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Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates

(atherosclerosis/immunohistochemistry/*in situ* hybridization)

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ABSTRACT **Atherosclerosis is marked by an overt inflammatory infiltrate, with enhanced recruitment of monocytes/macrophages observed in both human and experimental atherosclerosis. We previously determined that monocyte chemoattractant protein 1 (MCP-1) accounts for virtually all of the chemotactic activity produced by vascular (aortic) smooth muscle cells in culture. We now report that arteries from a primate model of atherosclerosis with dietary-induced hypercholesterolemia exhibit increased levels of MCP-1 mRNA expression *in vivo*, whereas their normal counterparts demonstrate minimal MCP-1 expression. Furthermore, immunohistochemistry and *in situ* hybridization clearly indicate that the expression of MCP-1 protein and mRNA is in the smooth muscle cells of the medial layer of the artery and in monocyte-like and smooth muscle-like cells found in the overlying intimal lesion. These studies indicate that one of the responses to dietary hypercholesterolemia is the expression of MCP-1 by vascular smooth muscle cells. This expression, when augmented with other cellular and molecular factors, could significantly contribute to the recruitment of monocytes/macrophages to the vessel wall.**

Atherosclerosis is a multistage disease involving invasion of the intima by smooth muscle cells (SMCs) from the media, followed by SMC proliferation and production of an extensive insoluble matrix including such connective tissue components as collagens, elastin, and proteoglycans (1). The two major lesions seen are the fatty streak and the atheromatous or fibrotic plaque. Evidence suggests that fatty streaks, seen in childhood, may progress to fibrotic lesions in adults. Both types of lesions are typified by macrophage invasion (2). Factors released by the macrophage have been shown to influence SMC migration, proliferation, and connective tissue gene expression (3–5). Thus a renewed appreciation of the role of the inflammatory process in coronary arteriosclerosis has emerged.

Monocyte chemoattractant protein 1 (MCP-1) is a monomeric polypeptide that migrates in a gel with an estimated molecular mass of 9–15 kDa (6, 7). The various forms of MCP-1 result from differences in O-linked glycosylation and do not affect chemotactic activity (8, 9). Unlike other chemoattractants, MCP-1 is relatively specific for monocytes. Lymphocytes and polymorphonuclear leukocytes lack MCP-1 receptors and do not respond to MCP-1 (10). Previous *in vitro* studies have shown that this protein can either be secreted by normal cells such as endothelial cells (11) and SMCs (12) or by many tumor cell lines (6, 13). MCP-1 expression in most normal cell types occurs in response to proinflammatory factors such as interleukin 1, tumor necrosis factor α, and interferon γ (11, 14). It has also been shown that minimally modified plasma low density lipoprotein stimulates MCP-1 production by human SMCs and endothelial cells *in vitro* (15). It has been suggested that macrophage/monocyte recruitment in atherosclerosis occurs in response to a gradient of chemoattractants released from cells of the vascular wall (16). We now report that arteries from primates with hypercholesterolemia exhibit increased MCP-1 mRNA expression *in vivo*. Furthermore, MCP-1 protein and mRNA are observed in the SMCs of the medial layer of the artery and in monocyte-like and smooth muscle-like cells found in the vessel wall.

MATERIALS AND METHODS

Cynomolgus Monkey Model. Hypercholesterolemia of seven nonhuman primates (*Macaca fascicularis*) was induced in the experimental group by feeding a normal diet supplemented with 2% (wt/wt) cholesterol and 10% (wt/wt) fat (17). Three primates were maintained on the diet for 6 months, one was on the diet for 11 months, and three were on the diet for 18 months. Serum cholesterol levels in the 18-month experimental group ranged from 544 to 901 mg/dl. Serum cholesterol levels in three control primates fed a normal diet ranged from 137 to 155 mg/dl.

