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Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions

(*in situ* hybridization/immunocytochemistry/RNA probes/mRNA/foam cells)

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ABSTRACT **The recruitment of monocyte–macrophages into the artery wall is one of the earliest events in the pathogenesis of atherosclerosis. Monocyte chemoattractant protein 1 (MCP-1) is a potent monocyte chemoattractant secreted by many cells *in vitro*, including vascular smooth muscle and endothelial cells. To test whether it is expressed in the artery *in vivo*, we used Northern blot analysis, *in situ* hybridization, and immunocytochemistry to study the expression of MCP-1 in normal and atherosclerotic human and rabbit arteries. Northern blot analysis showed that MCP-1 mRNA could be isolated from rabbit atherosclerotic lesions but not from the intima media of normal animals. Furthermore, MCP-1 mRNA was extracted from macrophage-derived foam cells isolated from arterial lesions of ballooned cholesterol-fed rabbits, whereas alveolar macrophages isolated simultaneously from the same rabbits did not express MCP-1 mRNA. MCP-1 mRNA was detected by *in situ* hybridization in macrophage-rich regions of both human and rabbit atherosclerotic lesions. No MCP-1 mRNA was found in sublesional medial smooth muscle cells or in normal arteries. By using immunocytochemistry, MCP-1 protein was demonstrated in human lesions, again only in macrophage-rich regions. Immunostaining of the serial sections with an antiserum against malondialdehyde-modified low density lipoprotein indicated the presence of oxidized low density lipoprotein and/or other oxidation-specific lipid-protein adducts in the same areas that contained macrophages and MCP-1. We conclude that (*i*) MCP-1 is strongly expressed in a small subset of cells in macrophage-rich regions of human and rabbit atherosclerotic lesions and (*ii*) MCP-1 may, therefore, play an important role in the ongoing recruitment of monocyte–macrophages into developing lesions *in vivo*.**

The earliest grossly visible atherosclerotic lesion is the fatty streak, characterized by the accumulation of lipid-loaded foam cells in the subendothelial space (1). Many of these foam cells are derived from circulating monocytes (2–4) that have penetrated into the subendothelial space and presumably taken up excess native and/or oxidized low density lipoprotein (LDL) (5–8). Thus, one of the important early events in the pathogenesis of atherosclerosis is the adherence of monocytes to the endothelium, followed by their migration into the subendothelial space (1, 3). The entry between endothelial cells presumably is in response to a gradient of one or more chemotactic factors. Several monocyte chemotactic factors have been described—produced by endothelial cells, by smooth muscle cells, or by macrophages (9–14)—but there is almost no information on which of these are important *in vivo*. Chemotactic activity may also be derived from the extracellular components of the artery wall. For example, proteolytic peptide fragments from several connective tissue matrix proteins are chemotactic for leukocytes (15–17). In addition, *in vitro*-oxidized LDL is chemotactic for circulating monocytes (18) and oxidatively modified LDL isolated from atherosclerotic lesions shows similar chemotactic activity for monocytes (8).

Recently, monocyte chemotactic protein 1 (MCP-1) was isolated and cloned from a human glioma cell line U-105MG, a line that constitutively secretes MCP-1 (13, 19). MCP-1 is secreted by stimulated human lymphocytes (19) and also accounts for most of the monocyte chemotactic activity secreted by endothelial cells and smooth muscle cells *in vitro* (20). The gene for human MCP-1 is the homologue of mouse gene *JE*, first found in platelet-derived growth factor-stimulated mouse fibroblasts (21) and also found in lipopolysaccharide-stimulated mouse peritoneal macrophages (22). Thus, many cells have the potential to secrete MCP-1 *in vitro*. However, it is not known whether MCP-1 is expressed by any cells of the atherosclerotic lesion *in vivo*.

The purpose of the present study was to determine whether MCP-1 is present in normal and atherosclerotic arteries and which cell types express it.

