Lipoprotein lipase enhances human monocyte adhesion to aortic endothelial cells

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Abstract Lipoprotein lipase (LPL)-mediated lipolysis of very low density lipoprotein (VLDL) has been demonstrated to increase U937 monocyte adhesion to endothelial cells. In the present study, we evaluated the ability of LPL to enhance human monocyte adhesion to bovine aortic endothelial cells (BAEC) in the absence of exogenous lipoproteins. Exposure of BAEC to 1 µg/ml LPL at 37°C resulted in a significant increase in monocyte adhesion over control values. Addition of VLDL in the culture media further enhanced the LPL effect. A significant increase in monocyte adhesion was also observed when BAEC were incubated with LPL at 4°C. Heparin or heparinase treatment of BAEC totally abolished the LPL stimulatory effect on monocyte adhesion. In addition, incubation of monocytes with heparinase suppressed the ability of LPL to stimulate monocyte adhesion to endothelial cells. These treatments also markedly decreased LPL binding to the monocyte and endothelial cell surfaces. In contrast to native LPL, heat-inactivated or phenylmethylsulfonyl fluoride (PMSF)-treated LPL did not increase monocyte adhesion to BAEC. Finally, incubation of LPL in the presence of the 5D2 antibody resulted in a total suppression of the LPL-induced monocyte adhesion to BAEC. ⬛ Taken together, these data demonstrate that LPL activity plays an important role in LPL-induced monocyte adhesion and that LPL binding to heparan sulfate proteoglycans expressed on both monocytes and endothelial cells surfaces is required for the enhanced monocyte adhesion. These results suggest a new mechanism by which LPL may promote the development of atherosclerosis, that of facilitating monocyte adhesion to the endothelium.—**Mamputu, J-C., A-C. Desfaits, and G. Renier.** Lipoprotein lipase enhances human monocyte adhesion to aortic endothelial cells. *J. Lipid Res.* 1997. **38:** 1722–1729.

Supplementary key words lipoprotein lipase • monocyte adhesion • endothelium • atherosclerosis

Lipoprotein lipase (LPL) is the key enzyme responsible for the hydrolysis of triglycerides present in chylomicrons and very low density lipoproteins (VLDL). It is normally synthesized by adipose cells, smooth muscle cells, and macrophages (1). After intracellular processing, LPL is transferred to binding sites at the luminal surface of vascular endothelium where it is bound to heparan sulfate proteoglycans (HSPG) (2). Depending on its location, LPL has been suggested to have a dual role in regard to atherogenesis (3). While by facilitating the hepatic uptake of atherogenic lipoproteins LPL seems to exert some important anti-atherogenic properties, it may act in the arterial wall as an atherogenic protein. Indeed, it has been shown that LPL, by stimulating the cellular binding and uptake of atherogenic lipoproteins by different vascular cell types including smooth muscle cells and macrophages, contributes to lipid accumulation within these cells (4, 5). In addition, besides its effect on arterial lipid metabolism, LPL also acts as an activator of macrophage function, inducing tumor necrosis factor alpha (TNF-α) and nitric oxide production (6, 7).

Monocyte–endothelial cell interactions play a crucial role in the pathogenesis of atherosclerosis. Enhanced adhesion of monocytes to aortic endothelium is believed to represent one of the earliest events in atherogenesis (8). It has been demonstrated that lipolysis of VLDL by LPL at the endothelial cell surface markedly enhances monocyte adhesion to endothelium (9). Because LPL binds to HSPG expressed on both monocytes (10) and endothelial cells, we examined in the present study the possibility that these LPL heparin binding properties may facilitate monocyte–endothelial cell interactions. Our results, which demonstrate that LPL binding to heparin-sensitive sites on these cells surfaces enhances human monocyte adhesion to endothelial

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cells, suggest a new mechanism by which LPL may promote atherogenesis.

