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Research Article

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A New Antiinflammatory Compound, Leumedin, Inhibits Modification of Low Density Lipoprotein and the Resulting Monocyte Transmigration into the Subendothelial Space of Cocultures of Human Aortic Wall Cells

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Abstract

Addition of leumedin, *N*-[9H-{2,7-dimethylfluorenyl-9-methoxy}carbonyl]-L-leucine at 30–60 μM together with LDL almost completely prevented the induction of monocyte chemotactic protein mRNA, reduced monocyte chemotactic protein 1 levels by 84%, and inhibited monocyte migration into the subendothelial space of cocultures of human aortic wall cells by ≤ 98%. LDL incubated with leumedin formed a stable complex that remained intact even after refloating in an ultracentrifuge. Leumedin at 50 μM did not change conjugated diene formation during coculture modification of LDL or Cu^{++} catalyzed oxidation of LDL. Unlike LDL from control rabbits, LDL isolated from rabbits that were injected with 20 mg/kg leumedin was remarkably resistant to modification by the coculture and did not induce monocyte migration to a significant degree. Moreover, HDL isolated from rabbits injected with leumedin was far more effective in protecting against LDL modification by the artery wall cocultures than HDL from control rabbits. We conclude that leumedins can associate with lipoproteins in vivo, rendering LDL resistant to biological modification and markedly amplifying the protective capacity of HDL against in vitro LDL oxidation by artery wall cells. (*J. Clin. Invest.* 1993. 91:1225–1230.) Key words: monocyte chemotactic protein 1 • human aorta • endothelial • smooth muscle • polymerase chain reaction • NPC 15669 • MCP-1

Introduction

Accumulating evidence suggests that oxidized lipoproteins may play a critical role in the development of atherosclerosis (1–6). We previously reported that incubation of LDL with multilayer cocultures of human aortic endothelial cells (HAEC)[1] and smooth muscle cells (HASMC) resulted in mild LDL modification and a marked increase in monocyte transmigration into the subendothelial space of cocultures (7). HDL and antioxidants prevented these LDL induced effects (7). A new class of compounds with potent antiinflammatory effects (8) and capable of inhibiting endotoxic shock in mice (9) termed "leumedins" were recently reported. We have examined the effect of leumedins on LDL modification and monocyte transmigration in cocultures of artery wall cells. We have found that leumedins associate with LDL and HDL in vivo and effectively inhibit the modification of LDL by human aortic wall cell cocultures, and thus prevent LDL induced monocyte transmigration into the subendothelial space of the cocultures.

Methods

Materials. Tissue culture media, serum, and supplements were obtained from sources reported (7, 10). Acetylsalycilic acid, ibuprofen, dexamethasone, indomethacin, and leucinamide were purchased from Sigma Chemical Co. (St. Louis, MO). Purified monocyte chemotactic protein (MCP-1) and antibody to MCP-1 were provided by Dr. Anthony J. Valente, University of Texas (San Antonio, TX) (11). Leumedins (NPC 15199 and NPC 15669; Fig. 1) were kindly provided by Dr. Ronald M. Burch of Nova Pharmaceuticals Corp. (Baltimore, MD). Recombinant human monocyte chemotactic and activating factor (MCAF/MCP-1) and monoclonal antibody to MCAF were obtained from Peprotech (Cherry Hill, NJ).

Cocultures. HAEC, HASMC, and human peripheral blood monocytes were isolated as previously described (10). A 24-well unit previously developed and described (7) or chamber slides (Costar, Cambridge, MA) were used for the study of monocyte transmigration. The wells were treated with 0.1–0.5% gelatin (tissue culture grade, G 9391; Sigma Chemical Co.) at 37°C overnight. HASMC were seeded in the units at a confluent density of 1×10^5 cells/cm^2 and cultured for 2 d, at which time they covered the entire surface of the well and had produced a substantial amount of extracellular matrix. HAEC were subsequently seeded on top of the matrix/HASMC at 2×10^5 cells/cm^2 and allowed to grow forming a complete monolayer of confluent endothelial cells in 24 h (7, 10). For some cocultures, the HASMC surface was treated with human fibronectin before seeding the HAEC. In these instances, the cell layer was covered with a solution of fibronectin, 20 μg/ml medium 199 for 10 min, which was removed before seeding with HAEC. This treatment prevented HAEC detachment that sometimes occurs during subsequent incubations in the presence of low serum concentrations (i.e., 5% human serum). In all experiments, HAEC and autologous HASMC (from the same donor) were used at passage levels of 3–7. The growth medium contained 10% heat-inactivated pooled human serum and 10% fetal bovine serum in medium 199. Identical results were obtained with cocultures formed in the dif-

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1. *Abbreviations used in this paper:* AID, antiinflammatory drugs; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; HPF, high power field; MCAF, monocyte chemotactic activating factor; LPDS, lipoprotein-deficient serum; MCP-1, monocyte chemoattractant protein 1.

