Dietary Cholesterol-Induced Changes in Macrophage Characteristics

Relationship to Atherosclerosis

KEM A. ROGERS, PhD, RICHARD L. HOOVER, PhD, JOHN J. CASTELLOT, Jr., PhD, JOHN M. ROBINSON, PhD, and MORRIS J. KARNOVSKY, MB, BCh, DSc

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts

In diet-induced hypercholesterolemia, circulating monocytes adhere to the endothelium of the vessel wall and emigrate into the intima. Atherosclerotic lesions may develop, characterized by the presence of lipid-laden macrophages and proliferating smooth muscle cells recruited from the media. Using rat peritoneal macrophages, the authors examined the influence of diet-induced hypercholesterolemia on several variables of macrophage function that may contribute to lesion formation, including adhesion to bovine aortic endothelial cells (BAECs) and vascular smooth muscle cells (VSMCs), the production of chemoattractants and mitogens for VSMCs, and the release of the reactive oxygen species, superoxide. In general, a hypercholesterolemia-induced augmentation of macrophage function was observed. In comparison with macrophages from normal animals (N MØs), macrophages from hypercholesterolemic animals (H MØs) were 50–80% more adhesive to BAECs and VSMCs. H MØ-secreted products increased VSMC migration 6 to 7-fold, whereas N M0s only stimulated motility 2.5-fold. In addition, H MØ-conditioned media produced increased VSMC growth 5-fold, compared with a 2.5-fold increase produced by N MØ-conditioned media. Although the production of superoxide was found to be the same for both N MØs and H MØs, the release of superoxide by macrophages found in the intima of hypercholesterolemic animals may contribute to the necrosis of cells in the developing lesion. These results suggest that dietary cholesterol may accelerate atherosclerotic lesion formation by inducing specific changes in the properties of circulating monocytes and intimal macrophages. (Am J Pathol 1986, 125:284–291)

THE "RESPONSE TO INJURY" hypothesis postulates that, after endothelial dysfunction or injury, macrophages and/or platelets accumulate at the injured site and release factors that promote atherosclerotic lesion formation.[1-5] Diet-induced hypercholesterolemia, which is thought to injure vascular endothelium,[4-6] is accompanied by the adhesion of monocytes to the endothelium of large arteries, followed by their invasion into the intima and modulation into lipid-laden macrophages, accumulations of which form the fatty-streak precursors of atherosclerotic lesions.[7-13] As the lesion progresses, vascular smooth muscle cells (VSMCs) migrate from the media into the intima, proliferate, and become filled with lipid. With time, foci of necrosis and inflammation characteristic of the fibrous plaque also may develop in the lesion (for reviews see Ross[5] and McGill[14]).

Recent evidence suggests that intimal macrophages may be responsible for the recruitment and subsequent proliferation of medial smooth muscle cells in the intima. *In vitro* experiments have identified smooth muscle cell chemotactic factors[15] that can be produced by macrophages.[16,17] In addition, cultured macrophages release mitogenic substances that stimulate the growth

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Address reprint requests to Morris J. Karnovsky, MB, BCh, DSc, Department of Pathology, Harvard Medical School, 25 Shattuck St., Boston, MA 02115.

of VSMCs.[18,19] These results suggest that the intimal macrophage is involved in orchestration of events that lead to the development of the mature atherosclerotic lesion.

The mechanism by which hypercholesterolemia contributes to atherogenesis remains unclear. In this article, we have examined the influence of hypercholesterolemia on a number of properties of elicited rat peritoneal macrophages, including adhesiveness to various substrata, release of substances that are either chemotactic or mitogenic for VSMC, and production of reactive oxygen species that may damage intimal cells.[20] The results suggest that the monocyte may provide an important link between disorders of lipid metabolism and the pathogenesis of atherosclerosis.