MATERIALS AND METHODS

Reagents

Dulbecco's minimal essential medium (DMEM) and L-glutamine were purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Fetal calf serum (FCS) was obtained from Hyclone Laboratories Inc. (Logan, UT). Penicillin-streptomycin was purchased from Flow (Mc Lean, VA). Bovine serum albumin (BSA) fatty acid-poor, endotoxin-free, fraction V, was purchased from Calbiochem (La Jolla, CA). Bovine LPL, heparin, heparinase III, human VLDL, phenylmethylsulfonyl fluoride (PMSF), and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. (St. Louis, MO). LPL was dialyzed against saline before use. Hanks' balanced salt solution (HBSS), polymyxin B sulfate, and RPMI-1640 medium were purchased from Gibco (Grand Island, NY). The monoclonal antibody 5D2, generated against bovine LPL, was kindly provided by Dr. J.D. Brunzell (University of Washington, Seattle, WA).

Endothelial cell cultures

Bovine aortic endothelial cells (BAEC) (19th passage) were grown to confluence in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (DMEM-FCS) at 37°C in 5% CO_2/95% air atmosphere for 6 days. The cells were then trypsinized and subcultured in 96-well culture plates (Costar) for 48 h, at which time cell confluence was reached. In all experiments, cells were used between the 20th and the 24th passage.

Isolation of human monocytes

Human monocytes were isolated from 100 ml anticoagulated (heparin sodium) whole blood collected from non-smoker healthy donors. First, peripheral blood mononuclear cells (PBMC) were obtained by density centrifugation using Ficoll (Nycomed Pharma As, Oslo, Norway). The cells collected from the interface were washed three times with HBSS, and allowed to aggregate in the presence of FCS. After further purification by rosetting technique and density centrifugation, recovery of highly purified monocytes (85–90%), as assessed by FCAS analysis, was obtained. Human monocytes were resuspended in FCS-free RPMI medium supplemented with penicillin-streptomycin.

Adhesion assay

On the day of the assay, BAEC culture medium was gently removed and the cells were washed twice with 200 µl of fresh FCS-free DMEM. The medium was then changed to DMEM containing 3% BSA and 100 ng/ml polymyxin B sulfate. Native or denatured LPL (1 µg/ml) was allowed to bind to BAEC for 1 h at 37°C. In some experiments, BAEC were incubated with heparin (50 U/ml) for 5 min at room temperature to remove LPL bound to their surface. In other experiments, BAEC were incubated with medium containing 2.5 U/ml heparinase for 1 h prior to LPL binding assay. Finally, in the experiments where the effect of the anti-LPL antibody was studied, LPL was incubated in the presence of the 5D2 anti-LPL antibody (10 µg/ml) before being allowed to bind to BAEC. At the end of the incubation period, the cells were washed twice with 200 µl of phosphate-buffered saline (PBS) to remove unbound LPL. Highly purified human monocytes (230,000) were then added to the wells and allowed to adhere to BAEC for 2 h. Non-adherent monocytes were removed by washing the cells with warm PBS (pH 6.0). Monocyte adhesion to BAEC was quantitated by measuring monocyte myeloperoxidase (MPO) activity as previously described by Wang, Beekhuizen, and van Furth (11).

LPL binding to endothelial cells and monocytes

LPL binding to endothelial cells and monocytes was determined by incubating LPL in presence of these cells for 1 h at 37°C. At the end of the incubation period, the medium was removed and the cells were washed twice with HBSS. Bound LPL was released by adding 50 U/ml of heparin in PBS for 5 min. LPL activity in the media and in the heparin-released fractions was determined using the kit Confluolip from Progen (Heidelberg, Germany). Binding of PMSF-treated LPL to endothelial cells was determined by Western blotting using the 5D2 anti-LPL antibody as previously described (12).

Determination of cell viability and total protein content

Cell viability after heparinase treatment was estimated using trypan blue exclusion and was consistently found to be higher than 95%. After extensive dialysis against saline, total protein content of the purified LPL preparation was measured according to the method of Bradford (13) using a colorimetric assay (Bio-Rad, Mississauga, ONT), and BSA as standard.

Determination of endotoxin content

Endotoxin content of the LPL preparation (1 µg/ml), was determined by the Limulus amebocyte lysate

assay (Sigma Chemical Co., St. Louis, MO) and was consistently found to be lower than 6 pg/ml.

