Monocyte Chemoattractant Protein-1 in Human Atheromatous Plaques

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Abstract

Monocytes appear to be central to atherogenesis both as the progenitors of foam cells and as a potential source of growth factors mediating intimal hyperplasia, but the chemical messages which stimulate the influx of monocytes into human atheroma remain unknown. Monocyte chemoattractant protein-1 (MCP-1) is a recently described molecule with powerful monocyte chemoattractant activity expressed by monocytes, vascular endothelial cells, and smooth muscle cells in culture. To begin to address the role of MCP-1 in vivo, we examined 10 normal arteries and 14 diseased human arteries for MCP-1 expression by in situ hybridization. MCP-1 mRNA was detected in 16% of 10,768 cells counted in human carotid endarterectomy specimens with highest expression seen in organizing thrombi (33%) and in macrophage rich areas bordering the necrotic lipid core (24%) as compared to the fibrous cap (8%) and the necrotic lipid core itself (5%). Based on immunohistochemical staining of serial sections and on cell morphology, MCP-1 mRNA appeared to be expressed by vascular smooth muscle cells (VSMC), mesenchymal appearing intimal cells (MICs), and macrophages. By contrast, few cells expressing MCP-1 mRNA were found in normal arteries (< 0.1%). These data suggest a potential role for MCP-1 in mediating monocytic infiltration of the artery wall. (J. Clin. Invest. 1991. 88:1121–1127.) Key words: In situ hybridization • vascular smooth muscle cells • vascular disease • macrophages • cytokines

Introduction

Monocyte-derived foam cells are a major component of atheroma, comprising as much as 60% of cells found in the necrotic lipid core, and 10–20% of cells in the fibrous cap (1). These cells have been demonstrated not only in mature plaques but also in early fatty streaks in animal models of atherogenesis and in human disease (2). It has been suggested that growth factors produced by plaque macrophages may be important in terms of plaque progression (3). The signals which attract monocytes into these lesions remain unknown. Monocyte chemoattractant protein-1 (MCP-1) is a secreted molecule with potent chemotactic activity for monocytes (4). MCP-1 is expressed by cultured glioma cells (4), fibroblasts (5), monocytes (6), lymphocytes (7), endothelial cells (8), and smooth muscle cells (9). TNFα (5, 8, 10), IL-1β (5, 8, 10), γIFN (8), and endotoxin (5, 10) have all been shown to increase MCP-1 mRNA levels in cultured endothelial cells and fibroblasts. In addition, increases in MCP-1-like activity and MCP-1 mRNA have been documented in supernatants of human umbilical vein endothelial cells (HUVE’s) stimulated with serum enriched with minimally oxidized LDL and β VLDL (11, 12). These in vitro studies suggest a potential role for MCP-1 in the recruitment of monocytes into the vessel wall in the setting of inflammation or hyperlipidemia.

To address whether MCP-1 may play a role in recruiting monocytes to the vessel wall in vivo, we set out to identify MCP-1 mRNA production in human atheroma using in situ hybridization. Atherosclerotic plaques exhibited marked hybridization, especially in macrophage-rich inflammatory regions. By comparison, minimal expression was noted in control arteries without atherosclerotic disease.

To identify possible regulators of MCP-1 production in cells derived from human atheroma, vascular smooth muscle cells (VSMCs) grown from human carotid endarterectomy specimens were stimulated with various cytokines and MCP-1 mRNA levels were determined by Northern blot. Tumor necrosis factor (TNFα), gamma interferon (γIFN), and supernatants from mixed lymphocyte culture were shown to stimulate production of MCP-1 mRNA, whereas 10% FCS did not.

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1. Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; MIC, mesenchymal intimal cells; PCR, polymerase chain reaction; VSMC, vascular smooth muscle cell.