Materials and Methods

Animals

Fifty male Sprague–Dawley rats (Charles River, Wilmington, Mass; CD strain), weighing 150–200 g, were fed a diet consisting of Purina standard laboratory rat chow with or without added cholesterol (4%) and cholic acid (1%) for a period ranging from 2 to 3 weeks. Twenty-five rats were used in each group. The diets were prepared by Teklad Diets (Madison, Wis). Total serum cholesterol levels were measured once a week spectrophotometrically with the use of Sigma kit # 351.

Cell Isolation and Culture

Macrophages

Elicited rat macrophages were obtained from the peritoneal cavity 4 days after 3–5 ml of sterile 4% thioglycollate broth was injected. Isolated cells were rinsed twice in Hanks balanced salt solution buffered with 15 mM HEPES, pH 7.4 (Hanks–HEPES). More than 90% of the cells thus obtained were determined to be macrophages by the cytochemical demonstration of alpha-naphthyl esterase in the cytoplasm.[21] Macrophage-conditioned media were prepared by the cells being plated at a concentration of 1×10^6/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. At 24 or 48 hours, the cell debris was removed by filtration of the material through a 0.45-μm filter, and the conditioned media were stored at −20 C until used.

Endothelial Cells

Bovine aortic endothelial cells (BAECs) were isolated according to the methods of Booyse et al.[22] Briefly, the aortas were washed and incubated with collagenase (1 mg/ml) for 20 minutes at 37 C, and the endothelial cells were washed off the vessel wall with calcium–magnesium-free Hanks balanced salt solution. The cells were then washed twice with Hanks-HEPES and plated into tissue culture dishes in RPMI 1640 supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone (Gibco). Primary cultures of the cells were used in all experiments; they were not passaged.

Vascular Smooth Muscle Cells

Rat VSMCs from the aortas of normocholesterolemic Sprague–Dawley rats were isolated, cultured, and characterized as previously described.[23,24] Briefly, the abdominal aorta was removed and the adventitia cleaned away with the aid of a dissecting microscope. The aorta was then cut longitudinally, and small pieces of media were stripped from the vessel wall and placed in 60-mm tissue culture dishes in RPMI 1640 supplemented with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Within 1–2 weeks, rat VSMCs, began to migrate from the explants and were passaged 1 week after the first appearance of cells. Cells were used between the first and fourth passage.

Cebus monkey VSMCs were isolated from explants of aorta, as described by Ross.[25] They were characterized in the same manner as rat VSMCs[23,24] and were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were used while between the first and fourth passage.

Mitogenesis Assays

The mitogenic activity of peritoneal macrophage-conditioned media was assayed as previously described for VSMCs.[26,27] Briefly, rat or cebus monkey VSMCs were plated into 24-well (16-mm) multiwell plates at 7.5×10^3 cells per well in normal growth media. After 24 hours, these media were replaced with RPMI 1640 (rat VSMCs) or DMEM (cebus monkey VSMCs) containing 0.4% FBS. This treatment was continued for 24–48 hours, at which time the cells are growth arrested in G_0, as determined by cytofluorimetic and other criteria[27,28] Control cultures were released from the G_0 block by exposure to normal growth medium (DMEM with 10% FBS). Experimental cultures were exposed to a 1:1 mixture of growth medium and macrophage-conditioned media (final FBS concentration: 10%). Estimates of cell number were made by the cells being trypsinized and counting them with a Coulter Counter at 1, 3, and 5 days after release from G_0.

Macrophage Adhesion Assay

Adhesion of peritoneal macrophages to various substrata was assessed with the use of the monolayer col-

lection assay developed by Walther et al.[29] Macrophages were isolated as described above, resuspended in Hanks–HEPES at a concentration of 5×10^6 cells/ml, labeled at 4 C for 1 hour with chromium-51 (20 μCi/ml of cell suspension), rinsed, and resuspended in cold buffer at the desired concentration. Chromium-51-labeled macrophages suspended in Hanks–HEPES at a concentration of 2×10^5 were added to confluent monolayers of rat VSMCs, BAECs, or tissue culture grade plastic, followed by incubation at 37 C for 5, 10, 15, 20, 30, and 45-minutes. The monolayers were rinsed twice with the same buffer and the percentage of adherent cells calculated as the ratio of the radioactive counts per minute remaining after washing to the total radioactive counts per minute added to each well.