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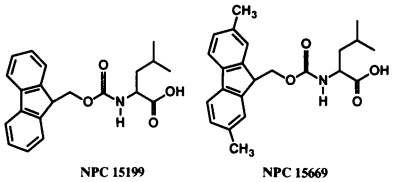

Figure 1. The chemical structure of leumedins. *N*-(fluorenyl-9-methoxycarbonyl) leucine, NPC 15199, and the methylated derivative NPC 15669.

ferent systems (i.e., polycarbonate membranes or chamber slides) and the results were independent of whether or not the HASMC surface was coated with fibronectin. Blood monocytes were obtained from individuals from a large pool of healthy donors by a modification of the Recalde procedure as previously described (12).

Lipoproteins. Very low density lipoprotein (d = 1.006–1.019 g/ml), LDL (d = 1.019–1.063 g/ml), HDL (d = 1.063–1.21 g/ml), and lipoprotein-deficient serum (LPDS) (d > 1.21 g/ml) were isolated from the sera of normal blood donors or New Zealand white rabbits by density gradient ultracentrifugation as described (13) and were used within 1–2 wk of isolation. The concentration of lipoproteins is expressed in terms of protein content throughout this report.

MCP-1 ELISA. A direct ELISA was developed and used to determine the MCP-1 levels in coculture supernatants based on a procedure reported previously (7). The monoclonal antibody to MCP-1 was used at 1:500 to 1:1,000 dilution.

Conjugated diene formation. The antioxidant effect of leumedin was examined by determining the pattern of formation of conjugated dienes during Cu^{++} catalyzed oxidation of LDL in the absence or presence of leumedin following the method of Esterbauer et al. (14) or during the modification of LDL by cocultures.

Monocyte transmigration assay. The cocultures were treated with native LDL (100–500 μg/ml), in the absence or presence of leumedin, or other antiinflammatory compounds. The culture supernatants were subsequently transferred to untreated cocultures and were incubated for an additional 24 h. In the later stages of these studies, leumedins were added in two divided doses; the first dose was included at the time of the addition of LDL and the second dose was added 12 h later. Monocytes were labeled with the fluorescent probe DiI at 4°C for 10 min (15), centrifuged, and the cell pellet was resuspended in medium 199. The labeled monocytes were either immediately used or were cryopreserved for later use. At the end of the 48 h treatment, the cocultures were washed with medium 199 at 37°C and DiI-labeled monocytes were added to cocultures in the multiwell units or chamber slides at 2.5×10^5 cells/cm^2 and were incubated for 40–60 min at 37°C. The medium used contained 0.5% heat-inactivated pooled human serum. The medium containing nonadherent leukocytes were then removed and cell layers were washed at 37°C to remove the loosely adherent monocytes on top of the endothelial cells. The cocultures on the polycarbonate membranes or on the slides were fixed with 10% neutral buffered formalin at room temperature for 24 h and were mounted with phosphate buffered glycerol. By this time, the HAEC monolayer had taken up a small amount of the fluorescent label from the monocytes above and below and had provided a detectable boundary that facilitated distinguishing the monocytes that were adhered above the HAEC from those that had migrated below the HAEC layer. The number of monocytes in the subendothelial space (beneath the HAECs) in a minimum of nine fields was determined under 50× oil objective magnification and fluorescence microscopy with a rhodamine filter set.

Association of leumedin and aspirin with LDL. LDL samples, 5 mg each, were incubated under argon with leumedin or aspirin at final concentrations of 30 or 60 μM, in 10 ml of LPDS, 4 mg/ml, for 16 h at 37°C on a rotator (30 rpm). LDL was subsequently isolated at d = 1.019–1.063 g/ml and was dialyzed extensively against 3 × 1,000 vol of phosphate buffer. The reisolated LDL preparations were studied in the coculture system for modification and induction of monocyte transmigration. Control LDL was prepared in parallel without the addition of leumedin or aspirin.