Statistical analysis

Statistical analysis of the results was performed using the unpaired Student's t test. Results are expressed as mean values $\pm$ SEM.

RESULTS

Effect of LPL on monocyte adhesion to endothelial cells

Confluent endothelial cell monolayers were incubated with LPL (1 µg/ml) for 1 h at 37°C prior to the addition of freshly isolated human monocytes. Treatment of endothelial cells with LPL led to a marked increase (163% over control values, $P < 0.001$) in human monocyte adhesion (**Fig. 1,** panel A). A 2.4-fold increase in monocyte adhesion to endothelial cells was also observed after exposure of the cells to 10 ng/ml LPS, used in these experiments as positive control (Fig. 1, panel A). A similar stimulatory effect of LPL on monocyte adhesion was also observed when incubation of BAEC with LPL was performed at 4°C (156% over control values, $P < 0.02$) (Fig. 1, panel B).

To address the possibility that, under our experimental conditions, an LPL-generated hydrolytic product could be responsible for the stimulatory effect of LPL on monocyte adhesion, we next measured monocyte adhesion to endothelial cells bound with LPL in the presence or absence of VLDL. As shown in **Fig. 2,** addition of VLDL to the culture media led to a further and significant increase in LPL-induced monocyte adhesion (monocyte adhesion (% over control values): LPL: 150 $\pm$ 4%; LPL + VLDL: 185 $\pm$ 2%, $P < 0.05$).

Effect of heparin treatment of endothelial cells on LPL-induced monocyte adhesion

To determine whether LPL binding to the endothelial cell surface is required for the stimulatory effect of LPL on monocyte adhesion, LPL-treated endothelial cells were incubated in the presence of heparin to remove endothelial cell surface-associated LPL and LPL activity was measured in the heparin-released fractions. Under these experimental conditions, incubation with heparin resulted in the release of 70% of bound LPL activity (data not shown) and a complete abrogation of the stimulatory effect of LPL on monocyte adhesion was observed (**Fig. 3**). Heparin treatment did not affect LPS-stimulated monocyte adhesion (LPS-stimulated monocyte adhesion (% over control values): control:
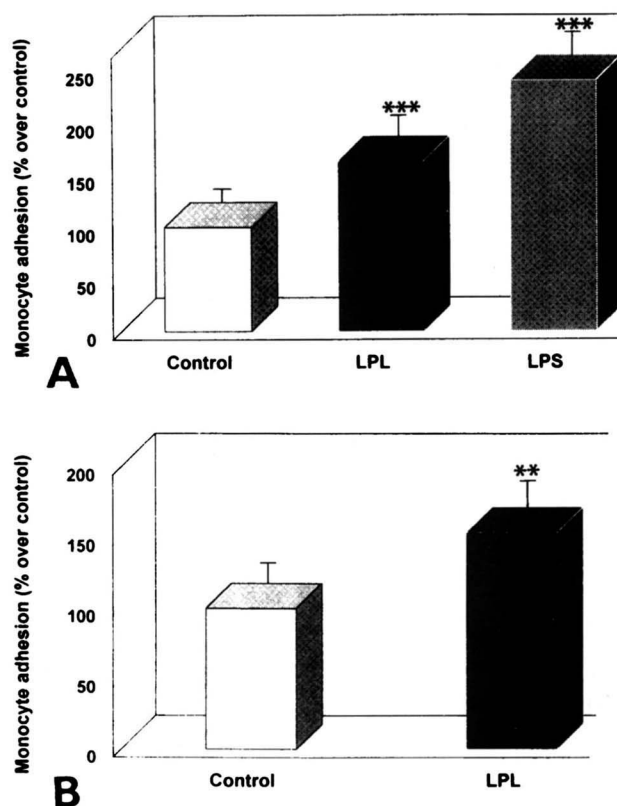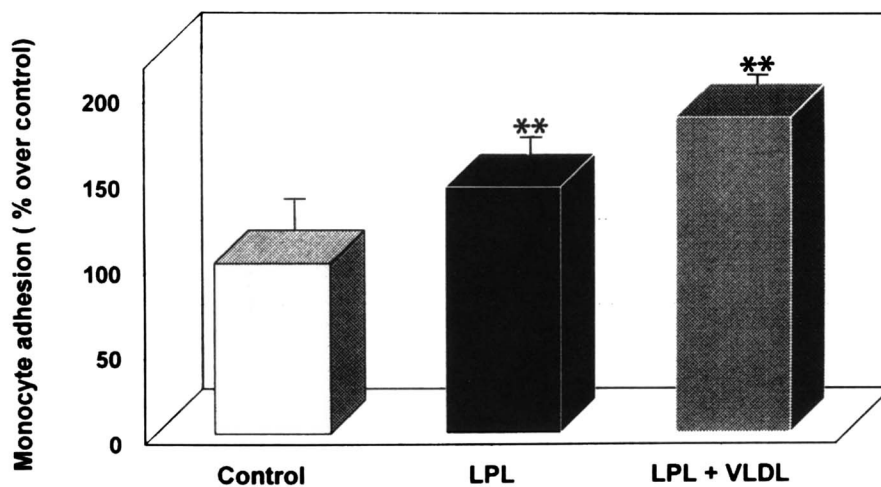