Chemotaxis Assays

Chemotaxis assays were performed with 25 μl modified blind-well Boyden chambers (Neuro Probe), as previously described.[30] Briefly, 5-μm-pore-diameter polycarbonate filters were soaked in 0.5 M acetic acid overnight at room temperature. The filters were washed with distilled water and incubated for at least 2 hours in 100 μg/ml gelatin solution at room temperature. The gelatin-coated filters were air dried and incubated in 10 μg/ml fibronectin solution (Collaborative Research) for 1–2 hours at room temperature and air dried. The test substance, which always contained 10% FBS, was placed in the bottom chamber. All of the serum was from the same lot. The filter was inserted, and freshly trypsinized VSMCs (5×10^4 per assay) in DMEM containing 10% FBS were added to the top chamber. The chambers were incubated for 3 hours at 37 C in a humidified 5% CO_2/95% air atmosphere. The filters were removed and fixed in methanol for 15 minutes at room temperature. We carefully removed the cells on the upper filter surface by rubbing them with a cotton swab. The filters were stained with 1% Giemsa and mounted on slides. The number of cells on the lower filter surface was determined by the counting of at least 10 high-power (×430) fields with the use of a light microscope.

To distinguish between chemotaxis and chemokinesis, we performed checkerboard analyses,[31] in which cells are exposed to the motility-stimulating substance both in the presence and absence of a gradient. In these experiments, different concentrations of conditioned medium were placed in the top and bottom chambers. In assays to which conditioned medium was added to the top chamber, the trypsinized VSMCs were taken up directly into the desired concentration of conditioned medium.

Table 1—Influence of Diet on Weight and Serum Cholesterol

	Normal diet		CC diet	
Week	Weight (g)*	Serum cholesterol (mg/dl)*	Weight (g)*	Serum cholesterol (mg/dl)*
0	158 ± 2	65 ± 2	158 ± 2	65 ± 2
1	217 ± 5	65 ± 5	232 ± 3	491 ± 26
2	271 ± 4	60 ± 3	271 ± 3	311 ± 21
3	314 ± 6	56 ± 2	317 ± 6	223 ± 19

* Plus or minus standard error of the mean.

Superoxide Release

Superoxide (O_2^-) release was measured as previously described.[32] Briefly, O_2^- release was measured continuously at 37 C with the use of the O_2^- dismutase-inhibitable reduction of ferricytochrome c at 550 nm in a double-beam spectrophotometer.[33,34] The standard assay mixture (1.0 ml) consisted of Hanks–HEPES containing 0.075 mM ferricytochrome c and macrophages at a concentration of 1.0×10^6/ml. Cells were incubated in the assay mixture for 3 minutes at 37 C before O_2^- release was initiated with the addition of phorbol-12-myristate-13-acetate (PMA) at a final concentration of 30 ng/ml. In addition to the standard assay mixture, the reference cuvette contained 25 μg of O_2^- dismutase. The rates of O_2^- release were calculated from the linear portion of the reaction progress curves.

Results

Serum Cholesterol Analysis

To ensure that rats on the cholesterol–cholic acid (CC) diet were indeed hypercholesterolemic but otherwise healthy, we measured serum cholesterol levels and weight at weekly intervals. Control rats and rats fed the CC diet gained weight steadily and at the same rate (Table 1). In the control rats, serum cholesterol remained at the normal level of 55–65 mg/dl. Marked hypercholesterolemia developed in the rats fed the CC diet after 1 week, with seven to eight times the serum cholesterol levels of control animals. Serum cholesterol levels in the CC-fed rats decreased slightly during the time course of the experiment but at 3 weeks were still four times that found in control animals (Table 1). Peritoneal macrophages were collected from control animals and animals maintained on the CC diet for 2 to 3 weeks.