METHODS

Tissue Samples. Human aortic samples were obtained from two organ donors for renal transplants (ages 28 and 33 years) and from two medicolegal autopsies (ages 37 and 41 years) 3.5 and 5 h postmortem. Samples were immersion-fixed overnight at 4°C in formal/sucrose [4% (wt/vol) paraformaldehyde/5% (wt/vol) sucrose/1 mM EDTA/50 μM butylated hydroxytoluene, pH 7.4] and embedded in paraffin (23).

Five control and 12 cholesterol-fed New Zealand White rabbits [2% (wt/wt) cholesterol diet for 10 weeks] were anesthetized with intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg), perfused 2 min with physiologic saline containing 1 mM EDTA and 50 μM butylated hydroxytoluene, and perfusion-fixed for 5 min with formal/sucrose (24). Aortic tissue was then removed, immersion-fixed in formal/sucrose for 6 h, and embedded in paraffin. In some experiments aortic tissue was not fixed but was used directly for the isolation of arterial RNA. All human studies were approved by the Human Subjects Committee and all animal studies by the Animal Subjects Committee of the University of California, San Diego.

Isolation of Arterial Foam Cells. Macrophage-derived foam cells were isolated from 14 New Zealand White rabbit arteries as described (25). Briefly, atherosclerotic lesions were induced by balloon deendothelialization followed by 10–11 weeks of a 2% cholesterol diet. Foam cells were obtained from isolated lesions by using collagenase and elastase di-

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Abbreviations: LDL, low density lipoprotein; MCP-1, monocyte chemotactic protein 1.

gestions, followed by a discontinuous metrizamide gradient centrifugation, and were shown to be free of any contaminating smooth muscle cells (25). All solutions and reagents were free of any significant endotoxin contamination according to the *Limulus* assay (Whittaker Bioproducts). Alveolar macrophages were isolated simultaneously from the same animals using bronchoalveolar lavage with phosphate-buffered saline. As a further control, the alveolar macrophages were treated with the same metrizamide or the collagenase/elastase mixture used to isolate arterial foam cells.

Probes. The following probes were subcloned into plasmid vectors (26) and used for *in situ* hybridization and Northern blot analysis: A 0.65-kilobase full-length rabbit MCP-1 cDNA (nucleotides −15 to 633) (27) was subcloned into pBluescript II (Stratagene) and a 0.55-kilobase *Bam*HI–*Pst* I fragment (nucleotides 1 to 547) of human retinoic acid receptor cDNA (28) was subcloned into pGEM-4Z (Promega). Because of the high homology between human and rabbit MCP-1 sequences (19, 27), the rabbit probe was considered suitable for studies with human tissue. This was confirmed by Northern blot analysis, which demonstrated that mRNA isolated from cultured human medial smooth muscle cells hybridized to the rabbit MCP-1 probe. Human retinoic acid receptor cDNA was used as an irrelevant control probe that did not hybridize to human or rabbit arterial tissue.

Antisense and sense RNA probes were synthesized using Sp6, T7, or T3 RNA polymerases with ^{35}S-labeled UTP (1200 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) (24, 29). The specific activity of the probes varied from 250 to 300 Ci/mmol. Full-length probes were used for Northern blot analysis. For *in situ* hybridization, probe sizes were reduced by alkaline hydrolysis to an average length of 250 nucleotides (23, 30). Probe sizes were confirmed by polyacrylamide gel electrophoresis and their specificity was tested by Northern blot analysis (26).

***In Situ* Hybridization.** All experiments were done on a set of serial paraffin-embedded sections (10 μm) with an antisense probe, a corresponding nonhybridizing sense probe, and an irrelevant nonhybridizing probe. In some experiments tissue sections were pretreated with RNase A, which abolished the signal. *In situ* hybridizations were done as described (23, 24). Briefly, tissue sections were deparaffinized, pretreated with proteinase K (1 μg/ml for 15 min at 37°C; Boehringer Mannheim), and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at 20°C). Sections were washed twice with 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0), dehydrated, and dried *in vacuo*. Slides were hybridized for 14 h at 50–52°C in a hybridization solution (see below) containing each probe at 3–6 × 10^6 cpm/ml (23, 24).