Fig. 1. Effect of native LPL on human monocyte adhesion to endothelial cells. Confluent monolayers of BAEC were bound with LPL (1 µg/ml) for 1 h at 37°C (panel A) or at 4°C (panel B). At the end of the incubation period, cells were washed and monocytes were added to BAEC to determine adhesion. In some experiments, BAEC were incubated in the presence of LPS (10 ng/ml), used as positive control. Data are expressed as percentage of adherent control monocytes and represent the mean $\pm$ SEM of 7 (panel A) and 3 (panel B) independent experiments. ** $P < 0.02$ vs. control; *** $P < 0.001$ vs. control.

180 $\pm$ 8%; heparin 161 $\pm$ 5%). These data demonstrate that LPL binding to heparin-sensitive sites on the endothelial cells is required for its stimulatory effect on monocyte adhesion.

Effect of heparinase treatment of endothelial cells and monocytes on LPL-induced monocyte adhesion

To investigate whether removal of HSPG expressed on endothelial cells may affect LPL-induced monocyte adhesion, endothelial cells were pretreated with heparinase (2.5 U/ml) for 1 h at 37°C before the addition of LPL (1 µg/ml). Pretreatment of endothelial cells with heparinase resulted in a 4-fold increase of LPL in the medium (data not shown) and totally abolished the LPL-induced increase in monocyte adhesion (**Fig. 4**). In contrast, no effect of heparinase treatment on LPS-stimulated monocyte adhesion was observed (LPS-stim-

Fig. 2. Effect of VLDL on LPL-induced human monocyte adhesion to endothelial cells. Confluent monolayers of BAEC were bound with LPL (1 μg/ml) and further incubated in the presence or absence of VLDL (20 μg protein/ml) at 37°C for 24 h. At the end of the incubation period, cells were washed and monocytes were added to BAEC to determine adhesion. Data are expressed as percentage of adherent control monocytes and represent the mean ± SEM of 3 independent experiments. ** $P < 0.02$ vs. control.

ulated monocyte adhesion (% over control values): control: $180 \pm 8\%$; heparinase: $168 \pm 5\%$).

To document the role of HSPG expressed on monocytes in LPL-induced monocyte adhesion to endothelial cells, human monocytes were pretreated with heparinase (0.01 U/ml) for 30 min at 37°C before being allowed to bind BAEC. Treatment of monocytes with heparinase led to a marked reduction of LPL binding to these cells (data not shown) and totally suppressed the ability of LPL to enhance monocyte adhesion to endothelial cells (**Fig. 5**). Taken together, these observa-

tions indicate that both endothelial and monocyte cell surface proteoglycans are obligatory participants in the stimulatory effect of LPL on monocyte adhesion.