Macrophage Adhesion

Monocyte adhesion to the vessel wall may be one of the first steps in the formation of diet-induced atherosclerotic lesions. For investigation of the influence of diet on macrophage–vascular cell interaction,

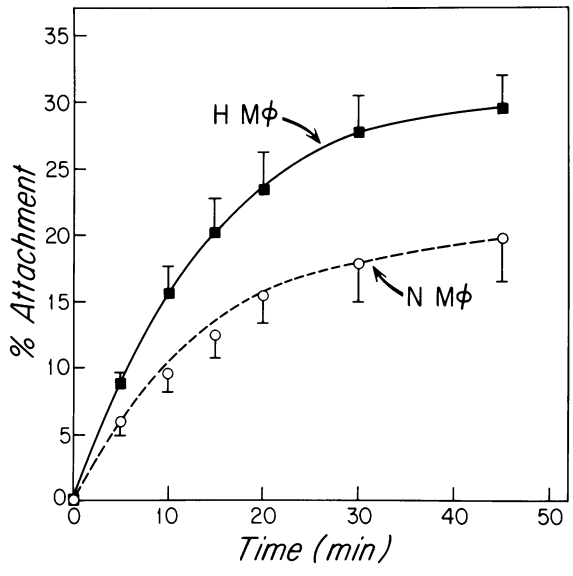

Figure 1—Time course of adhesion of N MØs (*open circles*) and H MØs (*squares*) from rats to BAECs *in vitro*. Chromium-51-labeled macrophages were plated onto confluent monolayers of BAECs and the nonadherent cells washed off at various times with buffer. Percentage attachment was calculated by division of the total radioactive counts per minute remaining in each well by the number of radioactive counts added. Note that H MØs adhere at a faster rate and to a greater extent than do N MØs. *Error bars* represent standard error of the mean.

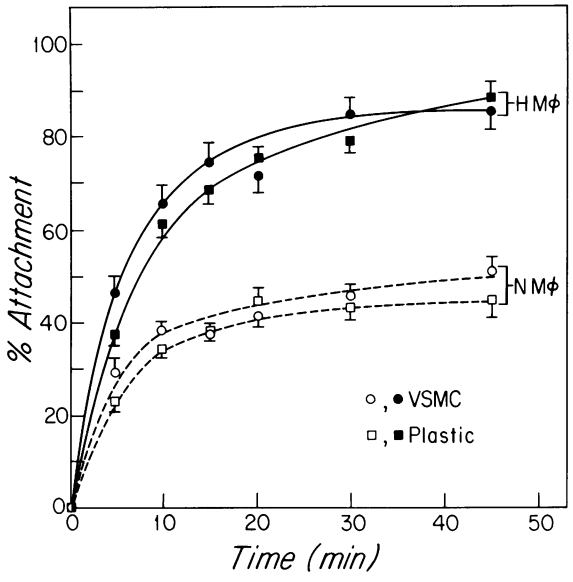

Figure 2—Time course of adhesion of N MØs (*open symbols*) and H MØs (*closed symbols*) from rats to rat VSMCs (*circles*) and tissue culture grade plastic (*squares*). The adhesion assay was carried out as described in **Figure 1**. H MØs adhered to both VSMCs and plastic at a faster rate and to a greater extent than did N MØs. Note that adherence of each cell type was not influenced by the substratum tested. *Error bars* represent standard error of the mean.

thioglycolate-elicited peritoneal macrophages from both normal (N MØs) and hypercholesterolemic (H MØs) rats were examined for their adhesiveness to BAECs, rat VSMCs, and plastic over a 45-minute time course. In general, the initial rate of attachment of H MØs was 40% higher than that found for N MØs (Figures 1 and 2). In addition, the total number of adherent H MØs at 45 minutes was 50% higher on BAECs (Figure 1) and 80% higher on plastic and rat VSMCs (Figure 2) than normal cells.

