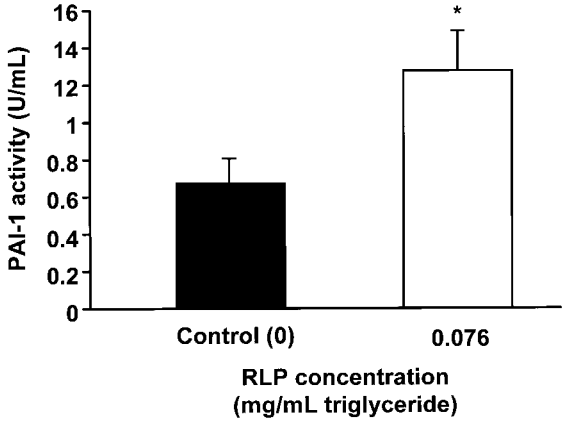

FIG. 3. Plasminogen activator inhibitor-1 activity is increased in endothelial cells exposed to RLPs (0.076 mg/mL triglyceride) compared to control ($*P = 0.008$). RLP, remnant lipoprotein.

Our principal finding is that RLPs from patients with type III hyperlipoproteinemia can impair fibrinolysis by inducing endothelial secretion of PAI-1. This effect may be, at least in part, transcriptional. Thus, RLPs may induce an antifibrinolytic state through interaction with the vascular endothelium in type III hyperlipoproteinemia. Such an antifibrinolytic state may potentially predispose to precipitation of acute coronary syndrome and cardiovascular morbidity in this condition. Further studies examining the concentrations of PAI-1 antigen and PAI-1 activity in patients with Type III hyperlipoproteinemia are indicated. Also, it would also be of interest to examine any potential associations of apoE isoforms and fibrinolytic potential *in vivo*.

The mechanism by which RLPs may induce a hypofibrinolytic state are not known. Metabolism of RLPs and PAI-1 occurs through a common receptor (44). The low-density lipoprotein receptor-related protein, which binds RLPs, also binds the inactive complex of PAI-1 and urokinase plasminogen activator, mediating cellular uptake and degradation of PAI-1. Thus RLPs could compete with PAI-1 for binding to the low-density lipoprotein receptor-related protein, resulting in decreased degradation of inactive PAI-1 and increased concentrations of PAI-1 antigen. However, we have shown in current experiments, that RLP exposure increases both PAI-1 antigen and activity through tran-

scriptional activation of endothelial cells. Thus, the RLP-associated increase in PAI-1 antigen is unlikely to be due to defective degradation of inactive PAI-1 secondary to competitive inhibition of low-density lipoprotein receptor-related protein.

Other lipoprotein receptors may regulate PAI-1 synthesis. For example, very low-density lipoproteins induce endothelial secretion of PAI-1 following binding to the VLDL receptor (40). Lipoprotein receptors responsible for the RLP-associated increase in PAI-1 secretion could include very low-density lipoprotein receptors, low-density lipoprotein receptors, membrane binding proteins 200 and 235, or cell-surface heparan sulfate proteoglycans (45, 46). Furthermore, a VLDL response element has been identified in the promoter region of PAI-1 (41). It is possible that the effect of remnant lipoproteins may be at the level of the VLDL response element or a similar response element in the PAI-1 promoter. Alternatively, auto-oxidation of RLPs could induce PAI-1 secretion, as oxidation of other lipoproteins such as low density lipoproteins or lipoprotein(a) is known to enhance endothelial PAI-1 secretion, possibly through activation of protein kinase C (38, 47). However, the latter mechanism is unlikely to explain our current observations because lipid hydroperoxide content of RLPs was not increased and inhibitors of auto-oxidation (EDTA and BHT) were used. The specific mechanism of RLP-associated induction of endothelial PAI-1 secretion is unknown and therefore requires further investigation.

In conclusion, a remnant-associated impairment in endothelium-dependent fibrinolysis may contribute to cardiovascular morbidity associated with elevated remnant lipoproteins. It is unknown whether routine measurement and treatment of an increase in RLPs may improve fibrinolysis and, most importantly, cardiovascular morbidity and mortality.

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