Effect of denatured LPL on monocyte adhesion

To assess whether the structural characteristics of LPL are involved in LPL-induced monocyte adhesion to endothelial cells, the effect of denatured LPL on monocyte adhesion was next evaluated. Heat-inactivated LPL was obtained by boiling the enzyme for 30 min. Treatment of BAEC with boiled LPL did not result
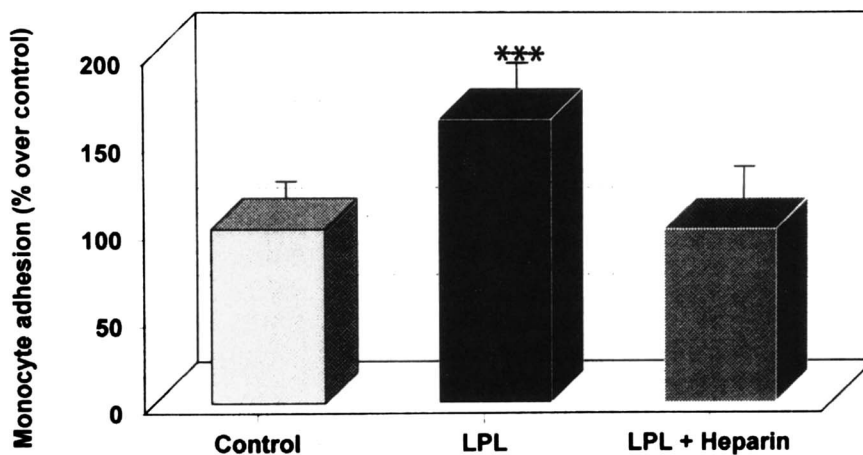
Fig. 3. Inhibition of LPL-induced human monocyte adhesion to BAEC by heparin. Confluent monolayers of BAEC were bound with LPL (1 μg/ml) for 1 h at 37°C. At the end of the incubation period, heparin (50 U/ml) was added to the wells for 5 min. Cells were then washed and incubated in the presence of human monocytes to determine adhesion. Data are expressed as percentage of adherent control monocytes and represent the mean ± SEM of 7 independent experiments. *** $P < 0.001$ vs. control.
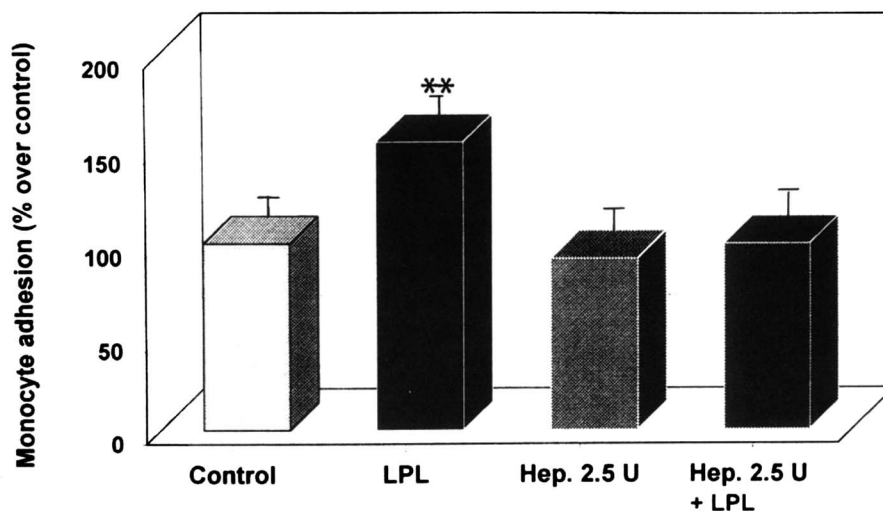
Fig. 4. Effect of heparinase treatment of endothelial cells on monocyte adhesion. Confluent monolayers of endothelial cells were treated with heparinase (2.5 U/ml) for 1 h at 37°C and after extensive washing were incubated for 1 h with LPL. At the end of the incubation period, cells were washed and monocytes were added to BAEC to determine adhesion. Data are expressed as percentage of adherent control monocytes and represent the mean ± SEM of 5 independent experiments. ** $P < 0.02$ vs. control.

in any enhancement of human monocyte adhesion to endothelium (**Fig. 6**), suggesting that the ability of LPL to stimulate monocyte adhesion is closely linked to the integrity of its native conformation.