After the hybridization the slides were washed for three 15-min periods in 4× SSC and digested with RNase A (20 μg/ml; Boehringer Mannheim) for 30 min at 37°C. The slides were then washed for two 15-min periods at 20°C with 2× SSC, 1× SSC, and then 0.5× SSC. The final wash was with 0.1× SSC at 60°C (rabbit tissue) or at 55°C (human tissue) for 60 min. Slides were then dehydrated, dipped in photographic emulsion (NTB-2; Eastman Kodak), and developed after 3–10 weeks exposure time (23, 24). After development the slides were counterstained with hematoxylin/eosin. *In situ* hybridization solution contained 50% (vol/vol) formamide (Fluka), 2× SSC, 20 mM Tris·HCl (pH 7.4), 1× Denhardt's solution, 1 mM EDTA, 10% (wt/vol) dextran sulfate (Pharmacia), 1 mM dithiothreitol, and yeast tRNA (0.5 mg/ml; Boehringer Mannheim). [1× Denhardt's solution contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (all from Sigma).]

Immunocytochemistry. Serial paraffin-embedded sections (10 μm) were used for immunostaining using the following characterized antibodies: a rabbit antiserum against human MCP-1 (31), a guinea pig antiserum against malondialdehyde-modified LDL (7, 32), a mouse monoclonal antibody against smooth muscle cells (HHF-35) (33), and mouse monoclonal antibodies against human (HAM-56) (34) and rabbit (RAM-11) (35) macrophages. An avidin–biotin-horseradish peroxidase system was used for the immunostaining (Vector Laboratories). After immunocytochemistry the slides were counterstained with methyl green.

Northern Blot Analysis. Total RNA was isolated from normal aortic intima-media of control New Zealand White rabbits [0.5 g (wet weight) per normal sample] and from several atherosclerotic lesions dissected from cholesterol-fed rabbits [0.5 g (wet weight) per lesion sample]. Freshly isolated macrophage-derived foam cells and alveolar macrophages (10–30 × 10^6 cells) from the ballooned cholesterol-fed animals (25) were also used for the isolation of cellular RNA. RNA was isolated from the samples using guanidinium isothiocyanate extraction followed by CsCl centrifugation (26). Ethanol-precipitated RNA was electrophoresed on 1% agarose/formaldehyde gels and transblotted to nylon membranes (Nytran, Schleicher & Schuell) (26). Membranes were hybridized overnight at 65°C with ^{35}S-labeled RNA probes (1 × 10^6 cpm/ml). Hybridizations and the following washes were done according to manufacturer's instructions. The final wash was with 0.1× SSC/0.5% SDS/1 mM dithiothreitol at 80°C for 60 min. Membranes were then dried and the signal was detected using autoradiography (Kodak XAR-5 film).

RESULTS

By using Northern blot analysis, MCP-1 mRNA was detected in atherosclerotic lesions of cholesterol-fed New Zealand White rabbits, whereas no message for MCP-1 was detected in normal aortic intima-media of control animals (Fig. 1). MCP-1 mRNA was also detected in freshly obtained macrophage-derived foam cells isolated from rabbit atherosclerotic lesions induced by balloon deendothelialization, fol-