Northern Blot Analysis. MCP-1 expression in the carotid arteries was examined at the RNA level by Northern blot analysis. Animals were anesthetized and segments of the carotid arteries were surgically isolated after sacrifice and placed in ice-cold saline. The vessels were gently stripped of the adventitial layer and snap-frozen in liquid nitrogen. For isolation of RNA, frozen tissue was homogenized in 4 M guanidine isothiocyanate with a Polytron homogenizer. The homogenate was passed twice through a 21-gauge needle, and the total RNA fraction was isolated by centrifugation for 20 h at 35,000 rpm in a 50 Ti rotor at 20°C through 5.7 M CsCl. RNA samples were fractionated on a 1% agarose/formaldehyde gel and transferred to GeneScreen*Plus* membrane (DuPont). A full-length human MCP-1 cDNA probe (7) was generously provided by E. Appella (Laboratory of Cell Biology, National Cancer Institute, Bethesda, MD). The cDNA probe was labeled by random priming as described (8). The membrane was hybridized and washed according to the manufacturer's directions. The poly(A) sequence content in the RNA preparations was determined as described (18).

Immunohistochemistry and *in Situ* Hybridization Analysis. For immunohistochemistry and *in situ* hybridization, seg-

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Abbreviations: SMC, smooth muscle cell; MCP-1, monocyte chemoattractant protein 1.

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ments of the arteries were fixed immediately after excision by immersion in ice-cold 4% (wt/vol) paraformaldehyde for 3 h. After fixation, the tissues were allowed to sink in 30% (wt/vol) sucrose in phosphate-buffered saline overnight at 4°C to decrease freezing artifacts. The frozen fixed tissues were then embedded in OCT compound (Miles) for cryostat sectioning (8-μm sections). For immunohistochemistry, fixed tissue sections were incubated with MCP-1 antiserum (1:500 dilution). MCP-1-specific antisera, raised against purified baboon SMC-derived MCP-1 in rabbits, has been described (6, 15). As a negative control, various serial sections were incubated with antiserum in the presence of excess purified MCP-1 protein as competitor. Antibodies were localized by an indirect immunoperoxidase technique (avidin–biotin–horseradish peroxidase complex) employing diaminobenzidine as a chromogen (Vector Laboratories). Cells were counterstained with hematoxylin.

For *in situ* hybridization, ^{35}S-labeled complementary "antisense" MCP-1 RNA probes were incubated with tissue sections as described (19, 20). As a negative control, another serial section from the specimen was incubated with the ^{35}S-labeled noncomplementary "sense" MCP-1 RNA probe. The RNA probes were transcribed from the full-length human cDNA probe.

RESULTS

Increased Arterial MCP-1 mRNA Levels in Hypercholesterolemic Animals. MCP-1 expression in the carotid arteries of monkeys (*M. fascicularis*) was examined at the RNA level by Northern blot analysis. Induction of MCP-1 in the carotid arteries of the three animals maintained on the hypercholesterolemic diet for 18 months was clearly evident, whereas little or no MCP-1 expression was detected in the carotid arteries of the three normocholesterolemic control monkeys (Fig. 1*A*). The observation that poly(A) sequences in the preparations from control and treated animals were equivalent (data not shown) suggests that the mRNA content was unaltered by the diet. Furthermore, the result that measurement of elastin mRNA showed a slight decrease in the hypercholesterolemic animals indicates that the increase in MCP-1 is selective (S.D., W.H., S.P., and G.E.S., unpublished results). It is evident from these studies that primates with hypercholesterolemia exhibit a much higher net level of MCP-1 expression in their arteries than do normocholesterolemic animals.