Methods

MCP-1 probe isolation. The cDNA for MCP-1 was cloned from stimulated human lung fibroblasts using polymerase chain reaction (PCR) amplification of cDNA made from cytoplasmic RNA. Human lung fibroblasts were obtained from explants obtained at autopsy. Cultures were maintained in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, and 25 mM Hepes, buffer pH 7.4. To increase MCP-1 mRNA levels, cells were stimulated with phorbol myristic acetate (100 ng/ml for 48 h), then with E. coli endotoxin (10 μg/ml, Sigma Corp., St. Louis, MO) in the presence of 1 μg/ml cycloheximide (Sigma Chemical Corp.) for 6 h (13). Total RNA was isolated using guanidium thiocyanate lysis and CsCl density gradient centrifugation (14). cDNA was then produced using 12–18mer oligo dT primers (Pharmacia Fine Chemicals, Piscataway, NJ) and reverse transcription followed by RTase A digestion. 30 cycles of PCR were performed (15) using 33mer oligonucleotide primers which primed the 5' and 3' ends of the coding region for MCP-1 and encoded protruding Xho I (5' primer) and Eco RI (3' primer) restriction sites. The PCR product was subcloned into
the Xho I and Eco RI sites of BlueScript SK-plasmid and its sequence
determined by dideoxy nucleotide sequencing (16). Primer sequences 
were 5' CCC CTC GAG TCC AGC ATG AAA GTC TCT GCC CTT 
for the 5' sequence, and 5' CCC GAA TTT TGT TCA AGT CTT CGG 
AGT TGG GTT for the 3' complementary sequence.

In situ hybridization. Human carotid endarterectomy specimens 
were obtained directly from the operating room, fixed for 2 h at 4°C in 
4% paraformaldehyde immediately upon surgical excision, cryopre-
served in 15% sucrose for 3 h and frozen in OCT at -70°C. To con-
struct riboprobes, Bluescript KS+ plasmid containing a full-length 
MCP-1 insert was made linear by restriction digestion with Eco RI or 
Xho I (for sense and antisense probes, respectively), then transcribed 
using T3 or T7 RNA polymerase (Stratagene Corp., La Jolla, CA) in 
the presence of 35S-UTP. In situ hybridization was performed on 10 μm 
frozen histologic sections as previously described (17, 18). After coating 
with photographic emulsion, sections were exposed for 4–12 wk. Ex-
posed sections were counterstained with hematoxylin and eosin.

The proportion of cells expressing MCP-1 was determined by 
counting 10,768 individual cells identified by nuclear staining at 400× 
in the 14 atherosclerotic specimens hybridized to the MCP-1 riboprobe 
(Fig. 1, e and f). A cell was considered positive if the number of silver 
grains associated with stained nuclei exceeded five times background 
over equivalent cell-sized areas without nuclei. For most sections we 
screened, cells with seven or more grains were considered positive.

Immunohistochemistry. Serial microscopic frozen sections imme-
diately adjacent to sections hybridized with the MCP-1 probe were used 
for immunohistochemical identification of cells in three atheroscle-
rotic specimens. Primary monoclonal antibodies included HAM56 
(Dako Corp., Carpinteria, CA) which reacts with ∼95% of the human 
monocyte/macrophage cell population (19). Cross-reactivity of this an-
tibody has been shown to capillary endothelium, hence, all slides 
stained with HAM56 were compared with sequential slides stained 
with Ulex europeaus lectin (Vector Laboratories, Inc., Burlingame, 
CA), which selectively stains endothelium (20). Vascular smooth mus-
cle cells were identified with HHF35 (Enzo Pharmaceuticals, New 
York, NY) to muscle actin which specifically reacts with cardiac, skele-
tal, and smooth muscle cells, but not fibroblasts, endothelium, neural 
tissue, or epithelium (21). T lymphocytes were identified with UCHL1 
(Dako Corp., Santa Barbara, CA) which reacts with CD45RO ex-
pressed by prestimulated and a subset of resting T cells (22). This anti-
body does not react with B cells or NK cells, although there is some 
cross-reactivity with cells of myoid and monocytic lineage (PMNs 
were rare in these specimens). Appropriate biotin-conjugated sec-
ondary antibodies were used and detected with avidin-alkaline phos-
phatase (ABC-AP kit; Vector Laboratories, Inc.) according to the manufac-
turer's instructions as reported previously (17, 18). Counterstaining 
was done with 0.4% methyl green (Sigma Corp.).

Cell culture. VSMCs were cultured in collagen matrix (Vitrogen; 
Collagen Corp., Palo Alto, CA) from human atheroma obtained at 
carotid endarterectomy using published techniques (23). Medium 
was changed twice weekly, and cells passed before reaching confluence. All 
cells were used within the first six passages.