To evaluate whether loss of LPL activity also affects the ability of LPL to stimulate monocyte adhesion, irreversible inhibition of the enzyme was obtained by incubating LPL (1 µg/ml) in presence of 1 mM PMSF. We found that PMSF-treated LPL was totally ineffective to increase monocyte adhesion to endothelium (Fig. 6). A

90% decrease in LPL binding capacity to endothelial cells, as assessed by Western blotting and LPL mass determination, was also observed after PMSF treatment of the enzyme (data not shown).

Effect of the anti-LPL monoclonal antibody 5D2 on LPL-induced monocyte adhesion

To assess the specifity of the effect of LPL on monocyte adhesion and to further document the structural characteristics of LPL that may account for the stimula-
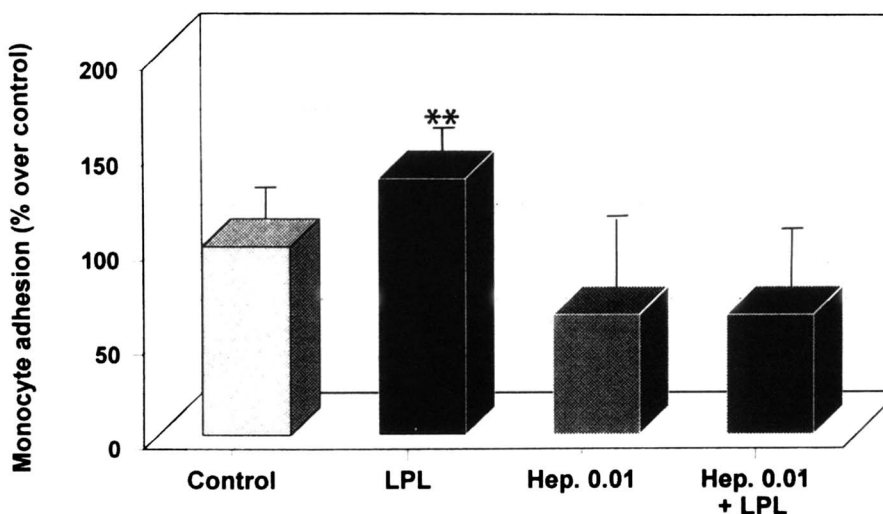
Fig. 5. Effect of heparinase treatment of human monocytes on their adhesion to LPL-treated endothelial cells. Freshly isolated monocytes were incubated with heparinase (0.01 U/ml) for 30 min at 37°C. At the end of the incubation period, monocytes were washed and added to LPL-treated endothelial cells for 1 h at 37°C to determine adhesion. Data are expressed as percentage of adherent control monocytes and represent the mean ± SEM of 4 independent experiments. ** $P < 0.02$ vs. control.
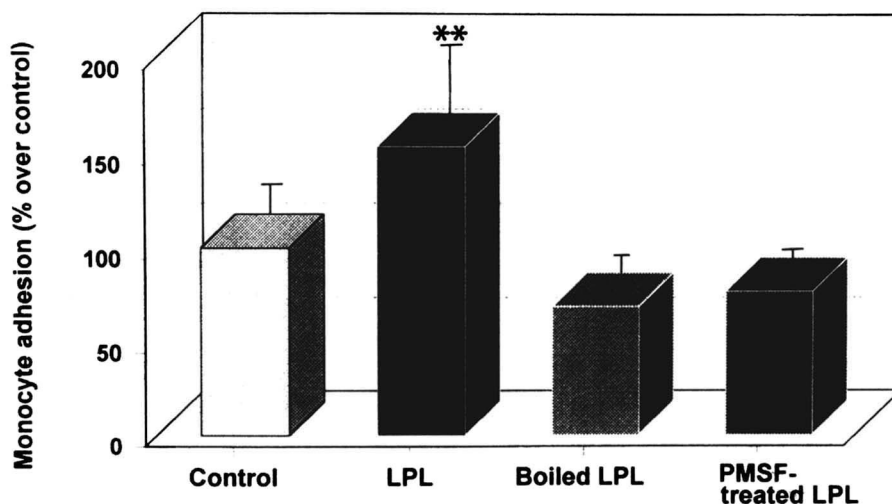
Fig. 6. Effect of denaturation of LPL on monocyte adhesion. Boiled and PMSF (1 mM)-treated LPL (1 μg/ml) were bound to confluent monolayers of BAEC for 1 h at 37°C. At the end of the incubation period, cells were washed and monocytes were added to BAEC to determine adhesion. Data are expressed as percent of adherent control monocytes and represent the mean ± SEM of 5 independent experiments. ** $P < 0.02$ vs. control.