The extent of macrophage adhesion also varied with the substratum used. Both N MØs and H MØs were 2.5–3.0 times more adherent to rat VSMCs than to BAECs (Figures 1 and 2). However, both the rate and extent of adhesion to plastic was indistinguishable from adhesion to rat VSMCs (Figure 2).

Smooth Muscle Cell Chemotaxis

Migration of VSMCs from the media to the intima contributes significantly to the development of myointimal thickenings in the vessel wall. For examination of the influence of hypercholesterolemia on the production of chemotactic factors by rat peritoneal macrophages, media conditioned for 24 or 48 hours with N MØs or H MØs were tested with the use of both rat and cebus monkey VSMCs as the target cells in a modified Boyden chamber assay.[30,31,35,36] VSMCs suspended in unconditioned media (DMEM supplemented with 10% FBS) were added to the top chamber, and the substance to be tested was placed below the filter in the bottom chamber. We counted the number of cells that moved to the lower filter surface when the bottom chamber also contained unconditioned media to determine background migration.

Migration of both cell types was enhanced 2.5-fold by N MØ-conditioned media. In contrast, H MØ-conditioned media increased VSMC migration 5.5- to 7-fold over background. No significant difference in cell migration was detected when media conditioned for 24 hours were compared with media conditioned for 48 hours in each of the two VSMC groups (Figure 3).

To determine if the enhancement of smooth muscle cell migration by H MØ-conditioned media resulted from stimulation of chemokinesis, chemotaxis, or both, we performed a checkerboard analysis in which rat VSMCs were exposed to the test substance both in the presence and absence of a concentration gradient. When the lower chamber contained unconditioned media, the number of cells on the lower surface of the filter decreased with increasing concentrations of conditioned media in the upper chamber. When the upper chamber contained unconditioned media, the number of cells on the lower surface of the filter increased with increasing concentrations of conditioned media in the lower chamber. When the top and bottom wells contained

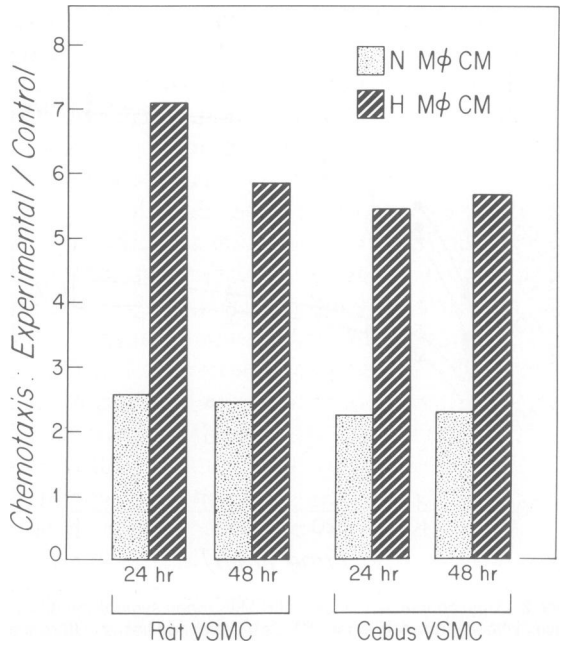

Figure 3—Chemotaxis of rat and cebus VSMCs in the presence of media conditioned by macrophages from normal (N MØ CM) or hypercholesterolemic rats (H MØ CM). The data are presented as the ratio of the number of cells migrating to the lower surface of the filter in the presence of N MØ CM or HMØCM to the number migrating in the presence of unconditioned media (*Experimental/Control*). The test media were conditioned for 24 to 48 hours. The data shown are derived from at least three separate experiments (each sample run in duplicate) with each of five different lots of MØ CM. In each case, H MØ CM were significantly more chemotactic than N MØ CM ($P < 0.01$).

equal concentrations of conditioned media, there was no increase in the number of cells on the lower surface of the filter in comparison with background. Thus, it appears that the enhancement of smooth muscle cell migration by conditioned media is a chemotactic phenomenon.

