CC Chemokine I-309 Is the Principal Monocyte Chemoattractant Induced by Apolipoprotein(a) in Human Vascular Endothelial Cells

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Background—Lipoprotein(a) [Lp(a)] is a risk factor for atherosclerosis; however, the mechanisms are unclear. We previously reported that Lp(a) stimulated human vascular endothelial cells to produce monocyte chemotactic activity. The apolipoprotein(a) [apo(a)] portion of Lp(a) was the active moiety.

Methods and Results—We now describe the identification of the chemotactic activity as being due to the CC chemokine I-309. The carboxy-terminal domain of apo(a) containing 6 type-4 kringles (types 5 to 10), kringle V, and the protease domain was demonstrated to contain the I-309–inducing portion. Polyclonal and monoclonal anti–I-309 antibodies as well as an antibody against a portion of the extracellular domain of CCR8, the I-309 receptor, inhibited the increase in monocyte chemotactic activity induced by apo(a). I-309 antisense oligonucleotides also inhibited the induction of endothelial monocyte chemotactic activity by apo(a). I-309 mRNA was identified in human umbilical vein endothelial cells. Apo(a) induced an increase in I-309 protein in the endothelial cytoplasm and in the conditioned medium. Immunohistochemical studies have identified I-309 in endothelium, macrophages, and extracellular areas of human atherosclerotic plaques and have found that I-309 colocalized with apo(a).

Conclusions—These data establish that I-309 is responsible for the monocyte chemotactic activity induced in human umbilical vein endothelial cells by Lp(a). The identification of the endothelial cell as a source for I-309 suggests that this chemokine may participate in vessel wall biology. Our data also suggest that I-309 may play a role in mediating the effects of Lp(a) in atherosclerosis. (Circulation. 2000;102:786-792.)

Key Words: lipoproteins ■ atherosclerosis ■ peptides

Most epidemiological and genetic studies have shown significant correlation between lipoprotein(a) [Lp(a)] blood levels and arteriosclerotic vascular disease and have established that Lp(a) is an independent risk factor for atherosclerosis. Lp(a) is composed of an LDL particle that is linked to apolipoprotein(a) [apo(a)], a glycoprotein of variable size that shares partial homology with plasminogen. The mechanisms by which Lp(a) participates in atherosclerosis are not clear. Because attraction of circulating monocytes to the vessel wall has been shown to occur throughout the development of the atherosclerotic plaque, our prior study examined whether Lp(a) could stimulate vascular endothelial cells to produce monocyte chemotactic activity (MCA). We found that Lp(a) stimulated human vascular endothelial cells, in a process requiring de novo transcription and protein synthesis, to produce MCA. The apo(a) portion of Lp(a) was identified as the structure associated with Lp(a) that served as the endothelial agonist. The monocyte chemoattractant, however, was not identified. The MCA was not due to monocyte chemoattractant protein-1 (MCP-1), shown to be induced in endothelial cells by oxidized LDL.

In the present study, we report that I-309, a CC chemokine previously reported to be produced by T lymphocytes and stimulated monocytes, is produced by endothelial cells and is responsible for the MCA induced by Lp(a).

Methods

Antibodies
The following were from were from R&D Systems, Inc: recombinant I-309; blocking polyclonal antibodies against granulocyte-macrophage colony–stimulating factor, interleukin-8, RANTES, I-309, and monocyte inflammatory protein-1α; and an anti–I-309 murine monoclonal antibody. Antibody against a peptide corresponding to the N-terminal extracellular domain sequence of human of CCR8 was from Alexis Biochemicals. The following antibodies were used in

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immunohistochemical studies: rabbit anti-human apo(a) (prepared as described\(^\text{10}\)), rabbit anti-human von Willebrand factor (vWF; A0082, Dako), monoclonal anti-human CD-68 pannumrophage antibody (KP-1, M814, Dako), and monoclonal antibody to smooth muscle \(\alpha\)-actin (1A4, Dako).