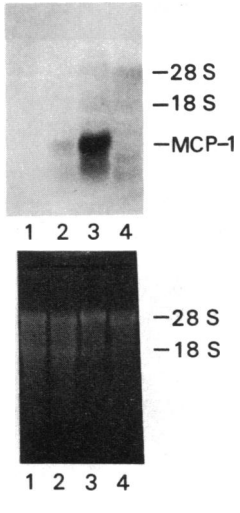

FIG. 1. Expression of MCP-1 mRNA in rabbit atherosclerotic lesions and in rabbit lesion macrophage-derived foam cells. Total RNA was isolated and subjected to Northern blot analysis (25 μg of total RNA per lane). (*Upper*) Northern blot with MCP-1 antisense RNA probe. Lanes: 1, normal rabbit aortic intima-media; 2, atherosclerotic lesions isolated from cholesterol-fed rabbit aorta; 3, macrophage-derived foam cells isolated from ballooned aorta of cholesterol-fed rabbits; 4, alveolar macrophages isolated simultaneously from the ballooned cholesterol-fed animals. (*Lower*) Ethidium bromide staining of the RNA gel. Lanes 1–4 are the same as in *Upper*. Positions of the 28S and 18S rRNA subunits are indicated.

lowed by diet-induced hypercholesterolemia. The foam cells were harvested 10–11 weeks after ballooning (25). To rule out a possible induction of MCP-1 mRNA expression in the foam cells during their isolation, alveolar macrophages were simultaneously isolated from the same ballooned animals using the same procedures. As shown in Fig. 1, very little, if any, MCP-1 mRNA was detected in the alveolar macrophages. *In situ* hybridization and immunohistochemistry, described below, shows that only macrophage-rich areas contain MCP-1 mRNA and protein. Thus, the weaker signal in the lesion RNA sample reflects in part the inclusion in the intima-media of RNA from smooth muscle cells and other cells that contain little or no MCP-1 mRNA.

In situ hybridization was used to further characterize the expression of MCP-1 mRNA *in vivo*. Hybridization of the sections with a rabbit MCP-1 antisense RNA probe showed MCP-1 mRNA expression in macrophage-rich regions of both human and rabbit atherosclerotic lesions. An example of the MCP-1 mRNA expression in a human fatty streak is seen in Fig. 2*A*. No hybridization was seen with the corresponding sense probe (Fig. 2*B*) or with an irrelevant antisense RNA probe (human retinoic acid receptor) (data not shown). Pretreatment of the slides with RNase A abolished the signal (data not shown).

Immunostaining of the serial sections with a rabbit antiserum against human MCP-1 showed that some intimal cells contained MCP-1 protein (Fig. 2*C*). Immunostaining patterns using a macrophage-specific antibody suggested that the cells responsible for the expression of MCP-1 were macrophages (Fig. 2*D*). The same areas also stained positively for epitopes characteristic of oxidized LDL (Fig. 2*E*). In some areas a weak positive *in situ* hybridization signal for MCP-1 mRNA was detected on the endothelium. There was no hybridization or immunostaining for MCP-1 detectable above background in medial smooth muscle cells (data not shown).

Similar positive *in situ* hybridization results for MCP-1 mRNA were seen in rabbit atherosclerotic lesions (Fig. 3*A*). Again, there were a few scattered cells showing a very strong signal. No signal was detected with the nonhybridizing MCP-1 sense probe (Fig. 3*B*). Use of a macrophage-specific antibody showed macrophages in regions where MCP-1 mRNA was detected (Fig. 3*C*). As with the human lesions,

