MCP-1 binds to oxidized LDL and is carried by lipoprotein(a) in human plasma

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Abstract  Lipoprotein oxidation plays an important role in pathogenesis of atherosclerosis. Oxidized low density lipoprotein (OxLDL) induces profound inflammatory responses in vascular cells, such as production of monocyte chemotactant protein-1 (MCP-1) [chemokine (C-C motif) ligand 2], a key chemokine in the initiation and progression of vascular inflammation. Here we demonstrate that OxLDL also binds MCP-1 and that the OxLDL-bound MCP-1 retains its ability to recruit monocytes. A human MCP-1 mutant in which basic amino acids Arg-18 and Lys-19 were replaced with Ala did not bind to OxLDL. The MCP-1 binding to OxLDL was inhibited by the monoclonal antibody E06, which binds oxidized phospholipids (OxPAPC) in OxLDL. Because OxPAPC are carried by lipoprotein(a) [Lp(a)] in human plasma, we tested to determine whether Lp(a) binds MCP-1. Recombinant wild-type but not mutant MCP-1 added to human plasma bound to Lp(a), and its binding was inhibited by E06. Lp(a) captured from human plasma contained MCP-1 and the Lp(a)-associated endogenous MCP-1 induced monocyte migration. These results demonstrate that OxLDL and Lp(a) bind MCP-1 in vitro and that OxPAPC are major determinants of the MCP-1 binding. The association of MCP-1 with OxLDL and Lp(a) may play a role in modulating monocyte trafficking during atherogenesis.—Wiesner, P., M. Tafelmeier, D. Chittka, S.-H. Choi, L. Zhang, Y.-S. Byun, F. Almazan, X. Yang, N. Iqbal, P. Chowdhury, A. Maisel, J. L. Witztum, T. M. Handel, S. Tsimikas, and Y. I. Miller. MCP-1 binds to oxidized LDL and is carried by lipoprotein(a) in human plasma. J. Lipid Res. 2013. 54: 1877–1883.

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Vascular cells secrete chemokines into the extravascular space. Glycosaminoglycans (GAGs) are expressed on the surface of endothelial cells, where they bind and transcytose chemokines to the luminal surface (1, 2). Monocyte chemotactant protein-1 (MCP-1) [synonym: chemokine (C-C motif) ligand 2 (CCL2)], is a major chemokine involved in development of atherosclerosis via monocyte recruitment to the vascular wall (3). Plasma levels of MCP-1 are associated with traditional risk factors for atherosclerosis in the general population and with an increased risk for death or myocardial infarction (MI) in patients with acute coronary syndrome (4–6). GAGs have been shown to play an important role in the in vivo activation and function of MCP-1 (7, 8). Earlier studies demonstrated that negatively charged GAGs bind to MCP-1 via the basic amino acids Arg-18 and Lys-19 in the MCP-1 molecule (9).

Oxidized low density lipoprotein (OxLDL) is an electronegative component of vascular lesions and an important pathogenic factor in the development of atherosclerosis (10). OxLDL activates vascular cells to secrete MCP-1 (11), leading to recruitment of monocytes, which differentiate into macrophages and internalize OxLDL. The resulting lipid-laden macrophage foam cells are a hallmark of atherosclerotic lesions that play a central role in atherosclerotic progression. We hypothesized that, similar to MCP-1 binding to GAGs, MCP-1 would also bind to electronegative OxLDL, which in turn would play a role in guiding monocyte recruitment.

Abbreviations:  CCL2, chemokine (C-C motif) ligand 2; CCR2, C-C chemokine receptor type 2; GAG, glycosaminoglycan; Lp(a), lipoprotein(a); mAb, monoclonal antibody; MCP-1, monocyte chemotactant protein-1; MI, myocardial infarction; nLDL, native LDL; OxLDL, oxidized low density lipoprotein; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; OxPL, oxidized phospholipid; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; RLU, relative light unit.