**Purification of Lp(a), Apo(a), and Recombinant Apo(a) Derivatives**

Lp(a) and apo(a) were prepared as detailed previously.\(^\text{5,11}\) Lys-plasminogen was from Immuno AG. Two recombinant apo(a) derivatives were assembled as detailed.\(^\text{12}\) The construction and expression of (1) pRK5ha17, which encoded a 17-kringle (17K) apo(a) containing type-4 kringles 1 to 10, with 8 type-2 repeats, kringle V, and the protease domain, and (2) pRK5ha5-p, which encoded the 6-kringle (6K) apo(a) containing type-4 kringles 5 to 10, kringle V, and the protease domain were as previously described.\(^\text{13,14}\) Their purification was performed as detailed.\(^\text{15}\)

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were harvested and cultured as described.\(^\text{16}\) For MCA assays, cells from passages 2 to 5 were grown in 6-well plates. All cell incubation mixtures contained 20 \(\mu\)g/mL polymyxin B (Sigma Chemical Co) to inhibit endotoxin.\(^\text{8}\) Aliquots of incubation mixtures were removed at the indicated time points and stored at \(-80^\circ\text{C}\) for further analysis.

**Monocyte Chemotactic Assay**

Monocyte Chemotactic Assay (MCA) was measured as described\(^\text{4}\) with the use of a NeuroProbe 5-\(\mu\)m (pore size) chamber (Nucleopore Corp). To determine the effect of antibodies against various human cytokines, chemokines, or CCR8 on the MCA, 0.2 mL of the conditioned medium (CM) was incubated with the IgG fraction of the respective antisera for 30 minutes at 37\(^\circ\text{C}\) (1 \(\mu\)g IgG/mL) and then tested for MCA. All samples were tested in triplicate.

**Synthetic Antisense Oligonucleotides**

The following phosphorothioated oligonucleotides were obtained from GeneLink Inc: AS1, an 18-mer flanking the initiation ATG codon of the I-309 cDNA (5'-GATGATGCTGACATTTCC-3'); AS2, an 18-mer flanking the sequence 13 bases downstream from the end of AS1 (5'-CACGACTGAGCCAGCAACA-3'); SC1, a scrambled AS1 sequence (5'-ACCACATTGTGTTATGCG-3'); SC2, a scrambled AS2 sequence (5'-ACACGATGCAGACAGAC-3'); and MCP-1 AS, an MCP-1 antisense oligonucleotide (5'-CGGCAGAGACTTTGATGC-3').\(^\text{17}\) To test the effect of the anti-sense oligonucleotides, HUVECs were preincubated with 30 \(\mu\)mol/L each of the various oligonucleotides in medium 199 (M199). After 6 hours of incubation with 6K in medium containing the various oligonucleotides, the CM was tested for MCA.

**Northern Blot Analysis**

RNA was prepared by use of the Qiagen Kit (Qiagen GmbH), and blot hybridization was performed as previously described.\(^\text{5,18}\) With the full-length I-309 cDNA kindly provided by Dr Michael S. Krangel, Duke University Medical Center, Durham, NC. As a loading control, filters were hybridized with cDNA encoding GAPDH. To quantify levels of mRNA, the bands were scanned and analyzed by NIH IMAGE (Version 1.57).

**Western Blot Analysis**

HUVECs grown to confluence in Petri dishes were incubated with M199 or with 6K apo(a) (20 \(\mu\)g/mL) for 6 hours. The CM was absorbed on heparin-Sepharose beads (Pharmacia) and eluted by boiling for 5 minutes in 2\% SDS. The samples were electrophoresed (8\% to 20\% linear gradient SDS-PAGE), then electrotransferred to a nylon membrane, and incubated with goat anti-I-309 followed by rabbit anti-goat IgG horseradish peroxidase (R&D Systems). The blot was developed by chemiluminescence.

**Results**

**Induction of MCA by Recombinant Apo(a) Derivatives**

HUVECs grown to confluence were incubated with 120 nmol/L 6K apo(a), 17K apo(a), or lys-plasminogen for 6 hours, and the CM was tested for MCA (Figure 1). The MCA induced by M199 incubated with HUVECs was subtracted from the MCA induced by the CM of HUVECs incubated with the various agonists. The 6K apo(a) stimulated the HUVECs to produce \(\sim46\%\) more MCA than did the 17K apo(a) (\(P<0.001\)). The native apo(a) and the 17K apo(a) induced similar amounts of MCA. Lys-plasminogen and other members of the kringle protein superfamily, including tissue plasminogen activator and human prothrombin (data not shown), failed to stimulate MCA when incubated with HUVECs. The apo(a) proteins were also added directly to the

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**Figure 1.** Recombinant apo(a) derivatives induce HUVECs to produce MCA. HUVECs were incubated with 6K apo(a), 17K apo(a), apo(a) purified from Lp(a), and lys-plasminogen (Pig) (120 nmol/L each). Apo(a) and 6K and 17K apo(a) were also added to CM harvested after incubation of M199 with HUVECs, and mixtures were tested for MCA. Background MCA of HUVECs incubated with medium (58.3±13.8 monocytes per high-power field [hpf]) was subtracted from activity in incubation mixtures containing various added proteins. All samples were tested for MCA in triplicate wells. Three hfps were counted for each well. Standard deviations are shown. \(P<0.001\) vs 17K apo(a). Inset, SDS-PAGE of apo(a), 17K apo(a), and 6K apo(a). Lines left of gel indicate location of molecular mass standards at 205, 120, and 87 kDa, respectively.