Smooth muscle cell phenotype was confirmed by immunohisto-
chemical staining using HHF35 and fluorescein secondary antibody. 
Cultured cells stained positive for actin in a spiculated pattern typical 
for smooth muscle cells in 83±5% (95% confidence interval) of 195 
cells counted (not shown).

Northern blotting. VSMCs grown to 90% confluence in 150 cm² 
flasks were made quiescent by incubating for 48 h with serum-free 
medium after washing 3× with PBS. Thereafter, they were either stimu-
lated for 4 or 24 h with the cytokines or growth factors used in each 
specific experiment. Total cellular RNA was isolated using the guian-
dium isothiocyanate method (14). 10 μg of glyoxal-treated RNA was 
thenn run in a 1% agarose gel and transferred to nylon reinforced nitro-
cellulose (Schleicher & Schuell Corp., Keene, NH) as described (14). 
32P-labeled MCP-1 probe was produced using the random primer tech-
nique and T7 DNA polymerase (Stratagene Random Primer kit; Strata-
agene Corp., La Jolla, CA). Nitrocellulose membranes were prehybri-
dized for 4 h and hybridized at 42°C over night at a concentration of 106 
cpm/ml in buffer containing 50% formamide. Two washes using 2× SSC 
with 0.1% SDS at 55° and two high-stringency washes using 0.1× 
SSC with 0.1% SDS at 55° were performed for 15 min each. Nitrocellu-
lose was then exposed to Kodak X-OMAT film with an intensifying 
screen at ~70° for variable periods of time depending on estimated 
radioactivity of the nitrocellulose membranes.

Results

In situ hybridization. 14 carotid endarterectomy specimens, 
eight normal coronary arteries, and two normal internal mammary 
arteries were examined by in situ hybridization. Positive 
hybridization for MCP-1 was clearly present in all atheroma 
examined (Fig. 1, a, c, e). No cellular hybridization was seen 
in control slides probed with MCP-1 sense riboprobe (data not 
shown).

Expression of MCP-1 in histologically defined regions of 
human atheroma. Positive hybridization was detected in 16% 
of 10,768 cells counted. Random high-power fields among rep-
resentative histologic regions were scored using a counting grid

Figure 1. (a) A portion of atherosclerotic plaque showing extensive hybridization of MCP-1 antisense probe at 8 wk exposure. Area of inflammatory cell infiltrate in organizing thrombus hybridizes most strongly in this section. Darkfield polarized light epiluminescence view enhances silver grains corresponding to areas of hybridization (8-wk exposure; magnification, 100). (b) Same field as a in brightfield stained with hematoxylin and eosin showing fibrous cap area (right) adjacent to organizing thrombus in intraplaque hemorrhage (left). Infiltration of inflammatory cells can be seen between these two areas (magnification, 100). (c) Darkfield polarized-light epiluminescence view of carotid atherosclerotic plaque showing hybridization for MCP-1 in the inflammatory cell-rich area underlying the necrotic lipid core abutting the arterial media (above) (8-wk exposure; magnification, 100). (d) Adjacent frozen section to c stained with antimacrophage monoclonal antibody (HAM56) showing correlation of immunohistochemical staining for macrophages with areas of intense hybridization seen in e. Positive staining appears red com-
pared with methyl green counterstain (magnification, 100). (e) High-power darkfield polarized light epiluminescence view of an atherosclerotic plaque showing MCP-1 hybridization in a VSMC in the fibrous cap region. Discrete vascular smooth muscle cells were shown to hybridize 
strongly and were often surrounded by morphologically similar cells which did not hybridize (8-wk exposure, magnification, 500). (f) Same field as c in lightfield view for comparison, showing an area with a high percentage of smooth muscle cells (positive HHF35 staining on serial section not shown). Note typical elongated nucleus and smooth muscle morphology in hybridized cell seen in e (g) Section of normal internal mammary artery obtained from coronary bypass surgical specimens hybridized with MCP-1 antisense probe. Very low levels of hybridization were seen in normal vessels. Examination of this section under high power revealed only four cells hybridizing to MCP-1 probe (arrows). The other bright areas in the adventitia represent refractive fibrous tissue, a normal feature of the adventitia when viewed by epiluminescence (8-wk exposure; magnification, 100). (h) Section of control coronary artery obtained from cardiomyopathy patient during heart transplantation (native heart) hybridized with MCP-1 antisense probe. Hybridization was rare in these specimens. One smooth muscle-appearing cell can be seen in the media 
with higher than background hybridization (arrow). Concentric fibrous intimal thickening was a universal finding in adult coronary specimens, however, it was not associated with hybridization of probe (8-wk exposure; magnification, 125).