tory effect of this enzyme on monocyte adhesion, additional experiments were performed in the presence of the monoclonal antibody 5D2 which recognizes an epitope located in the C-terminal domain of LPL. Pretreatment of LPL with 10 μg/ml of this anti-LPL antibody totally suppressed LPL-induced monocyte adhesion to endothelial cells (**Fig. 7**), although it did not affect the LPS-induced monocyte adhesion (LPS-stimulated monocyte adhesion (% over control values): control: 180 ± 8%; anti-LPL: 172 ± 5%).

DISCUSSION

It has been previously shown that LPL-mediated lipolysis of VLDL at the endothelial cell surface increases the adhesion of U937 monocytes to aortic endothelial cells (9). This effect has been proposed to be due to the hydrolytic activity of the enzyme and to correlate with the generation of LPL-derived lipolytic products (9). The present study demonstrates that incubation of aortic endothelial cells with LPL, in the absence of any
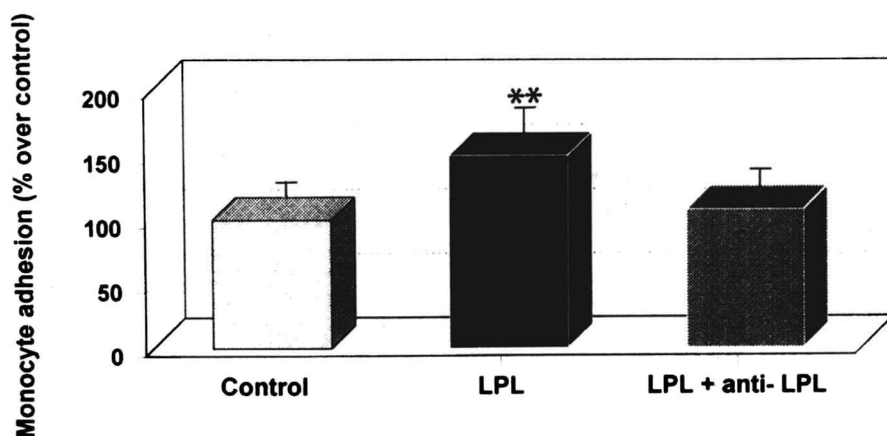
Fig. 7. Effect of anti-LPL antibody on LPL-induced human monocyte adhesion to endothelial cells. LPL (1 μg/ml) was incubated in the presence of the 5D2 anti-LPL monoclonal antibody (10 μg/ml) prior being added to BAEC. After a 1 h incubation period at 37°C, cells were washed and monocytes were added to BAEC to determine adhesion. Data are expressed as percentage of adherent control monocytes and represent the mean ± SEM of 5 independent experiments. ** $P < 0.02$ vs. control.

added exogenous lipoproteins, also leads to enhanced human monocyte adhesion to endothelium. Results generated in this work point to the conclusion that the activity of the lipase plays an important role in the LPL-induced monocyte adhesion even in the absence of exogenous substrate, and more so in its presence. Indeed, we found that inhibitors of the enzymatic activity of LPL block the induced monocyte adhesion to endothelial cells and that addition of exogenous lipoproteins to the incubation medium further enhances the LPL stimulatory effect.