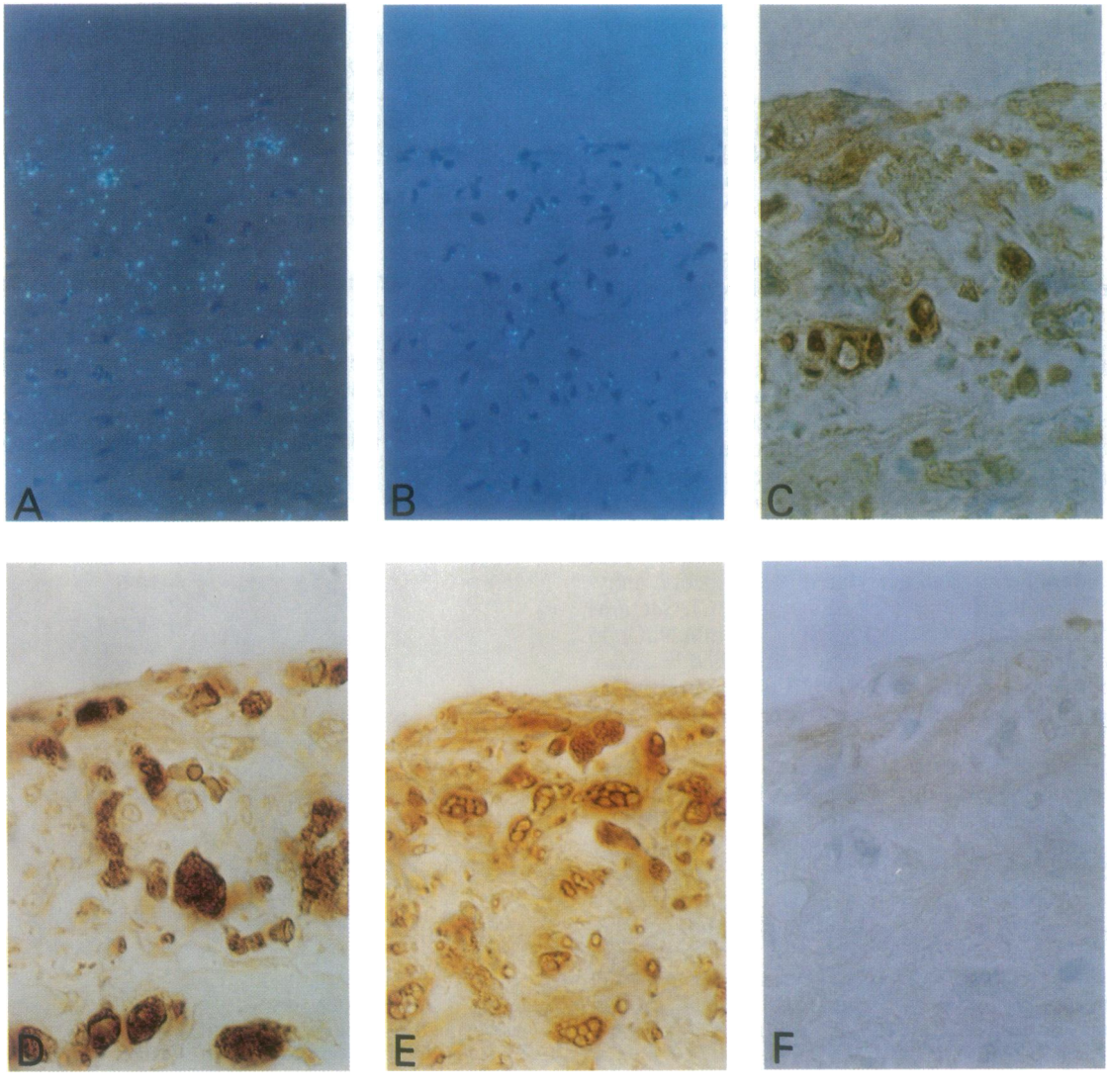

FIG. 2. MCP-1 expression in a human fatty streak (abdominal aorta; 41-year-old male; postmortem time, 3.5 h). (*A* and *B*) *In situ* hybridization of the serial sections. (*A*) Rabbit MCP-1 antisense probe. (*B*) Rabbit MCP-1 nonhybridizing sense probe. Exposure time in *A* and *B* was 8 weeks. (*C–F*) Immunostaining (avidin–biotin-horseradish peroxidase system) of the serial sections. (*C*) Antibody against human MCP-1 protein (rabbit antiserum, 1:250 dilution). (*D*) Antibody against human macrophages (HAM-56, 1:1000 dilution). (*E*) Antibody against malondialdehyde-modified LDL (MAL-2, 1:500 dilution). (*F*) Nonimmune control for the immunostaining. Hematoxylin/eosin counter stain in *A* and *B* and methyl green counter stain in *C–F*. Photographs in *A* and *B* were taken using polarized light epiluminescence; photographs in *C–F* were taken using bright-field illumination. (*A* and *B*, ×220; *C–F*, ×550.)