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Table I. Comparison of Percentage Nuclei Associated with In Situ Hybridization to MCP-1 mRNA

<table>
<thead>
<tr>
<th>Morphologic subdivision</th>
<th>Positive cells/total cells</th>
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<tbody>
<tr>
<td>Fibrous cap (uniform portion)</td>
<td>76/1471 (5%)</td>
</tr>
<tr>
<td>Fibrous cap (pleomorphic portion)</td>
<td>288/2871 (10%)</td>
</tr>
<tr>
<td>Inflammatory area underlying necrotic core</td>
<td>482/1999 (24%)</td>
</tr>
<tr>
<td>Necrotic core</td>
<td>28/475 (6%)</td>
</tr>
<tr>
<td>Organizing thrombus</td>
<td>342/1035 (33%)</td>
</tr>
<tr>
<td>Immunohistochemical subdivision</td>
<td></td>
</tr>
<tr>
<td>HAM56 positive cells</td>
<td>439/1163 (38%)</td>
</tr>
<tr>
<td>HHF35 positive cells</td>
<td>82/1754 (5%)</td>
</tr>
<tr>
<td>Total</td>
<td>1737/10768 (16%)</td>
</tr>
</tbody>
</table>

A cell was considered positive if the number of silver grains associated with stained nuclei exceeded five times background over equivalent cell-sized areas. For most sections we screened, cells with seven or more grains were considered positive. Both morphologic and immunohistochemical criteria were used for subdivision of tissues. For the morphologic sub divisions, uniform and pleomorphic portions of the fibrous cap were seen to have different morphologic features and to hybridize to different degrees (see text for details). For the immunohistochemical sub divisions, HAM56 identified cells of monocyte lineage (primarily macrophages), and HHF35 identified VSMCs. Results show a preponderance of hybridization to macrophages, inflammatory areas underlying the necrotic lipid core, and organizing thrombus. Vascular smooth muscle, however, was also seen to hybridize but to a lesser degree.

Regions of the fibrous cap with uniform spindle-shaped cells generally showed uniform staining with the HHF35 antibody. Fibrous cap regions with pleomorphic nuclei often exhibited fewer VSMCs staining with HHF35 and frequently contained HAM56 and a few UCHL1-positive cells as well. MCP-1-positive cells in the fibrous cap included a population of mesenchymal appearing intim al cells (MIC) often in pleomorphic zones which did not co-stain with any of the antibodies used in this study (Fig. 2). These cells had large pale nuclei distinct from the typical spindle-shaped smooth muscle cell nuclei, a stellate cytoplasm, and were morphologically similar to the platelet-derived growth factor (17) or tissue factor (18) mRNA-containing MICs described previously in atherosclerotic plaques. Many areas within the atherosclerotic plaques examined contained numerous cells meeting these criteria especially in the pleomorphic regions of the fibrous cap and areas of organizing thrombi. It is difficult to estimate the proportion of positive MICs because their detection is by a process of exclusion rather than direct identification.

**Organizing thrombus.** Many cells hybridized strongly to the MCP-1 probe in areas of focal organizing thrombi (33% of 1,035 cells; Fig. 1, a and b). These regions often contained many HAM56-positive cells with some UCHL1-positive T cells. Spatial correlation of HAM56-positive cells and cells hybridizing for MCP-1 strongly suggests that macrophages express MCP-1. We cannot exclude the possibility that some expression in these areas is by MICs because these cells are often found in areas of focal organizing thrombus (24, 25).