**Immunohistochemistry**

Tissue specimens were processed as previously described\(^\text{10}\) and immunostained for I-309, vWF, apo(a), CD-68, and \(\alpha\)-actin. HUVECs grown on fibronectin-coated plastic slides were incubated with 120 nmol/L 6K apo(a) for 6 hours at 37\(^\circ\text{C}\) and stained for I-309 and vWF. Positive control, nonimmune negative, and processing control slides were prepared for each antigen stain. Preabsorption of the apo(a) antibody yielded negative staining. Absorption of the I-309 antibody with recombinant I-309 removed all of the staining activity in atherosclerotic plaque tissue sections.

**Statistical Analysis**

For studies involving the inhibitory effect of antibodies or antisense oligonucleotides on MCA, 2-way ANOVA was used. The Bonferroni correction was used to guard against multiple findings of significance due to the use of multiple antibodies. To compare the MCA of various kringle proteins, 1-way ANOVA followed by the Bonferroni correction was used.
CM collected from HUVECs incubated in M199 to test whether these proteins might act synergistically with the constitutive activity to increase MCA. As shown in Figure 1, these mixtures did not stimulate additional MCA, thus confirming that the exposure of HUVECs to apo(a) or to its recombinant derivatives was necessary for increased MCA.

Antibody Against I-309 Inhibits MCA Induced in HUVECs by 6K Apo(a)

Blocking antibodies against several cytokines were used to identify the MCA induced in HUVECs by apo(a). HUVECs were incubated with M199 or with 6K apo(a) for 6 hours. The antibody against I-309 completely inhibited the increase in MCA produced by 6K apo(a) (*P < 0.001), whereas antibodies against granulocyte-macrophage colony-stimulating factor, interleukin-8, RANTES, MCP-1, and monocyte inflammatory protein-1α (MIP-1α) did not (Figure 2).

A monoclonal antibody against human recombinant I-309 was also tested for its capacity to inhibit the MCA of recombinant I-309 and HUVEC-conditioned medium (Figure 3). Anti-I-309 monoclonal antibody inhibited both the recombinant I-309 and the 6K apo(a)–conditioned medium equivalently.

I-309 Antisense Oligonucleotides Inhibit MCA Induced in HUVECs by 6K Apo(a)

To provide further evidence that the MCA secreted by Lp(a)-stimulated HUVECs was largely attributable to I-309, the endothelial cells were incubated with antisense oligonucleotides to I-309 mRNA. As shown in Figure 4, an antisense oligonucleotide (AS1), flanking the initiation ATG codon, abolished the increase in MCA but did not inhibit the constitutive MCA (data not shown). A second antisense oligonucleotide (AS2), beginning 13 bases downstream from the end of AS1, was equally effective in inhibiting the increase in MCA induced by 6K apo(a) but had no effect on the constitutive MCA (data not shown). Scrambled oligonucleotides (SC1 and SC2), based on AS1 and AS2 sequences, were ineffective in inhibiting the increase in MCA. In addition, an antisense oligonucleotide for the monocyte chemoattractant MCP-1 did not inhibit the apo(a)-stimulated increase in MCA. These data suggest that the chemotactic activity induced in HUVECs by apo(a) is derived primarily from the de novo synthesis of I-309.
Antibody Against N-Terminal Domain of CCR8 Inhibits MCA Induced in HUVECs by Lp(a)

To define whether the I-309 receptor, CCR8, was involved in the chemotactic response of monocytes to Lp(a)-conditioned medium, an antibody against the N-terminal extracellular domain of CCR8, I-309 receptor (+CCR8), or normal goat IgG (+IgG) was added to CM before testing for MCA. Recombinant I-309 (100 ng/mL) was also tested under identical conditions (I-309). Background activity of HUVECs (40.2 ± 7.8 monocytes/hpf) was subtracted from that obtained after stimulation with Lp(a). In the case of the effect of CCR8 antibody on migration induced by I-309, monocyte migration induced by M199 alone (7.8 ± 2.8 monocytes/hpf) was subtracted. Standard deviations are shown. *P<0.002; **P<0.01.