Association of leumedin with serum components. ^{3}H-leumedin 15669, with a specific activity of 140×10^3 cpm/ng, was incubated with 10 ml of normal human serum for 6–16 h at 37°C on a rotator. Lipoproteins and LPDS were subsequently isolated by ultracentrifugation, were extensively dialyzed against three changes of phosphate buffer, 4 liters each, and the radioactivity associated with each fraction was determined.

PCR. Reverse transcription of cellular RNA. In preparation for amplification of cDNA, 1 μl containing 1 μg of total cellular RNA extracted from the cells was mixed with 4 μl of reverse transcription cocktail so that the final reagent concentrations in 5 μl were 5 mM $MgCl_2$; 50 mmol/liter tris-HCl, pH 8.3; 75 mmol/liter KCl; 10 mmol/liter DTT; 0.1 g BSA/liter (Gibco-BRL, Gaithersburg, MD); 2.5 U reverse transcriptase; 5 mg oligo-dT/liter (Pharmacia-LKB, Piscataway, NJ); and 1 mmol/liter of each: dCTP, dTTP, dATP, and dGTP (Perkin-Elmer-Cetus). Samples were then overlaid with mineral oil to prevent high temperature evaporation, and the reverse transcription reaction was carried out in a thermal cycler (Eppendorf Inc., Fremont, CA) as follows: 42°C for 60 min, 99°C for 5 min, and 4°C for 5 min.

Amplification of cDNA by PCR. The PCR reaction was carried out in a final volume of 25 μl in the same tube from the reverse transcriptase reaction. Twenty μl of PCR reaction mix was added to each tube to have final concentrations of 10 mmol/liter Tris-HCl pH 8.3, 50 mmol/liter KCl, 2 mM $MgCl_2$, 1 U *Taq* polymerase, and 0.15 mM of each primer. The reaction was carried out using the following step sequence for denaturation, primer annealing and extension respectively for 35 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. The computer program OLIGO™ for the MacIntosh was used to construct optimal oligonucleotide primer pairs for MCP-1 and α-tubulin used in the PCR reaction.

For quantitation of the PCR products, radioactive oligonucleotides were prepared by end-labeling with polynucleotide kinase using [^{32}P]γATP, and each radioactive oligonucleotide was purified by centrifugation through a chromatography column (BioSpin; Bio-Rad Labs, Richmond, CA). 1×10^6 cpm of each of the primer pairs used was added along with the reaction mix. After the reaction was completed, 20 μl of each sample were applied to acrylamide gels to separate PCR products from unincorporated oligonucleotide. Acrylamide gels were fixed, dried, and scanned (Ambis Image Acquisition and Analysis; AMBIS Inc.); the AMBIS system determines the radioactive profile of the gel and digitally quantitates the radioactivity in each band. The amount of radioactivity in the PCR product for MCP-1 was divided by that in the α-tubulin PCR product to normalize the data.

In vivo association of leumedin with lipoproteins. Male New Zealand white rabbits, 2–3 kg body wt, were injected with leumedin 15669 (20 mg/kg body wt). Briefly, leumedin was dissolved in 500 μl of dimethylsulfoxide and was injected into the ear vein, followed by injection of 2 ml of warm saline. Control rabbits were injected with dimethylsulfoxide vehicle and saline. LDL and HDL were isolated from the blood of rabbits obtained 24–36 h after leumedin administration and were tested in the coculture system.

Other procedures. Measurement of cell protein content and those of lipoproteins was performed by the method of Lowry et al. (16) and determination of cell number was carried out by standard procedures (17).

Results

Cocultures of HAEC and HASMC were incubated in the presence of 5–10% pooled human serum and some were supplemented with 100–500 μg/ml of LDL in the absence or presence of leumedins at different concentrations. After 24 h, the culture supernatant was removed and was incubated with untreated cocultures for an additional 24 h. The medium was then removed and human monocytes were added to the cocultures and their transmigration into the subendothelial space was determined. As shown in Fig. 2 *A*, incubation of LDL with cocultures, induced a 5.7-fold increase in monocyte migration. This effect was comparable to that induced by pure MCP-1