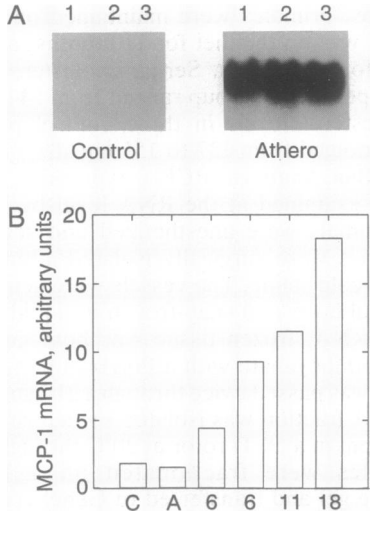

FIG. 1. Northern blot analysis of MCP-1 expression in hypercholesterolemic monkeys (*M. fascicularis*). (*A*) Adult male monkeys were fed chow (control) or chow supplemented with 2% cholesterol and 10% fat for 18 months (hypercholesterolemic or athero group). RNA was isolated from carotid arteries of three experimental and three control animals and analyzed on a Northern blot using an MCP-1 cDNA probe. (*B*) Adult male monkeys were treated as in *A* for 6, 11, or 18 months, and carotid RNA was isolated and subjected to Northern blot analysis as above. RNA, isolated from either the carotid artery (C) or the aortic artery (A) of a control animal, was similarly analyzed. The resulting autoradiogram was scanned on a Molecular Dynamics densitometer and the results were normalized to rRNA, quantified by ethidium bromide staining.

MCP-1 expression was further examined by assessing MCP-1 mRNA levels in primates that were hypercholesterolemic for 6, 11, and 18 months. The resulting Northern blot was scanned, and the density of each MCP-1 band was normalized to the amount of rRNA (Fig. 1*B*). An increase in MCP-1 expression is evident even by 6 months. These results indicate that carotid arteries in hypercholesterolemic primates and atherosclerotic lesions exhibit much higher levels of MCP-1 expression than normal untreated primates with normal serum cholesterol levels.

Immunohistochemical Detection of MCP-1 Protein in SMCs. To identify the cells in the artery responsible for MCP-1 expression, immunohistochemical studies were carried out using a monospecific polyclonal antibody prepared against baboon SMC-derived MCP-1. Cryostat sections of arterial tissue were immunostained by incubation with the MCP-1 antiserum followed by incubation with a biotinylated secondary antibody and avidin–biotin–horseradish peroxidase complex. As negative controls, serial sections were treated at the first step with MCP-1 antiserum in the presence of excess purified antigen. A heavily lesioned section of the carotid artery taken from an animal maintained on the hypercholesterolemic diet for 11 months is shown in Fig. 2. Fig. 2 represents an analysis of an early lesion with a clear thickening of the intima in relationship to the inner elastic lamina. Strongly positive expression of MCP-1 (Fig. 2*A*) is apparent in the SMCs of the tunica media, which can be unambiguously identified by their location, i.e., medial to the inner elastic lamina. In addition, mononuclear cells and smooth muscle-like cells are shown to express MCP-1 in the intima. These observations are consistent with *in vitro* studies that have shown MCP-1 expression in both vascular smooth muscle cells and stimulated peripheral blood monocytes (7, 12). The specificity of the MCP-1 immunostaining is demonstrated by absence of the chromogen in tissue sections incubated with MCP-1 antiserum in the presence of excess antigen (Fig. 2*B*). We also examined some nonovertly lesioned sites from the same heavily affected carotid artery. Specific MCP-1 expression was associated with medial SMCs in these regions (Fig. 2 *C* and *D*). Thus immunohistochemistry localizes the expressed MCP-1 to the SMCs and monocyte-like cells within the vessel wall.

***In Situ* Localization of MCP-1 mRNA in SMCs.** To confirm MCP-1 expression by the vascular SMCs and blood-derived mononuclear cells, *in situ* hybridization studies were performed. Fig. 3 shows a heavily lesioned section of carotid artery from a monkey on the hypercholesterolemic diet for 6 months. Fig. 3*A* demonstrates expression of MCP-1 in an intimal lesion by monocyte-like and smooth muscle-like cells. Higher magnification indicates MCP-1 expression by SMCs from the tunica media (Fig. 3*C*). Positive hybridization with the MCP-1 "antisense" complementary RNA probe is demonstrated by the clustering of silver grains on a SMC. There was little or no hybridization with the negative control, the MCP-1 "sense" noncomplementary RNA probe (Fig. 3 *B* and *D*). Thus, *in situ* hybridization substantiates the immunohistochemistry and further localizes the site of MCP-1 synthesis to the SMCs and the monocyte-like cells.