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I-309 mRNA Is Expressed in HUVECs

RNA Northern blot analysis of I-309 mRNA in HUVECs identified I-309 mRNA in Jurkat cells and in 6K apo(a)–stimulated and unstimulated cells (Figure 6). We identified 2 transcripts, a major 2.4-kb and a minor 0.55-kb transcript, as has been previously reported in stimulated lymphocytes. Densitometric scanning with normalization by use of the signal produced by GAPDH showed a 2-fold increase in I-309 mRNA after 6K apo(a) stimulation. Reverse transcriptase–polymerase chain reaction amplification of RNA isolated from Lp(a)-stimulated HUVECs generated the expected 290-bp fragment with primers specific for I-309 mRNA (data not shown). The identity of the 290-bp fragment as I-309 mRNA was confirmed by sequence analysis (GenBank Accession No. M57506). No sequences were identified in the absence of reverse transcriptase or in the presence of DNase.

Identification of I-309 Protein in CM of HUVECs by Western Blot Analysis

I-309 was identified in the medium of unstimulated and 6K apo(a)–stimulated HUVECs by Western blot analysis; however, the amount of I-309 was increased after stimulation with 6K apo(a) (Figure 7). Densitometric analysis of serial dilutions of both CM indicated a 4-fold increase in I-309 antigen in the 6K apo(a)–stimulated CM (data not shown).

HUVECs Express I-309 in Their Cytoplasm, and Amount Is Increased by 6K Apo(a)

To confirm the presence of I-309 antigen in endothelial cells, HUVECs were grown to confluence on plastic slides and treated for 6 hours either with M199 or with 6K apo(a). Compared with the untreated control cells (Figure 8A), the cells exposed to 6K apo(a) showed increased staining for I-309 (Figure 8B). The cells incubated in the absence of specific anti-I-309 did not stain (Figure 8C). The immunochemical stain for vWF antigen (Figure 8D) was strongly positive, indicating that the cells containing I-309 were all of endothelial origin.
Human Coronary and Carotid Atherosclerotic Plaques Contain I-309, Which Colocalizes With Apo(a)

Human carotid and coronary endarterectomy specimens stained positively for both apo(a) and I-309 (Figure 9). In general, there was colocalization of apo(a) and I-309 (Figure 9A and 9B) within large plaque areas in the fibrotic cap and necrotic core. Endothelial cells, identified by their staining for vWF antigen, stain positively for I-309 (Figure 9C). No Lp(a) was identified in association with this endo-

Figure 8. Photomicrographs of immunohistochemically stained HUVECs showing increase in I-309 in cytoplasm with 6K apo(a) stimulation. A and B, Cells incubated in M199 show some staining for I-309 (A) in contrast to cells incubated with 6K apo(a), which show intense I-309 staining (B). C, Cells were stained with an irrelevant antibody. D, Positive granular staining of HUVECs with vWF antibody is shown. Magnification ×100 (diaminobenzidine-peroxidase and hematoxylin counterstain)

Figure 9. Photomicrographs of immunohistochemically stained human atherosclerotic plaques. Low-magnification comparison of I-309–stained and apo(a)–stained sequential sections of coronary endarterectomy specimen shows general colocalization of their staining (A and B). fc indicates fibrous cap; nc, necrotic core; and m, media. Endothelial cells in the coronary lesion, identified by their staining for vWF antigen, stain positively for I-309 (C; arrow). No Lp(a) is observed in serial section of endothelial surface (D, arrow.) Macrophage-rich (KP-1 positive, not shown) region from carotid endarterectomy shows intense I-309 staining of macrophage cytoplasm (E, arrow) and intense staining for apo(a) in the immediately surrounding extracellular spaces (F, arrow). In acellular fibrotic regions of the plaque, I-309 and apo(a) show colocalization (G and H, arrows). Magnifications ×12.5 (A and B) and ×200 (C through H) (diaminobenzidine-peroxidase, hematoxylin counterstain).
thelium (Figure 9D). Macrophages were often surrounded by extracellular apo(a) staining (Figure 9F), whereas I-309 staining was mainly in the cytoplasm (Figure 9E). In areas of acellular matrix, apo(a), and I-309, the staining coincided exactly (Figure 9G and 9H).