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METHODS

Lipoproteins and human plasma samples

Native LDL (nLDL) (density = 1.019–1.063 g/ml) was isolated from plasma of normolipidemic donors by sequential ultracentrifugation (12). Contamination of native and modified LDL preparations by endotoxin was assessed with a LAL QCL-1000 kit (Lonza). LDL preparations with LPS higher than 50 pg/mg protein were discarded. To produce OxLDL, 0.1 mg/ml of nLDL was incubated with 10 μM CuSO4 for 18 h at 37°C (13). The extent of LDL oxidation was assessed by measuring thiobarbituric acid-reactive substances (typically, more than 30 nmol/mg protein), and OxLDL was concentrated to 1 mg/ml using a 100 kDa cut-off centrifugal concentrator (Millipore) and sterile filtered (0.22 μm).

Plasma samples (n = 127) were collected from patients presenting with chest pain and suspected acute coronary syndromes (ST-segment elevation MI; non-ST-segment elevation MI and unstable angina) on admission to the Veteran’s Affairs Medical Center San Diego. Patients that ultimately ruled out for MI by clinical criteria and myocardial enzyme biomarkers were included as controls. The blood was immediately spun down in EDTA and the plasma separated and stored at –70°C. The collection of human plasma and assays on these samples were approved by the Veteran’s Affairs Medical Center and the University of California, San Diego Human Research Subjects Protection Programs, respectively, and all participants gave written informed consent.

Transgenic mice

C57BL/6/J mice were wild type or transgenic expressing human apoB-100, human apo(a), or lipoprotein(a) [Lp(a)], i.e., both apoB-100 and apo(a), as previously reported (14–16). Mice were housed in a barrier facility with a 12 h light/12 h dark cycle, and fed normal mouse chow containing 4.5% fat (Harlan Teklad). All animal experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Recombinant MCP-1

Wild-type and R18A/K19A mutant MCP-1 constructs were expressed in Escherichia coli and purified by reverse-phase HPLC as previously described (9, 17). The MCP-1 preparations were tested for endotoxin contamination with a LAL QCL-1000 kit (Lonza). Endotoxin concentrations were below detectable range (<50 pg/mg) in all MCP-1 preparations.

Size exclusion chromatography

nLDL and OxLDL samples (30 μg/ml) were incubated with 380 ng/ml MCP-1 (wild type) for 30 min at 37°C before they were loaded (200 μl) on a Superdex 200 column (GE Healthcare) and eluted at 0.5 ml/min using an FPLC system (Pharmacia). Twenty fractions of 1.5 ml each were collected and assayed for MCP-1 and apoB-100 concentrations using ELISA as described below.

Native gel electrophoresis and immunoblotting

Samples of OxLDL, preincubated with either wild-type MCP-1, mutant MCP-1, E06 (18) and/or isotype control, nonspecific IgM (eBioscience), were run on a 3–8% precast Tris-glycine polyacrylamide gel (Invitrogen) with Tris-glycine buffer for 18 h at 100 mV. No SDS was present in the sample buffer or the gel. The proteins were transferred to a PVDF membrane, the membrane was blocked with 5% dry milk in PBS, washed, and subsequently incubated with an anti-MCP-1 antibody (R & D Systems) or an anti-apoB-100 antibody [mouse monoclonal antibody (mAb) MB47 (19) specific for human apoB-100]. The membrane was then washed and incubated with a secondary HRP-conjugated antibody directed against the respective primary antibody, incubated with ECL-plus (GE Healthcare) for 5 min, and visualized with an OptiChemHR Imaging System (UVP).

Microplate-based immunoassay

In Lp(a) binding experiments, Microfluor 96-well microtiter plates (Thermo Scientific) were coated with 5 μg/ml anti-apo(a) antibody LPA4 (20) overnight at 4°C. Plates were washed and blocked with 1% BSA/TBS for 45 min. Plasma samples (diluted 1:50 for human or 1:100 for mouse plasma) were plated in triplicates and incubated for 75 min at room temperature. Plates were washed three times and incubated with 50 ng/ml biotinylated goat anti-MCP-1 antibody (R and D Systems) for 60 min at room temperature. Plates were washed three times, incubated with alkaline phosphatase-conjugated NeutrAvidin (Thermo Scientific, 1:40,000 dilution) for 60 min at room temperature, washed, and incubated with Lumi-Phos-530 (Lumigen, 1:1 dilution in water) for 75 min at room temperature. The plates were read with an MLX Microtiter Plate Luminometer (Dynex Technologies) and results were displayed as relative light units (RLU) per 100 ms.