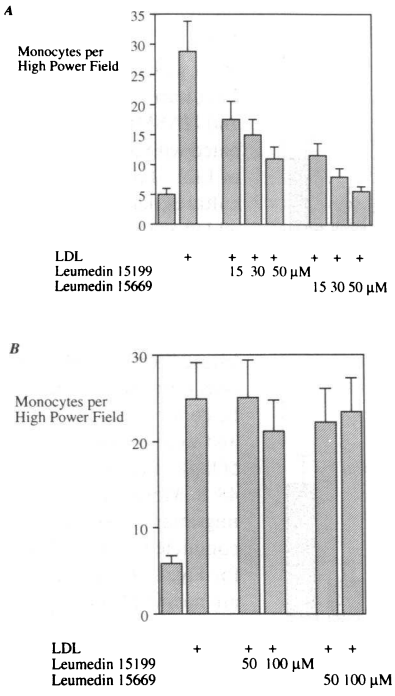

Figure 2. (*A*) Effect of leumedins on monocyte transmigration. Cocultures of HAEC and HASMC on polycarbonate membranes or in chamber slides were incubated with 100–500 μg/ml of LDL in the presence of 5–10% serum. Other cocultures contained LDL and leumedin 15199 or 15669 at various concentrations. After 24 h, the supernatants were transferred to fresh cocultures and were incubated for an additional 24 h. The culture medium was then removed, the cell layers were washed at 37°C and 150 or 500 μl (for chamber slides and 24-well units, respectively) of a suspension of fluorescently labeled human monocytes was added at 2.5×10^5 cells/cm^2. After 45 min at 37°C, the media were removed, the cocultures were washed at 37°C and were fixed with 10% neutral buffered formalin for 24 h. The membranes or slides were mounted and the subendothelial monocytes were enumerated under a total magnification of 625. The values are mean±SD of monocytes from 36 fields in quadruple cocultures. This figure is representative of three to seven separate experiments. (*B*) The effect of leumedins on cell-modified LDL. After 24 h of incubation with cocultures, LDL was reisolated from the conditioned medium by density gradient centrifugation and was exposed to fresh target cocultures at 250 μg LDL/ml for 24 h (*LDL*). Other target cocultures received leumedin 15199 or 15669 in addition to the cell-modified LDL, designated as *LDL + Leumedin 15199* and *LDL + Leumedin 15669*, respectively. Monocyte migration was evaluated as described for *A*. The values are mean±SD of number of monocytes in 36 fields in triplicate cocultures in each treatment in three separate experiments.

incubated with cocultures 2–4 h before the monocyte migration assay and with that induced by the potent chemoattractant FMLP (data not shown). Treatment of the cocultures with an antibody to MCP-1 for 2–4 h inhibited the LDL induced monocyte migration by 89% (data not shown). Inclusion of HDL (250 μg/ml) along with LDL in the cocultures inhibited the induced increase in monocyte migration by 92% (data not shown). The presence of leumedin 15669 at 15–50 μM reduced the induced monocyte migration by 72–98%. Incubation in the presence of leumedin 15199 at 15–50 μM was less effective in inhibiting the induced monocyte migration reducing it to 47–75% of the control value (compare *LDL + Leumedin 15199* and *LDL + Leumedin 15669* with the *LDL* alone). Similar results were obtained when the coculture supernatants were tested for their activity in an assay based on transmigration of monocytes across a polycarbonate filter (10) (data not shown). Addition of leumedins did not affect monocyte transmigration itself since the inclusion of these compounds in cocultures containing added FMLP, purified MCP-1, or recombinant human monocyte chemotactic and activating factor/MCP-1 did not reduce the number of monocytes that migrated into the subendothelial space (data not shown).

In other experiments, native LDL at 500 μg/ml was incubated with cocultures for 24 h and LDL was reisolated from the resulting conditioned medium by density gradient ultracentrifugation. This coculture treated LDL was subsequently incubated (at 250 μg LDL/ml) with new cocultures in 5% human serum for 24 h followed by monocyte migration assay. As seen in Fig. 2 *B*, there was a 4.3-fold increase in the number of monocytes that migrated into the subendothelial space of cocultures incubated with this cell-modified LDL. Coincubation of the reisolated LDL with leumedin 15669 or 15199 at concentrations as high as 100 μM did not prevent the LDL induced monocyte migration (Fig. 2 *B*). Similar results were obtained when LDL was incubated with cocultures and the resulting conditioned medium, without reisolating its LDL, was transferred to and incubated with fresh cocultures (data not shown).