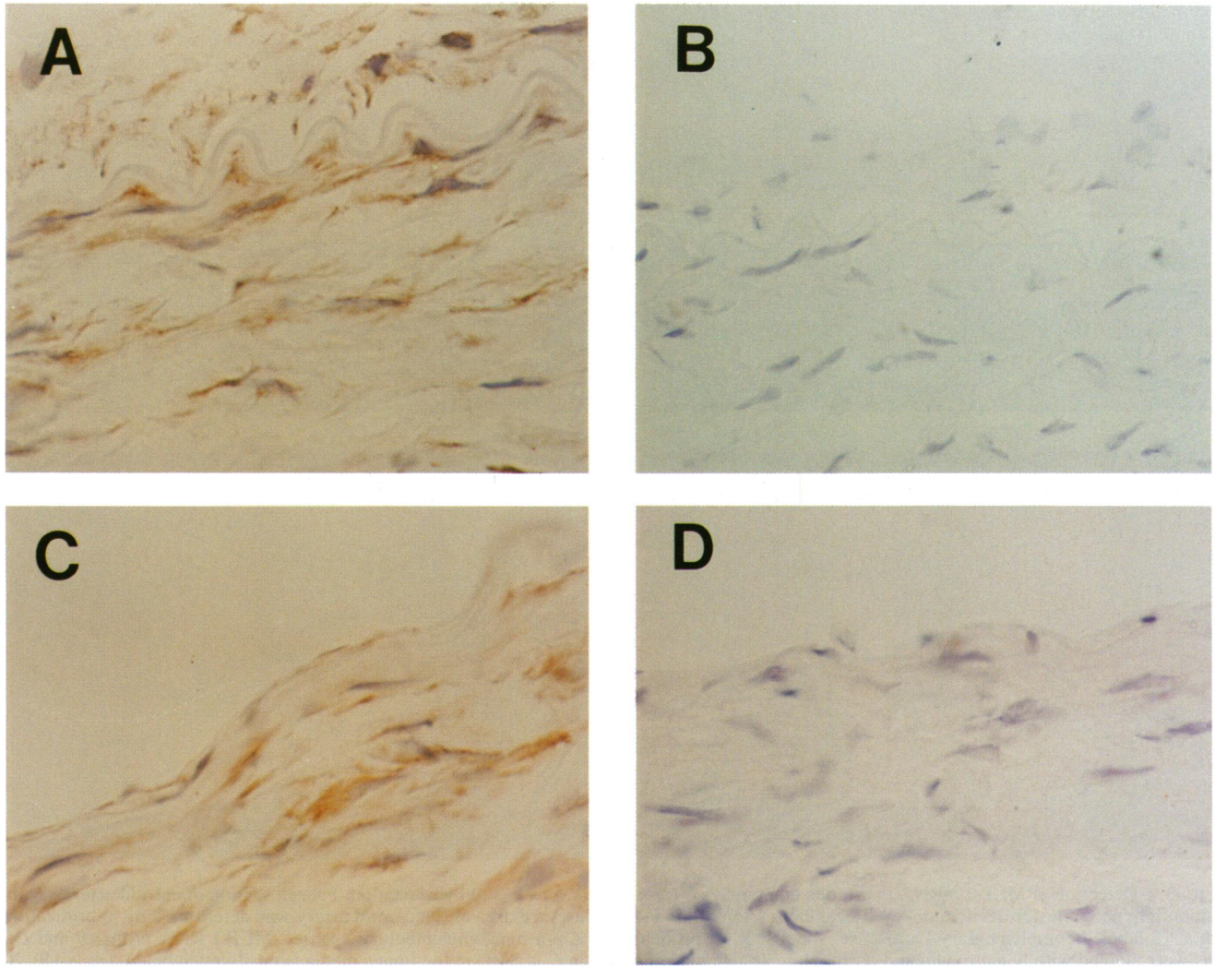

FIG. 2. Detection of MCP-1 protein in arteries of monkeys (*M. fascicularis*). Frozen sections of carotid arteries from an 11-month hypercholesterolemic monkey with (*A* and *B*) or without (*C* and *D*) visible intimal lesions were incubated with MCP-1 antisera (*A* and *C*) or MCP-1 antiserum plus excess purified MCP-1 protein as control (*B* and *D*). Sections were counterstained with hematoxylin. Antibodies were localized by immunoperoxidase staining. Positive staining appears brown in comparison with blue hematoxylin counterstain. Note typical spindle-shaped nucleus and smooth muscle morphology in positively stained cells in tunica media (lower portion of panels). (×640.)