Smooth Muscle Cell Mitogenesis

VSMC proliferation is thought to be one of the most important events in the development of the atherosclerotic plaque.[4,5] It has been shown *in vitro* that macrophages produce mitogenic factors for VSMCs.[18,19,37] To allow us to determine whether hypercholesterolemia influences the production of these mitogens, media conditioned for 24 or 48 hours with N MØs or H MØs were examined for their ability to stimulate VSMC proliferation. The results are expressed as a ratio of cell growth in conditioned media to cell growth in nonconditioned media.

Rat VSMC growth was similar in both 24-hour and 48-hour macrophage-conditioned media (Figure 4). N MØ-conditioned media stimulated cell growth 1.5- to 2-fold on Day 1 and 2- to 2.5-fold on Days 3 and 5. In comparison, H MØ-conditioned media stimulated cell growth 2.5- to 3-fold on Day 1; 3- to 4-fold on Day 3, and more than 5-fold on Day 5. Therefore, in comparison with N MØ-conditioned medium, H MØconditioned medium was at least twofold more mitogenic for rat VSMCs.

The mitogenic effects of 24-hour N MØ-conditioned media on cebus monkey VSMCs was the same as that described for rat VSMCs (Figure 5). Although 48-hour H MØ-conditioned media were somewhat less mitogenic, they were still 1.6 times as effective as 48-hour N MØ-conditioned media in stimulation of cebus monkey VSMC growth on Day 5.

O_2^- Release by Peritoneal Macrophages

Because macrophages are capable of producing reactive oxygen species, compounds that can damage cells,[38] it is possible that O_2^- release plays a role in the necrosis of cells present in atherosclerotic lesions. Peritoneal macrophages release O_2^- *in vitro* only when they are stimulated with a suitable agent, such as PMA.[39] After an initial lag phase of about 15 seconds, there is a maximal linear phase of O_2^- release that lasts 2–3 minutes. The maximal rates of O_2^- release after PMA stimula-

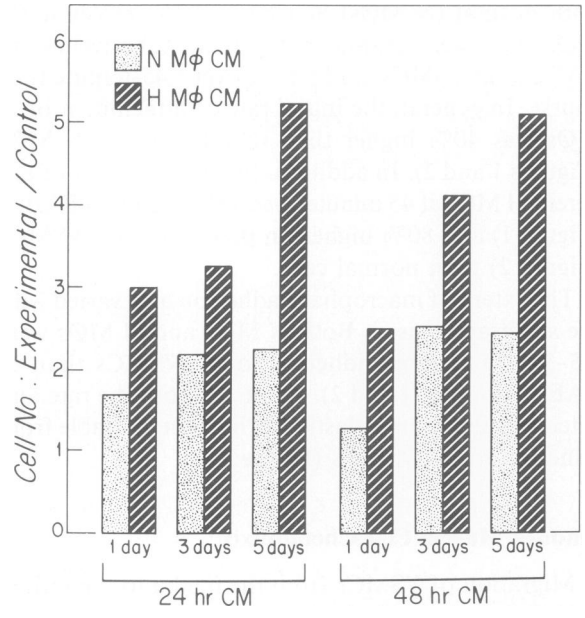

Figure 4—Effect of media conditioned by peritoneal macrophages from normal (N MØ CM) or hypercholesterolemic (H MØ CM) rats on rat VSMCs growth. The test media were conditioned for 24 to 48 hours and added to rat VSMC growth arrested in G_0. The data are presented as a ratio of the cell number in the conditioned media to the cell number in unconditioned media (*Experimental/Control*) on Days 1, 3, and 5. The starting cell number in each sample averaged 12×10^3. In the control cultures, the cell number on Days 1, 3, and 5 averaged 18.3×10^3, 51.6×10^3, and 66.4×10^3, respectively. The data shown are derived from at least three separate experiments. All of the values for H MØ CM were significantly greater than N MØ CM control ($P < 0.01$).