LDL modification by cocultures resulted in 170% and 173% increases in the mRNA and protein levels for MCP-1, respectively (Fig. 3, *A* and *B*). Inclusion of leumedin 15669 together with LDL prevented the LDL-induced increases in the levels of MCP-1 message and the MCP-1 protein by 84% and 87%, respectively (Fig. 3, *A* and *B*).

To investigate the potential antioxidant activity of leumedin, the rate and pattern of formation of conjugated dienes was determined in samples of LDL from two different subjects during modification in cocultures using cells from three different donors or by Cu^{++} catalyzed oxidation. Presence of leumedin

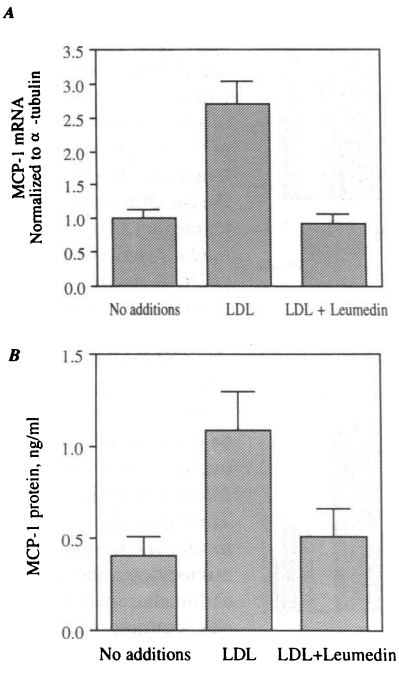

Figure 3. (*A*) The effect of leumedin on LDL induced MCP-1 gene expression. HAEC were plated in 60-mm dishes and cultured for 2 d in M199 containing 10% FBS. HAEC were subsequently seeded on HASMC and were allowed to grow for 1 d. The medium was then changed to one containing LDL at 500 μg/ml in the presence or absence of leumedin, and incubation was continued for an additional 16 h. The media were then transferred to new sets of cocultures and were incubated for 8 h. Total RNA was extracted and reverse transcribed for DNA amplification and analysis by PCR using primer pairs for MCP-1 and for α-tubulin as an internal standard. Values shown are the mean±SD from triplicate determinations. (*B*) The effect of leumedin on MCP-1 protein levels. Cocultures of HAEC and HASMC were incubated with LDL at 500 μg/ml with or without leumedin at 50 μM. After 24 h, the cultures were washed with M199 and were incubated in serum-free medium for 16 h. The supernatants were then analyzed in an ELISA for levels of MCP-1 protein. The values are mean±SD of triplicate cocultures in two separate experiments.

15669 together with LDL did not effect conjugated diene formation. Neither the duration of the lag phase, the slope of the propagation curve, nor the maximum level of conjugated dienes were significantly altered by presence of leumedin 15669 (data not shown). These results indicated that unlike α-tocopherol, butylated hydroxytoluene and probucol (18), leumedin is not a potent general antioxidant agent.

The antiinflammatory drugs (AID), aspirin, dexamethasone, ibuprofen, and indomethacin were also tested for their effects on LDL modification by the cells of the cocultures. At concentrations of 30 μM or less, AID by themselves did not have a significant effect on the induced monocyte transmigration (data not shown). The dose response for the effect of aspirin on LDL induced monocyte migration is shown in Fig. 4 *A*. Leumedin 15669 produced a significant 55% inhibition at 15 μM (compare *LDL* with *LDL + Leumedin 15669, 15 μM*). Significant inhibition of LDL modification with aspirin, however, was achieved only at concentrations of 50 μM or higher (compare *LDL* with *LDL + Aspirin, 50 μM*). Leucinamide at a concentration of 100 μM (or higher) did not have any effect on LDL induced monocyte migration. As seen in Fig. 4 *B*, simultaneous addition of leumedin and AID to the cocultures produced an additive or even synergistic inhibitory effect on the modification of LDL and the subsequent LDL induced monocyte migration. At low concentrations of the compounds, the presence of indomethacin together with leumedin was the most effective in preventing LDL modification (compare *LDL + Leumedin + Indomethacin*, with *LDL + Leumedin*, and with *LDL + Indomethacin*). It should be emphasized that in the experiments described in Fig. 4, the compounds were added to the cocultures simultaneously with the LDL.