DISCUSSION

In this primate model of atherosclerosis, dietary-induced hypercholesterolemia results in strong arterial expression of MCP-1 mRNA compared to normal control animals. Immunohistochemistry and *in situ* hybridization of carotid arteries demonstrate clearly that MCP-1 is synthesized by SMCs in the tunica media, identified by their location, at very early stages of formation of a lesion. For example, in the monkeys kept on the high cholesterol diet for 6–11 months, MCP-1 production was observed in nonovertly and overtly lesioned areas of the carotid. MCP-1 expression was also detected in monocyte-like and smooth muscle-like cells in the thickened intima. Intimal monocyte/macrophage infiltration and accumulation is a feature of both human and experimental atherosclerosis (21–24). Our results suggest that the intimal recruitment of the peripheral blood monocytes from the circulation is likely to occur, in part, as a response to a gradient of MCP-1, a major chemotactic substance for these cells, originating from SMCs within prelesioned areas of the vessel wall. Similar conclusions were drawn from a recent study by Nelken *et al.* (25) in which they observed elevated expression of MCP-1 by SMCs within human carotid endarterectomy specimens (25).

We have observed (17) that hypercholesterolemia in monkeys consistently led to formation of atherosclerotic lesions. Thus, hypercholesterolemia may either directly or secondarily induce expression of MCP-1. Studies *in vitro* have shown that MCP-1 mRNA and protein synthesis is increased by treatment of SMCs with minimally modified plasma low density lipoprotein (15). Other factors found to regulate MCP-1 expression that may play a role include growth factors and the inflammatory cytokines (11). In addition, other components of recruitment are likely to involve hemodynamic factors, plasma proteins, and local generation of cytokines, permeability factors, and cell adhesion molecules (26, 27). Secretion of additional MCP-1 by the infiltrating monocyte/macrophage or by the SMCs or endothelial cells in response to factors secreted by these inflammatory cells could then lead to amplification of the recruitment signal.

Whereas the results of our study and that of Nelken *et al.* (25) yielded similar findings, in a recent *in vivo* study by Ylä-Herttuala *et al.* (28), positive MCP-1 expression was detected only within the monocyte-derived macrophage of the intimal lesion in a rabbit hypercholesterolemia model and in human specimens from either organ donors or from medicolegal autopsy samples. The failure of Ylä-Herttuala *et al.* (28) to detect MCP-1 expression in SMCs in human