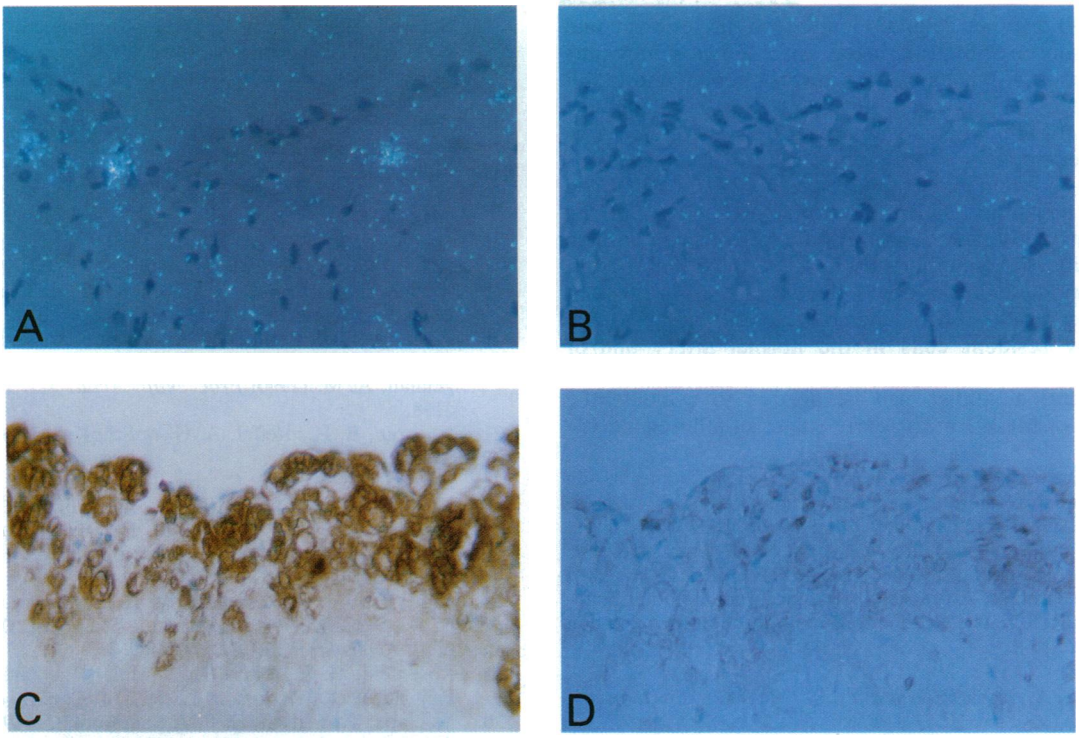

FIG. 3. MCP-1 mRNA expression in a rabbit atherosclerotic plaque (thoracic aorta, cholesterol-fed New Zealand White rabbit). (*A* and *B*) *In situ* hybridization of the serial sections. (*A*) Rabbit MCP-1 antisense probe. (*B*) Rabbit MCP-1 nonhybridizing sense probe. Exposure time in *A* and *B* was 8 weeks. (*C* and *D*) Immunostaining of the serial sections. (*C*) Antibody specific for macrophages (RAM-11; 1:1000 dilution). (*D*) Antibody specific for smooth muscle cells (HHF-35; 1:1000 dilution). Nonimmune controls for the immunostainings were negative (data not shown). Hematoxylin/eosin counter stain in *A* and *B*; methyl green counter stain in *C* and *D*. Photographs in *A* and *B* were taken using polarized light epiluminescence; photographs in *C* and *D* were taken using bright-field illumination. (×220.)

we cannot rule out the possibility that some MCP-1 is derived from smooth muscle cells present in the lesions (Fig. 3*D*), since *in situ* hybridization and immunostaining studies were not done on the same tissue section. However, in no case was MCP-1 mRNA detectable in normal artery wall or in the medial smooth muscle cells beneath the lesions (data not shown).