**Discussion**

We have reported that the apo(a) portion of Lp(a) induced human vascular endothelial cells to produce MCA.5 We now show that this activity is due to the CC chemokine, I-309. Both polyclonal and monoclonal antibodies against I-309 were found to strongly inhibit the MCA induced in HUVECs by apo(a). Two different antisense oligonucleotides against I-309 inhibited the endothelial production of MCA, whereas scrambled antisense oligonucleotides and an antisense oligonucleotide against MCP-1 were ineffective. In addition, an antibody against the N-terminal extracellular domain of CCR8, the recently described I-309 receptor,8,9 strongly blocked the monocyte chemotactic response to apo(a)-HUVEC–conditioned medium. These results argue that I-309 is the predominant chemokine induced in endothelial cells by Lp(a).

This is the first report demonstrating that I-309 is expressed in endothelial cells. We have identified I-309 mRNA by Northern blot analysis and have confirmed by sequence analysis that I-309 message is present in HUVECs. I-309 was induced in HUVECs, as documented by immunohistochemical staining and by Western blot analysis, inasmuch as 6K apo(a) induced a 4-fold increase in I-309 in the CM. I-309 was also detected in the CM from unstimulated HUVECs. In view of our observations that anti-I-309 antibody or I-309 antisense oligonucleotides failed to inhibit the constitutive expression of I-309 message in HUVECs, it is likely that insufficient I-309 was produced by the unstimulated HUVECs to stimulate monocyte chemotaxis.

Chemokines are low molecular weight proteins that attract leukocytes.20 Chemokines are classified into 2 major families that are distinguished by the positions of the first 2 of 4 conserved cysteines (CXC and CC). I-309 is a CC chemokine that is secreted by activated T lymphocytes19 and monocytes.21 I-309 stimulates human monocyte and Th2 lymphocyte chemotaxis.22,23 CCR8 has been identified as the receptor for I-309/TCA-3 and is expressed on monocytes and Th2 lymphocytes in both mouse and human cells.24,25 CCR8 serves as a coreceptor for diverse HIV-1 strains, and I-309 was found to be an inhibitor of HIV-1 envelope–mediated cell-cell fusion and virus infection.8

Attention has focused on MCP-1, a member of the CC chemokine family, because cytokines26 and minimally oxidized LDL6 stimulate vascular endothelial cells to produce MCP-1 in vitro. The oxidized phospholipids responsible have been characterized.27 In the case of Lp(a), lipid peroxidation was not responsible for its endothelial stimulating activity, because the protein portion of Lp(a), and not the lipid-containing LDL portion, was found to be the active agent.5 This finding has been extended in the present study by the demonstration that the recombinant 6K apo(a), containing 6 type-4 kringle (types 5 to 10), kringle V, and the protease domain, representing the carboxy-terminal portion of apo(a), was a potent stimulator of endothelial I-309. Thus, although oxidation of LDL is a requirement for this lipoprotein to stimulate endothelial cell MCA, Lp(a), through its apo(a) structure, has the intrinsic capacity to induce endothelial MCA. In view of the present findings, it is of interest that MCP-1 was the major monocyte chemoattractant produced by HUVECs when these cells were stimulated with oxidized LDL. Our finding that Lp(a) induces principally I-309 and not MCP-1 provides evidence that different pathways and mechanisms are involved.

We also report for the first time that I-309 is widely distributed in the human atherosclerotic plaque. I-309 was found in all 25 coronary and carotid endarterectomy specimens analyzed. In tissues in which the luminal endothelium was present, I-309 was present on the endothelium, as identified by vWF staining on serial sections. I-309 was also identified in plaque macrophages and was found in the extracellular matrix. These findings support the concept that I-309 may participate in the atherosclerotic process. The colocalization of apo(a) and I-309 in the plaque raises the possibility that apo(a) is active in inducing I-309 in the vessel wall.

The induction of endothelial I-309 in vitro by apo(a) supports the concept that chronic stimulation of the vascular luminal endothelium by elevated levels of Lp(a) may stimulate the production of I-309 and the attraction of monocytes to the vessel wall. This mechanism may explain, in part, the association between Lp(a) and atherosclerosis. It has been postulated that if dysregulated, I-309, similar to other chemokines, has the capacity to induce inflammation and tissue injury.25 Thus, the present study extends the repertoire of inflammatory agonists in the endothelial cell and documents a novel pathway through which a lipoprotein may induce a chemotactic signal for monocytes.

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**References**


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