In other experiments, PAPC (1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine) or oxidized 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine (OxPAPC) were directly plated on
inserts were fixed in ice-cold methanol and stained with crystal violet. Cells were counted in 150 fields of view, covering the whole insert. Experiments were performed in biological triplicates and repeated three to five times.

In a separate set of experiments, Lp(a) was isolated from human plasma using the apo(a)-specific antibody LPA4 (20) immobilized on agarose beads. Some plasma samples were spiked with 400 ng/ml of recombinant MCP-1 before the Lp(a) pull down. In brief, Protein A/G beads (GE Healthcare) were added to human plasma and incubated for 2 h at 4°C to remove endogenous immunoglobulins. Immunoglobulin-depleted plasma (500 μl; 1:1 diluted in chemotaxis buffer) was then incubated with 2 μg of the monoclonal anti-human apo(a) antibody LPA4 (20) overnight at 4°C, with gentle shaking, followed by a 1 h incubation with 50 μl of Protein A/G beads at 4°C. The beads were washed and used in the migration assay as a chemoattractant in the bottom well. To maintain beads in suspension, they were gently stirred every 10 min for the duration of the migration assay. Because of this interruption in the migration process, the number of cells migrated toward MCP-1 (positive control) was different in Figs. 2 and 5B.

**Statistical analysis**

Each experiment was repeated at least three times. ELISA and migration assays were performed in triplicates, and the results are presented as mean ± SD. Results of migration assays were analyzed by Student’s t-test, and results of dose-dependent MCP-1 binding were analyzed by two-way ANOVA, with Bonferroni posttest. Differences with \( P < 0.05 \) were considered statistically significant.

**RESULTS**

**OxLDL binds MCP-1**

To test the hypothesis that MCP-1 binds to OxLDL, MCP-1 was combined with OxLDL or nLDL, loaded onto a size exclusion column, and eluted fractions were analyzed for apoB-100 and MCP-1 content. As shown in Fig. 1, MCP-1 bound OxLDL to a greater degree compared with nLDL. MCP-1 associated with OxLDL also retained its capacity to induce migration of THP-1 monocytes (Fig. 2),
and the number of cells migrating in response to OxLDL-bound MCP-1 was significantly higher than in response to nLDL-bound MCP-1. The specificity of MCP-1-induced monocyte migration was validated in experiments with an antagonist of the MCP-1 receptor CCR2.

MCP-1 binding to OxLDL was confirmed in a native gel electrophoresis experiment in which MCP-1 migrated with the apoB band (Fig. 3A). In contrast to wild-type MCP-1, an MCP-1 mutant in which the basic amino acids Arg-18 and Lys-19 were replaced with nonpolar Ala, did not bind to OxLDL. Furthermore, the mAb E06, which binds and neutralizes oxidized phospholipids (OxPLs) on the surface of OxLDL (18), diminished binding of wild-type MCP-1 to OxLDL. To directly demonstrate that MCP-1 binds to OxPLs, we added MCP-1 to microtiter wells in which either PAPC or OxPAPC were plated. As shown in Fig. 3B, MCP-1 directly bound to OxPAPC, representing a mixture of several OxPL molecular species (21), but not to nonoxidized PAPC.