DISCUSSION

The mechanism(s) responsible for the recruitment of monocytes into the artery wall during atherogenesis has not been completely defined, although it appears to involve the adherence of monocytes to the endothelium, followed by their subsequent migration into the subendothelial space. The adherence step is likely to involve interactions between circulating monocytes and specific adhesion molecules expressed on the endothelial cells (36, 37). After the attachment monocytes migrate across the endothelium, which suggests the presence of chemotactic factors within the intima derived from cells already present within the artery wall.

Recent evidence suggests that MCP-1 may play an important role in the migration of monocytes into the subendothelial space: cytokine-activated endothelial cells secrete MCP-1 into the growth medium (38) and MCP-1 accounts for most of the monocyte chemotactic activity constitutively released into the growth medium by endothelial cells and smooth muscle cells (20). Since atherosclerotic lesions contain oxidatively modified LDL (6–8), it is interesting to note that minimally oxidized LDL can induce MCP-1 expression in endothelial cells and smooth muscle cells *in vitro* (20).

In the present studies, using Northern blot analysis, MCP-1 mRNA was identified in cholesterol-fed rabbit atherosclerotic lesions but not in normal intima-media (Fig. 1). Freshly isolated macrophage-derived foam cells were also positive for MCP-1 mRNA, whereas alveolar macrophages isolated simultaneously from the same animals did not contain measurable levels of MCP-1 message. This seems to rule out the possibility that MCP-1 mRNA was induced by the cholesterol feeding *per se* or during the isolation of the foam cells. The content of endotoxin during the isolation procedure was <0.1 ng/ml, which is unlikely to cause the activation of MCP-1 gene expression in the isolated foam cells. These findings imply that something unique to the microenvironment of the arterial foam cells or something linked to their accumulation of lipids specifically stimulates MCP-1 expression in arterial macrophages. As mentioned above, minimally oxidized LDL can induce MCP-1 expression in cultured endothelial cells and smooth muscle cells (20). If macrophages show a similar response, then oxidized LDL might be an important factor in this artery-specific expression. There is a large body of evidence that oxidized LDL is present in arterial lesions (6–8, 25).

Although both endothelial cells and smooth muscle cells are capable of synthesizing MCP-1 *in vitro* (11–13, 20, 38), *in situ* hybridization and immunostaining failed to detect MCP-1 message or protein in medial smooth muscle cells. The apparent absence of MCP-1 in medial smooth muscle cells, which secrete it under culture conditions (12, 20), implies growth-related or site-specific control of its expression. The issue of endothelial expression *in vivo* is not resolved by the present studies. A weak signal was detected in some sections but cell-specific markers were not used and the possibility of edge artifacts cannot be ruled out.

Only a minority of the cells in macrophage-rich areas of the atherosclerotic lesions expressed MCP-1 mRNA. The identity of these cells has not yet been established. However, the presence of MCP-1 mRNA in macrophage-derived foam cells of rabbit atherosclerotic lesions suggests that the cells could represent a subpopulation of macrophages. However, lym-

phocytes (39) or other cellular elements in the lesions could also be the source of MCP-1. In any case, the present results indicate that MCP-1 is expressed in early human and rabbit atherosclerotic lesions and thus could play a role in the recruitment of monocyte–macrophages in lesion areas *in vivo*. The cell-specific expression of MCP-1 in later lesions remains to be evaluated. It must be emphasized that the apparently negative results in normal artery and in the endothelium overlying lesions may only reflect a limitation in the sensitivity of the methods used. Also, the initial recruitment of monocytes into a newly developing lesion presumably reflects production of chemoattractants not by foam cells but by resident cells in the normal artery and/or monocytes traversing the intima in a "surveillance mode." *In vivo* intervention studies will be needed to evaluate the significance of MCP-1 in the early stages of atherogenesis.

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