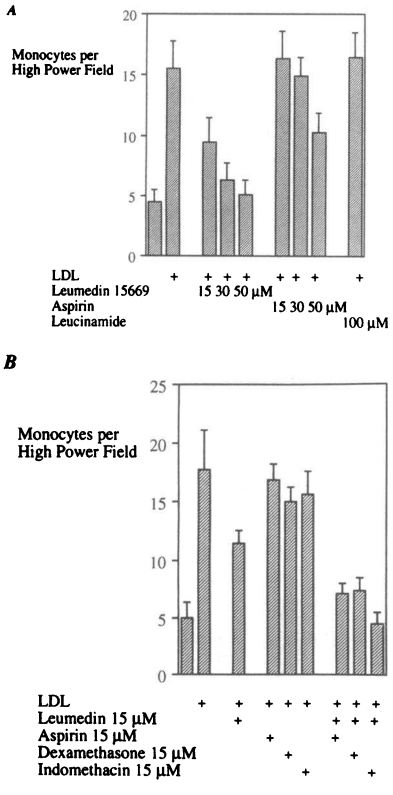

Figure 4. The effect of AID on LDL modification. (*A*) Dose-response. Cocultures of HAEC and HASMC were incubated with 500 μg/ml of LDL in the presence of 5–10% serum (designated as LDL). Some of the cocultures contained leumedin (*LDL + Leumedin 15669*) or aspirin (*LDL + Aspirin*) at various concentrations. Additional cocultures received leucinamide at 100 μM (*LDL + Leucinamide*). After 48 h, monocyte transmigration was tested as described for Fig. 2 *A*. The values are mean±SD of number of monocytes in the subendothelial space of 12–48 cocultures in two to six separate experiments. (*B*) Effect of combinations of leumedin and AID. Cocultures of HAEC and HASMC were incubated with 500 μg/ml of LDL in the presence of 5–10% serum. Some cocultures received in addition to LDL, leumedin 15669, aspirin, dexamethasone, or indomethacin, separately or in combination. After 48 h, monocyte transmigration assay was conducted as described for Fig. 2 *A*. The values are mean±SD of number of subendothelial monocytes in 27 high power fields in triplicate cocultures in three separate experiments.

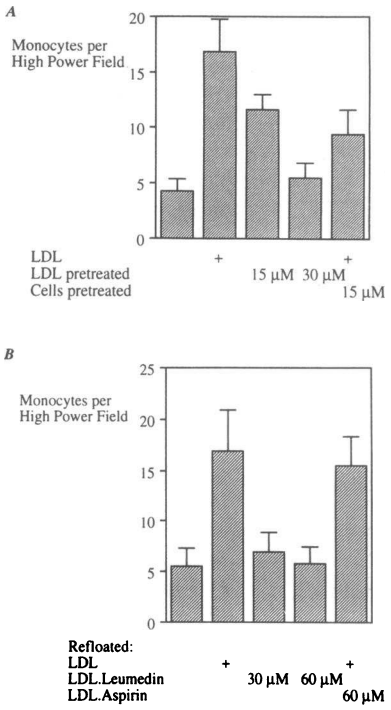

Figure 5. (*A*) Pretreatment with leumedins. Cocultures of HAEC and HASMC were incubated with 500 μg/ml of LDL. For some cocultures, leumedin was added at 15 or 30 μM together with LDL. In other cocultures, the cells were preincubated at 37°C for 16 h with leumedin at 15 μM. The cells were then washed twice for 1 h at 37°C and were incubated with 500 μg/ml of LDL for 48 h. Monocyte transmigration assay was conducted as described for Fig. 2 *A*. The values are mean±SD of number of subendothelial monocytes in 27 high power fields in triplicate cocultures in three separate experiments. (*B*) Association of leumedin and aspirin with LDL. LDL samples, 5 mg each, were incubated for 16 h at 37°C, with leumedin or aspirin at final concentrations of 30 or 60 μM, in 10 ml of LPDS at 4 mg/ml on a rotator. LDL preparations were subsequently isolated by ultracentrifugation at d = 1.019–1.063 and were extensively dialyzed. The reisolated LDL preparations were added to cocultures at 500 μg/ml of LDL and were incubated for 48 h at 37°C. Control LDL preparations, without the addition of leumedin or aspirin, were treated and reisolated in parallel (designated as LDL). Monocyte migration was examined as described for Fig. 2 *A*. The values are mean±SD of number of subendothelial monocytes in 36 high power fields in triplicate cocultures in four separate experiments.