Previous studies have shown that heparin treatment decreases LPL binding to the endothelial cell surface (14). Degradation of endothelial cell surface HSPG with heparinase has also been found to reduce LPL binding to these cells (15). Evidence that the stimulatory effect of LPL on monocyte adhesion requires LPL interaction with HSPG expressed on endothelial cells is 3-fold. First, we found that LPL detachment from the endothelium by heparin completely abolished the ability of LPL to enhance monocyte adhesion. Second, we demonstrated that heparinase-induced degradation of endothelial cell surface HSPG suppressed the LPL-induced monocyte adhesion. Third, we observed that LPL inactivation, which is known to reduce LPL binding to HSPG (16), resulted in a total abrogation of the enhancing effect of LPL on monocyte adhesion. LPL binding to HSPG expressed on the monocyte cell surface has been previously reported by Edwards et al. (10). Our data, which demonstrate that heparinase treatment of monocytes also prevents LPL-induced monocyte adhesion, clearly indicate that LPL binding to HSPG expressed on these cells is involved in the LPL effect on monocyte adhesion.

The monoclonal anti-LPL antibody 5D2 has been used for analysis of structure–function relationships of LPL (12, 17). Its epitope on LPL has been mapped in the C-terminal domain, particularly within residues 396–405, Ala 400 having been shown to be the critical amino acid residue conferring epitope specificity (18). The C-terminal domain of LPL has been suggested to contain both a lipid and a heparin/heparan sulfate-binding region (17, 19). While the role of the lipid-binding region in the initial interaction and binding specificity of LPL with lipoprotein particles has been demonstrated in some investigations (20, 21), the role of the C-terminal domain in the binding of LPL to HSPG is still controversial (22, 23). Our results demonstrate unequivocally that the anti-LPL antibody 5D2 abolishes LPL-induced monocyte adhesion to endothelial cells. One possible explanation for this effect is that this antibody, by inhibiting LPL activity, may have blocked LPL binding to cell-surface proteoglycans in these experiments. This hypothesis is supported by the finding of Chappell et al. (24) who found that another monoclonal antibody against LPL (MAB-7) totally prevented LPL-induced catabolism of lipoproteins and who proposed, on the basis of this observation, that anti-LPL antibodies could block LPL binding to cell-surface proteoglycans. However, arguing against this possibility, is the recent observation of Wong et al. (17) who reported that the 5D2 antibody-reacted LPL and the native LPL do not differ in their heparin affinity, and who therefore suggested that the epitope of the 5D2 antibody is distinct from the heparin-binding region of the molecule. From these results, it clearly appears that further studies are needed to elucidate the mechanism(s) that are involved in the suppressive effect of the 5D2 antibody on LPL-stimulated monocyte binding. Direct evidence that the 5D2 antibody-treated LPL binds as effectively to HSPG as native LPL would be of particular interest in this regard.

The results presented here indicate that LPL enhances human monocyte adhesion to endothelial cells both in the presence and absence of exogenous lipoproteins. These observations indicate that LPL-mediated lipolysis of lipoproteins may not represent the sole mechanism responsible for the stimulatory effect of LPL on monocyte adhesion to endothelium. Although very difficult to prove, one may suggest that LPL may exert its effect by hydrolyzing another substrate. Another possibility is that LPL may act as a protein bridge between monocytes and endothelial cells. Supporting this hypothesis are our data demonstrating that LPL also enhances monocyte adhesion even when bound at 4°C at the endothelial cell surface. Heparinase, by preventing LPL binding to HSPG expressed on these cell surfaces, may avoid the formation of such a bridge.

A crucial question that emerges from the present study is whether LPL-induced monocyte adhesion is of physiological importance. The physiological level of LPL in postheparin plasma approximates 200 ng/ml in normal subjects (25). Considering that, in our system, approximatively 10% of added exogenous LPL binds in vitro to endothelial cells, the effective LPL concentration that stimulates monocyte adhesion may be evaluated to 100 ng/ml, i.e., within the physiological range of LPL levels. It is thus possible that, under physiological conditions, the endothelial cell surface-associated LPL may facilitate the interaction of monocytes with the endothelium. We believe, however, that our observation may be most particularly relevant to some pathological situations, such as atherosclerosis, where lesion macrophages produce substantial amounts of LPL and where monocytes are attracted in large numbers towards the subendothelial matrix. Under this condition, macrophage LPL production in the arterial wall may represent a major source of LPL for the endothelial cells that

do not synthesize this enzyme. The resulting enhanced LPL expression on the endothelial cell surface, by facilitating monocyte binding to endothelium, may contribute to the progression of the atherogenic process.▪

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