**Necrotic lipid core.** Discrete cells in the necrotic lipid core were markedly positive for MCP-1 mRNA (6% of 475 cells) and were often adjacent to nonhybridizing cells. The necrotic cores exhibited diminished cellular density and contained predominantly necrotic debris, foam cell deposits, cholesterol clefts, and calcification. In agreement with previous studies macrophages were the predominant cell type found in the necrotic core although UCHL1-positive cells were also identified in these regions (1).

**Inflammatory regions bordering the necrotic lipid core.** Of all the regions studied the most intensely hybridizing MCP-1-positive cells were found in inflammatory regions bordering the necrotic core. Positive cells were relatively abundant, representing 24% of 1,999 cells counted. These inflammatory regions contained many HAM56-positive cells with a few scattered UCHL1-positive cells. Histologically identified foam cells were often observed to hybridize to the MCP-1 probe in these areas.

**Expression of MCP-1 by cell type.** To identify specific cell types associated with MCP-1 production, serial microscopic sections immediately adjacent to sections hybridized with the MCP-1 probe were used for immunohistochemical staining with HAM56, UCHL1, HHF35, and Ulex lectin in three representative specimens. Carefully chosen regions which stained exclusively with HAM56 were found to be strongly positive for

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**Figure 2.** (a) Brightfield epiluminescent view of cellular area with a high proportion of immunohistochemically unstained mesenchymal appearing intimal cells (MICs) in carotid atherosclerotic plaque (arrows). Nuclei are visible as the dark hematoxylin-stained bodies surrounded by bright silver grains. Hematoxylin and eosin section; magnification, 200. (b) Adjacent section to a stained with HAM56, showing lack of tissue macrophages in this section (typical cross-reactivity to endothelium by this antibody is also illustrated). (c) Serial section stained with HHF35 for actin-containing VSMCs. Some positive cells were noted, but do not appear to be related to those hybridizing for MCP-1 in this section. (d) Serial section stained with UCHL1 showing absence of T cells in this section. Positive staining for each monoclonal antibody appears red, compared with methyl green counterstaining. Lack of immunohistochemical staining in areas of marked hybridization indicates the presence of MICs.
MCP-1 (38% of 1,754 cells). Cells meeting histologic criteria for macrophages, including large pale nuclei with foamy cytoplasm, clearly hybridized with MCP-1 riboprobe, whereas hemosiderin containing macrophages did not. Fewer MCP-1 positive cells were found in regions staining exclusively with HHF35 (5% of 1,163 cells). These areas showed no HAM56 staining. Despite the low percentage of cells hybridizing in these areas, individual cells clearly meeting histologic criteria for VSMCs, including spindle-shaped nuclei and elongated cytoplasm, hybridized for MCP-1 (Fig. 1, e and f). It is not possible to comment on the colocalization of T cell staining with MCP-1 hybridization because no region was found to stain exclusively with UCHL1 in the absence of HAM56 staining. These data suggest that both VSMCs and macrophages can express MCP-1 in vivo. In addition, cells not staining with HAM56 or HHF35, meeting the histologic criteria for MICs were also seen to hybridize strongly for MCP-1.

We failed to detect MCP-1 hybridization to endothelial cells lining the vasa vasorum or on the luminal surface of the plaques. Given the fact that the total amount of endothelium seen in these specimens was small due to transverse sectioning, lack of endothelium in portions of the plaque and in some cases loss of endothelium in preparation, conclusions regarding MCP-1 expressed by the endothelium in vivo cannot be drawn from this study.

Normal, nondiseased internal mammary artery specimens obtained from coronary bypass operations exhibited little production of MCP-1 mRNA as determined by in situ hybridization. Hybridizing cells, if present, tended to be found in the adventitia (Fig. 1 g). Occasional macrophage staining was seen in the adventitia suggesting that hybridizing cells may be scattered tissue macrophages. Like the internal mammary arteries, normal coronary vessels obtained from explanted hearts also showed only minimal MCP-1 expression (Fig. 1 h). These specimens were obtained from nonatherosclerotic coronary arteries in patients with idiopathic cardiomyopathies undergoing heart transplantation. A small amount of concentric intimal thickening which was invariably present in these specimens but no advanced atherosclerotic lesions were seen. Hybridization was found in < 0.1% of cells counted in normal vessels.