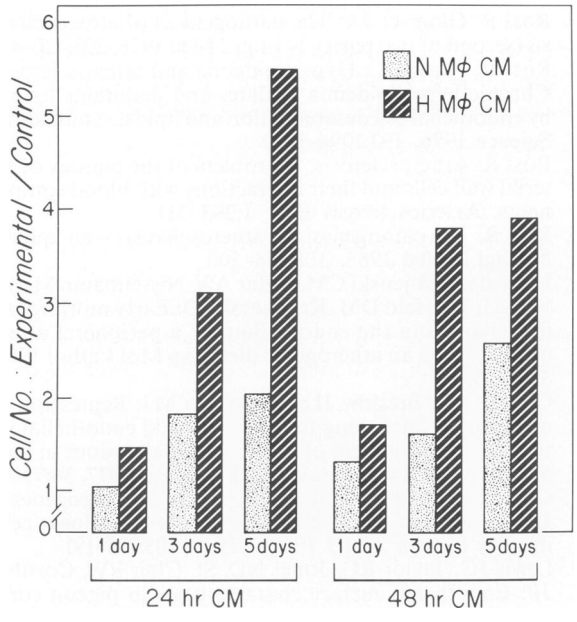

Figure 5—Effect of media conditioned by peritoneal macrophages from normal (N MØ CM) or hypercholesterolemic (H MØ CM) rats on cebus VSMC growth. The test media were conditioned for 24 to 48 hours and added to cebus VSMC growth arrested in G_0. The data are presented as a ratio of the cell number in the conditioned to the cell number in unconditioned media (*Experimental/Control*) on Days 1, 3, and 5. In the control cultures, the cell number on Days 1, 3, and 5 averaged 11.5×10^3, 29.9×10^3 and 35.8×10^3, respectively. The data shown are derived from at least three separate experiments. All of the values for H MØ CM were significantly greater than N MØ CM control ($P < 0.01$).

tion by peritoneal macrophages from normal rats was 11.2 ± 0.7 nm O_2^-/min/10^7 cells, while hypercholesterolemic rats released 12.5 ± 1.2 nm O_2^-/min/10^7 cells.

Discussion

The adhesion of monocytes to the vessel wall and their subsequent emigration into the intima appears to be a key early step in the formation of atherosclerotic lesions.[7,8,10] However, the role these cells play in the maturation of the lesion has not been clearly defined. In this article, we have compared four properties of rat peritoneal macrophages that are thought to be related to the development of atherosclerotic lesions: adhesion, production of chemotactic factors, secretion of mitogenic-activity, and the release of reactive oxygen species.

The causes of increased monocyte adhesion to large-vessel endothelium observed in hypercholesterolemic animals have not been identified. In fact, it is not clear whether it is changes in the monocyte, in the endothelial cell, or in both cell types that lead to the increased sticking of these leukocytes *in vivo*.[10] We compared the *in vitro* adhesive properties of N MØs and H MØs, using BAECs, rat VSMCs, and plastic as substrata, and found that H MØs adhered at a faster rate and to a greater extent than did N MØs. This result indicates that functional changes in macrophages from hypercholesterolemic rats may be associated with their increased adherence to the aortic endothelium. Similar functional changes in circulating monocytes from hypercholesterolemic rats may account for the increased adhesion observed in *in vivo* studies.[10] Our data do not rule out the possibility that functional changes in the endothelial lining also contribute to increased adhesion *in vivo*.