Preincubation of LDL with leumedins at 30 μM for 5 h under argon inhibited the induced monocyte migration by 90% (Fig. 5 *A*). Preincubation of the cocultures for 16 h with leumedins at 15 μM prevented the LDL induced monocyte migration by 58% (Fig. 5 *A*).

To examine the degree of association and the stability of leumedin or aspirin with LDL, samples of LDL from several donors were incubated with or without leumedin or aspirin under argon for 6–16 h at 37°C on a rotator. The LDL preparations were reisolated and used in cocultures for the studies of LDL modification and induction of monocyte migration. As shown in Fig. 5 *B*, preincubation of LDL with leumedin 15669 at 30 or 60 μM prevented the induced monocyte migration by 88–97% (compare *LDL.Leumedin* with *LDL*). Preincubation of LDL with aspirin, however, had no significant effect on the induced monocyte migration (compare *LDL.Aspirin* with *LDL*).

To examine the degree of association of leumedin with serum lipoproteins, freshly isolated human serum from four

different normal donors was incubated with ^{3}H-leumedin 15669. Subsequent isolation of the lipoprotein density classes demonstrated that ^{3}H-leumedin associated with lipoproteins to a degree that was 35-fold higher than that with the nonlipoprotein fraction (Fig. 6, *A*, compare *ALL Lipoproteins* with *LPDS*). Distribution among very low density lipoprotein, LDL, and HDL was similar (Fig. 6 *A*).

To investigate the in vivo incorporation and association of leumedin with LDL and HDL, rabbits were injected with leumedin and subsequently the rabbit LDL and HDL were isolated and tested in the human artery wall cell cocultures. LDL isolated from control rabbits induced a 3.5-fold increase in monocyte migration (Fig. 6 *B*). LDL isolated from rabbits injected with leumedin 15669 at 20 mg/kg body wt, however, was almost completely resistant to LDL modification and did not induce monocyte migration (Fig. 6 *B*). To examine the protective effect of HDL against LDL modification, human LDL was used, which is more readily obtainable. HDL isolated from control rabbits prevented modification of human LDL in cocultures to a modest degree and reduced the increase in monocyte migration only by 38% (Fig. 6 *B*). HDL from leumedin-injected rabbits, however, completely protected against LDL modification and hence prevented an increase in monocyte migration (Fig. 6 *B*).

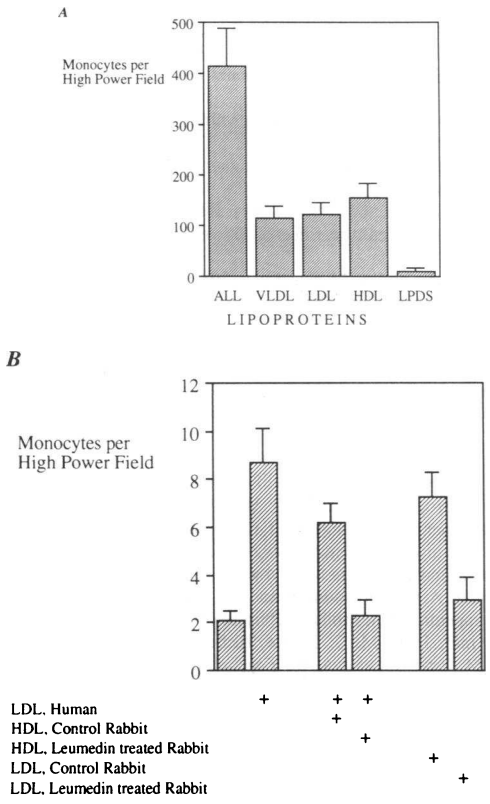

Figure 6. (*A*) Association of leumedin with serum lipoproteins. ^{3}H-leumedin 15669, specific activity = 140×10^3 cpm/ng, was incubated with 10 ml of freshly prepared human serum for 6–16 h at 37°C on a rotator. Lipoprotein classes and LPDS were isolated in an ultracentrifuge, were extensively dialyzed against phosphate buffer and the radioactivity associated with each fraction was determined. The values are the mean±SD of four determinations in three separate experiments using sera from four different normal donors. (*B*) Effect of injected leumedin on LDL and HDL in rabbits. Six New Zealand white rabbits were injected intravenously with leumedin 15669 (20 mg/kg body wt). Five additional rabbits (control) received the vehicle and saline. Blood was collected 24–36 h later, and LDL and HDL were isolated and were examined in monocyte migration assay in cocultures. The values are the mean±SD of 27 high power fields in quadruple cocultures in two separate experiments.