**Lp(a) is a carrier of MCP-1 in human plasma**

Whereas OxLDL occurs in vivo in the artery wall, it is unlikely that such extensively OxLDL occurs in the plasma. However, we have shown that E06-detectable OxPLs are found in plasma and that Lp(a) is the lipoprotein that carries a major fraction of OxPLs in human plasma (24, 25). Because the anti-OxPL antibody E06 blocked MCP-1 binding to OxLDL and MCP-1 directly bound to OxPLs (Fig. 3), we tested to determine whether Lp(a) binds MCP-1 as well. First, we tested plasma samples from transgenic mice expressing human apoB-100, human apo(a), or both were incubated with 250 ng/ml recombinant MCP-1 (wild-type) for 30 min at 37°C, and then subjected to ELISA with a capture apo(a) antibody and a detection MCP-1 antibody. A human plasma sample with an Lp(a) concentration of 109 mg/dl was diluted 1:50 and incubated with wild-type (wt) or mutant (mu) MCP-1 for 30 min at 37°C, and then subjected to ELISA as in panel (A). Results are presented as mean ± SD of technical triplicates; ***P < 0.001. The experiment was repeated with two other plasma samples from different donors, with similar results. C: A human plasma sample with an Lp(a) concentration of 97 mg/dl was diluted 1:50 and incubated with 35 μg/ml E06 or IgM isotype control and then with wild-type MCP-1 for 30 min at 37°C. The samples were subjected to ELISA as in panel (A). Results are presented as mean ± SD of technical triplicates; ***P < 0.001. The experiment was repeated with two other plasma samples from different donors, with similar results. D: A human plasma sample with an Lp(a) concentration of 190 mg/dl was diluted 1:50 and incubated with 500 ng/ml of wild-type MCP-1 for 30 min at 37°C, in the presence of 0, 0.1, 1, 10, or 100 μg/ml heparin. The samples were subjected to ELISA as in panel (A). Results are presented as mean ± SD of technical triplicates. The experiment was repeated with two other plasma samples from different donors, with similar results.
Because MCP-1 binds to GAGs (1), we tested to determine whether MCP-1 binding to Lp(a) can be completed by unfractionated heparin, a soluble GAG. Adding heparin to human plasma inhibited MCP-1 binding to Lp(a) in the human plasma (Fig. 4C).

However, after adding recombinant MCP-1 to mouse plasma samples, we detected MCP-1 only on apo(a)-containing lipoproteins (Fig. 4A). Using the same assay with human plasma samples, we also detected binding of recombinant wild-type MCP-1, but not mutant R18A/K19A MCP-1, to Lp(a) (Fig. 4B). Addition of mAb E06 inhibited MCP-1 binding to Lp(a) in the human plasma (Fig. 4C).

Because MCP-1 binds to GAGs (1), we tested to determine whether MCP-1 binding to Lp(a) can be completed by unfractionated heparin, a soluble GAG. Adding heparin to human plasma inhibited MCP-1 binding to Lp(a) in a dose-dependent manner (Fig. 4D). This result suggests that GAGs and Lp(a) may compete for an MCP-1 pool and together determine compartmentalization of the chemokine.

Endogenous MCP-1 associated with Lp(a) was assayed in a cohort of patients presenting with chest pain, using the same assay in which we captured Lp(a) from human plasma and then examined for the presence of MCP-1. The values of MCP-1/Lp(a) in individual samples varied as much as 1,000-fold, ranging from 3 to 3,335 (measured in RLU of the chemiluminescent immunoassay) (Fig. 5A).

We next measured to determine whether the Lp(a)-associated endogenous MCP-1 induced monocyte migration. Lp(a) was pulled from plasma with agarose beads coated with an apo(a) mAb, and the beads were used in a THP-1 monocyte migration assay. Migration of monocytes toward Lp(a) isolated from a high MCP-1/Lp(a) sample was significantly higher than the migration toward Lp(a) from a low MCP-1/Lp(a) sample (Fig. 5B). Adding a CCR2 antagonist to THP-1 cells inhibited monocyte migration toward Lp(a) isolated from the high MCP-1/Lp(a) plasma. Conversely, adding recombinant MCP-1 to the low MCP-1/Lp(a) sample increased monocyte migration. The MCP-1 bound to the Lp(a) beads was able to dissociate from the beads under the conditions of the assay (Fig. 6) and thus establish a gradient necessary for monocyte migration. These results suggest that MCP-1 carried by Lp(a) in human plasma is an active chemokine.