**Cytokine stimulation of VSMCs in culture.** The finding that VSMCs expressed MCP-1 in vivo suggested a possible role for smooth muscle–produced MCP-1 in initiating or enhancing monotypic infiltration of the vessel wall. To begin to examine factors potentially responsible for regulation of MCP-1 expression by VSMCs, Northern blot analysis was performed on cytokine-treated human VSMCs in culture. Northern blot analysis of 10 μg of total cellular RNA per sample revealed a very low level of detectable MCP-1 RNA in resting cells as well as those stimulated with 10% FCS. Supernatants from a mixed lymphocyte culture (donated by Bruce Hall, Stanford University, Palo Alto, CA) however, produced a marked increase in MCP-1 mRNA detected at 4 and 24 h (data not shown). Inflammatory regulators TNFα (200 U/ml) and γIFN (50 U/ml) were then investigated alone and in the presence of 5% FCS. Both cytokines stimulated marked increases in the level of MCP-1 RNA (Fig. 3). TNFα elicited greater increases at 24 h than at 4 h, whereas γIFN generated maximal response at 4 h with diminution over time. The differing tempos of MCP-1 induction after TNFα and γIFN stimulation presumably reflect different mechanisms of action. There was no appreciable difference in the responses to cytokines seen in the presence or absence of fetal calf serum.

**Discussion**

Human monocyte chemoattractant protein-1 (MCP-1) is a secreted peptide which exhibits powerful monocyte chemoattractant properties at nanomolar concentrations. It has been detected in smooth muscle cells, endothelium, stimulated monocytes, lymphocytes, fibroblasts, and various tumor cell lines (6). Because the signals which attract monocytes to the atherosclerotic plaque remain unknown, we examined arteries for MCP-1 production in vivo. We have shown that there is easily detectable MCP-1 expression by multiple cell types in various regions of the atherosclerotic plaque, but little expression in normal vessels.

The majority of MCP-1 production was seen in macrophages. MCP-1 was noted in all regions of the plaque with high concentrations of macrophages, especially underlying necrotic lipid cores and in organizing thrombi. The production of MCP-1 by macrophages may represent an amplification mechanism for the recruitment of additional macrophages to the atherosclerotic plaque.

In contrast to macrophages, VSMCs less frequently hybridized with MCP-1 probe. Individual VSMCs that hybridized strongly were sometimes surrounded by nonexpressing cells. Expression in these cells was not dependent on the proximity of tissue macrophages; hybridizing VSMCs were found in areas...
both with and without HAM56 staining. Because signals which stimulate MCP-1 production in VSMCs are not known and because MCP-1 production by VSMCs may be involved in the initial recruitment of macrophages to the vessel wall, we explored cultured atheroma-derived VSMCs' TNFα and γIFN, then determined MCP-1 mRNA levels by Northern blot. The increase in MCP-1 expression induced by γIFN and supernatants of mixed lymphocyte culture suggest a possible role for activated T lymphocytes in stimulation of vascular smooth muscle MCP-1 production.

The production of MCP-1 by MICS adds to the increasing evidence suggesting that these cells secrete a number of bioactive factors (17, 18). The origin of MICS and their role in atherogenesis remain unknown.

The identification of VSMCs and MICS as sources of MCP-1 in atherosclerotic plaque suggests that this molecule may potentially play a role in monocyte infiltration in the early development of the plaque. Mediators stimulating monocyte infiltration into early fatty streaks are unknown, but these lesions have been identified as possible precursors to mature atherosclerotic plaques (3). It is tempting to speculate that MCP-1 production by VSMCs or MICS may be elicited by lipopolysaccharide (11) or other stimuli in early atherosclerotic lesions. With infiltration of monocytes, a positive feedback mechanism might exist whereby infiltrating monocyte/macrophages secrete MCP-1 in large amounts, thereby attracting more peripheral blood monocytes to the developing atherosclerotic plaque. Antagonists of MCP-1 action will be needed to evaluate any causal role for MCP-1 in vivo.

In summary, we have shown that MCP-1 mRNA is expressed in atherosclerotic plaques in vivo. To our knowledge, this is the first study to demonstrate the production of a specific monocyte chemotactic agent in human atheroma.

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