It is suspected that intimal macrophages are involved in the recruitment of medial smooth muscle cells into the intima.[5,14] We compared the level of VSMC chemoattractants in media conditioned with either N MØs or H MØs and found that the latter was three to four times more effective in stimulating VSMC migration. The chemoattractant present in the conditioned media was not species specific, because both rat and cebus monkey VSMCs responded in a similar fashion. Checkerboard analysis indicated that the response was a chemotactic one and not simply chemokinetic. The chemical nature of the chemoattractant remains to be determined. It has been reported that 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), a major product of the lipoxygenase pathway secreted by peritoneal macrophages cultured *in vitro*,[16,17] is a potent stimulator of VSMC chemokinesis.[15] Interestingly, Mathur et al[40] have recently reported that cholesterol enrichment of mouse peritoneal macrophages *in vitro* increases the production of 12-HETE 2.5-fold. In addition, activated macrophages have been shown to release a platelet-derived growth factor- (PDGF) like chemoattractant for VSMCs in culture.[37] It should also be noted that macrophages cultured *in vitro* secrete chemoactive factors for monocytes,[41] which suggests that intimal macrophages present in a developing lesion are capable of supporting the recruitment of circulating monocytes.

Recent evidence suggests that VSMCs play a role in the margination and emigration of monocytes from the lumen into the vessel wall. Although not a focus of the present study, it should be noted that hypercholesterolemia can affect monocyte emigration. For example, monocyte/macrophage-specific chemotactic substances have been identified in lesion-prone areas of the aorta in both white carneau pigeons and swine fed an atherogenic diet.[42,43] Interestingly, in the swine model, only monocytes obtained from hypercholesterolemic animals and not normal animals responded to the vessel wall chemoattractant, which suggests that the atherogenic diet caused functional changes in the circulating monocytes. Although the source of the chemotactic substance in the vessel wall has not yet been fully characterized, it is possible that medial VSMCs are involved in its production, because these cells secrete a similar

chemoattractant substance when they are cultured *in vitro*.[41,44,45]

VSMC proliferation can be stimulated by mitogens derived from several sources[18,19,46-49] and is one of the principal cellular responses in the early pathogenesis of atherosclerosis.[4,5,14] We have shown that the *in vitro* production of smooth muscle cell mitogens by macrophages is enhanced threefold by hypercholesterolemia. If intimal macrophages behave in a similar manner, such an increase in mitogen production could contribute directly to the proliferation of smooth muscle cells, thus accelerating the development of the atherosclerotic lesion. Although macrophage-derived VSMC mitogens have been reported by several groups,[18,19,47,49] this is the first demonstration that hypercholesterolemia substantially increases the VSMC mitogenic activity secreted by macrophages.

The production of reactive oxygen species such as O_2^- and H_2O_2 is associated with the destruction of cells by various macrophage systems *in vivo*.[38] It has been suggested that the release of reactive oxygen species by intimal macrophages may damage both lipid-laden VSMCs and foam cell macrophages, which leads to the formation of the necrotic lipid core characteristic of the fibrous plaque.[4] Leukocyte-mediated endothelial damage has been demonstrated in many other systems.[50,51] Monocyte/macrophage-induced damage to endothelium by toxic oxygen products or some other mechanism could lead to the direct exposure of the subendothelial matrix to blood-borne components that could enhance lesion development.[4] Hypercholesterolemia does not appear to alter the ability of thioglycolate-elicited macrophages to release O_2^- during phorbol ester stimulation. However, oxygen-derived products may be of importance in lesion formation in the hypercholesterolemic model, because increased numbers of macrophages are present in affected sites.

In this article, we have examined the influence of hypercholesterolemia on several parameters of monocyte/macrophage function that may contribute to the development of atherosclerotic lesions, which include adhesion, production of VSMC mitogens and chemotactic agents, and release of O_2^-. Hypercholesterolemia-induced augmentation of these responses was observed in all instances except O_2^- release, in which case there was no change. While further experimentation is required for more precise definition of the cellular and biochemical mechanisms involved, our data provide an experimental link between an important risk factor for heart disease—high serum cholesterol—and the "response to injury hypothesis" of atherosclerosis.[1,2,4,5]

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