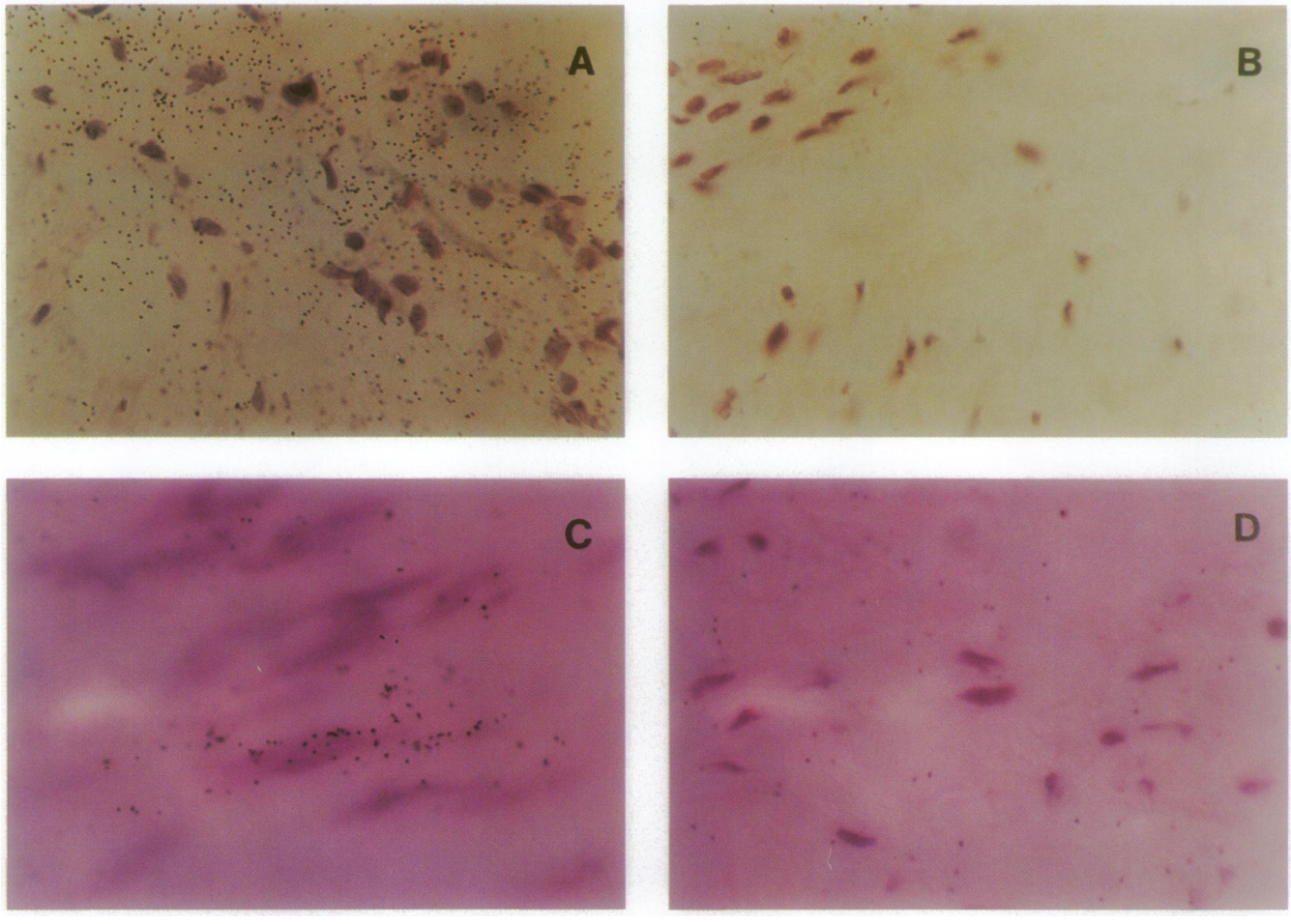

FIG. 3. Detection of MCP-1 mRNA in arteries of monkeys (*M. fascicularis*) by *in situ* hybridization. Frozen section of carotid arteries from a 6-month hypercholesterolemic monkey. Focal expression of MCP-1 in a lesioned area of the carotid artery was determined by hybridization with a ^{35}S-labeled complementary "antisense" MCP-1 RNA probe (*A* and *C*) or noncomplementary "sense" MCP-1 RNA probe (*B* and *D*). Cells were stained with hematoxylin and eosin. Note at high magnification in *C*, a SMC in the tunica media is positively labeled. (*A*, ×440; *B*, ×700.)

arteries with fatty streaks may be due to the fact that they did not examine patients with progressive coronary artery disease. These authors also did not detect MCP-1 expression in vascular SMCs of rabbits with diet-induced hypercholesterolemia. Our data demonstrated that in the hypercholesterolemic primate model, the SMCs of the tunica media express MCP-1 at both the transcriptional and translational levels. The discrepancy with our study might be due to the different animal models used for investigation. The species of animals and the specific arteries examined in these two studies are different. Differences in preparation of tissue for immunohistochemistry and in the cDNA probe for *in situ* hybridization may also contribute to the different conclusion reached. The results presented here clearly suggest that monocyte recruitment to the artery wall may be mediated by SMC secretion of MCP-1 that is induced by hypercholesterolemia during some or all stages of the disease.

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