**DISCUSSION**

LDL cholesterol is considered a major causal risk factor in development of atherosclerosis (2, 10). Realization that LDL can be oxidized in vivo and that oxidation changes the mode of LDL interaction with vascular cells led to subsequent studies of specific proatherogenic effects of OxLDL (27, 28). These effects include the excessive accumulation of OxLDL in macrophages and vascular smooth muscle cells, and the activation of proinflammatory signaling pathways (29, 30). These properties of OxLDL are predominantly attributed to the presence of oxidized lipids and oxidized lipid-modified apoB-100. The present study suggests that, in addition to oxidized lipids, OxLDL can also be a carrier of other proinflammatory molecules, such as the chemokine MCP-1. We demonstrate that in vitro generated OxLDL is capable of binding MCP-1 and becomes a monocyte-attracting lipoprotein. Our laboratory had previously reported that in vitro generated OxLDL had some monocyte chemotactic activity, due in part to the content of lysophosphatidylcholine generated in the OxLDL (31, 32). However, MCP-1 binding to OxLDL results in a significantly stronger chemotactic effect on monocytes than that induced by OxLDL alone.

Fully oxidized LDL is not thought to exist in significant quantities in plasma but instead is present mainly in the vessel wall (28). However, our studies demonstrate that the major lipoprotein carrier of OxPLs in human plasma, Lp(a), also binds MCP-1. As with OxLDL, addition of recombinant MCP-1 to Lp(a) turns it into a chemotactant. More remarkable is the observation that endogenous MCP-1 is present on circulating Lp(a) in human plasma and that Lp(a) isolated from the plasma with a high MCP-1/Lp(a) value is a stronger chemotactant than the Lp(a) from low MCP-1/Lp(a) plasma. These results suggest that Lp(a) in plasma may serve as a carrier for OxPLs and Lp(a) bind MCP-1/CCL2 1881
MCP-1 and that once it has entered the arterial intima with its associated MCP-1, it may subsequently enhance the trafficking of monocytes to the vascular wall. Lp(a) is found in the human atheroma and a higher content of Lp(a) correlates with the severity of the clinical presentation (33, 34). Upon penetration into the vascular wall, Lp(a) binds to lysine groups of various proteins via its multiple high-affinity lysine binding sites. This results in retention and concentration of Lp(a) in the vascular wall (25), where its OxPL content may mediate a number of proinflammatory effects and also may induce apoptosis of endoplasmic reticulum-stressed macrophages (35). In addition, Lp(a)-associated MCP-1 may also contribute to recruitment of additional monocytes to the lesion site and thereby exacerbate lesion progression. Thus, the Lp(a) content of MCP-1 may be added to the list of biological properties of Lp(a) that make it a proatherogenic lipoprotein (36). In the small clinical cohort that we evaluated, there was no clear relationship between MCP-1/Lp(a) levels and clinical presentation. We plan to conduct large event-powered studies to evaluate the role of MCP-1/Lp(a) in cardiovascular disease and test the hypothesis that this novel biomarker predicts clinical outcomes.

The mechanism of MCP-1 binding with OxLDL and Lp(a) is likely similar to that with GAGs and relies on ionic interactions. Both GAGs and OxLDL are electronegative and the replacement of basic Arg-18 and Lys-19 in MCP-1 with nonpolar Ala renders it incapable of binding to either GAGs or OxLDL. Many if not all chemokines, which tend to be highly basic proteins, bind to GAGs (1). This provides a mechanism to localize and present chemokines on cell surfaces and/or on the extracellular matrix and thus to support directional motility of monocytes and other leukocytes. The GAG interactions also facilitate chemokine transcytosis, provide protection from hydrolysis, and coreceptor and signaling functions (1). Although in vitro the GAG binding deficient MCP-1 mutant R18A/K19A, which was also used in this study, has only minimal reduction in the CCR2 (MCP-1 receptor) binding, in vivo it is unable to recruit monocytes when administered intraperitoneally (7). These results support the importance of GAG binding for in vivo MCP-1 function. Our results showing that GAGs and Lp(a) compete for the MCP-1 binding add to the complexity of MCP-1 in vivo compartmentalization and function. Future studies will demonstrate whether binding of MCP-1 to Lp(a) and OxLDL is important for the function of MCP-1 in the development of atherosclerosis, as suggested in the present study.

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