Discussion

Leumedins appear to be very effective in preventing human artery wall cells from modifying LDL to a biologically active molecule (Figs. 2, 3, 4, 5, and 6 *B*). The effect of leumedins appeared to be confined to the events that occurred during the initial steps of LDL interaction with the artery wall cells, since leumedins did not prevent the induction of monocyte transmigration brought about by LDL that had previously been modified by a first set of cocultures and subsequently tested in a second set of untreated cocultures (Fig. 2 *B*). Fig. 3 demonstrates that the LDL-induced increase in MCP-1 message and protein is completely blocked by leumedin. Based on our previous findings (7), it appears likely that LDL is modified in the microenvironments formed by the extracellular matrix components produced by the interaction of HAEC and HASMC (19–21). Antioxidants present in the serum are apparently excluded from this space. The coculture modified LDL then induces MCP-1 production by HAEC and HASMC (Fig. 3 and reference 7), which results in the establishment of a chemotactic gradient across the endothelial monolayer in the coculture (7). The mild degree of modification produced in the coculture system is presumably a result of the action of prooxidants in the microenvironments largely sequestered from the effect of antioxidants present in the serum in the coculture (2, 7, 21). The mechanism of LDL modification in the artery wall is not known. The modification might result from the release of superoxide anions from the artery wall cells, the action of membrane bound enzymes on LDL, and/or the transfer of cellular lipid peroxides to LDL (2, 22, 23). The loss of antioxidants such as α-tocopherol and the peroxidation of polyunsaturated fatty acids in the LDL lipids appears to be the initiation step in the modification of LDL (23). Preincubation of the cells with leumedins before the incubation with LDL significantly inhibited LDL modification (Fig. 5 *A*). This may suggest that the artery wall cells in culture were capable of storing sufficient quantities of leumedins to prevent the subsequent release of reactive oxygen species or to inhibit the production of specific oxidized cellular lipids that have been proposed as potential contributors to the initiation of lipid oxidation in LDL (2, 23). While not as potent, inhibitors of the cyclooxygenase pathway (aspirin and indomethacin) and the antiinflammatory agent dexamethasone when added to the cocultures partially prevented LDL modification (Fig. 4 *B*). We conclude that this effect was due in part to the ability of these compounds to partially prevent the cellular generation of reactive oxygen species. In this regard it should be noted that leumedin in combination with indomethacin was the most potent in preventing LDL modification by the coculture (Fig. 4 *B*). Injection of leumedin into rabbits or in vitro incubation of LDL with leumedin produced a highly stable complex that withstood the high salt concentration and high centrifugal force applied during the isolation of the LDL in the ultracentrifuge. The nature of the association of leumedin and LDL could not be determined from the present study nor do the experiments pre-

sented here allow us to deduce with certainty the mechanism(s) by which leumedins prevent LDL modification. The failure of leumedin to protect against the formation of conjugated dienes in LDL by either the coculture or metal ion oxidation strongly suggests that the action of leumedin is not that of a general antioxidant. Regardless of this, the leumedins are the only known compounds other than probucol that we have studied that associate intimately with LDL and prevent its oxidative modification. Aspirin at concentrations that are pharmacologically achievable in plasma in vivo, namely 20–30 μM, was not able to render LDL resistant to modification by the coculture and could not inhibit the resulting monocyte migration. It is well established that aspirin is rapidly metabolized in the plasma and only a small quantity of aspirin binds to albumin (24). The complete protection of human LDL produced by in vitro preincubation of LDL with leumedins and the complete resistance of LDL isolated from leumedin injected rabbits to oxidative modification together with, complete protection against LDL modification provided by HDL from these rabbits demonstrates the remarkable association of leumedin with lipoproteins and may have significant pharmacological implications. If LDL modification is, as it appears to be (1, 2, 25–30), a key event in the initiation of the inflammatory reaction in atherogenesis, the association of leumedins with LDL and the resultant inhibitory effect on LDL modification observed in the present study may prove to be an effective means of preventing or reducing this modification and the resulting monocyte localization in the subendothelium. Studies in laboratory animals are underway to explore this question.

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