Validation of the Circulating Monocyte Being Representative of the Cholesterol-Loaded Macrophage Biomediator Activity

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Context.—Inflammation is pivotal to atherosclerosis. The monocyte-macrophage, a crucial cell in atherogenesis, is present during all stages of atherosclerosis. However, there is a paucity of data comparing circulating monocytes to cholesterol-laden macrophages (foam cells), with regard to their atherogenic properties, especially in subjects with established risk factors such as hyperlipidemia.

Objective.—To determine whether the circulating blood monocyte is representative of the cholesterol-loaded macrophage with regard to its proatherogenicity in healthy controls and hyperlipidemic patients.

Design.—Fasting blood was drawn from 32 subjects (n = 16 controls and n = 16 hyperlipidemic patients), and peripheral blood monocytes were obtained. Also, macrophages were cultured and loaded with acetyl low-density lipoprotein on day 10. Day 1 peripheral blood monocytes and day 11 cholesterol-loaded macrophages were assessed for release of superoxide anion and cytokines (interleukin 1, interleukin 6, tumor necrosis factor α); surface expression of CD11b, VLA-4, and CD40; and adhesion to human endothelium.

Results.—Monocyte and cholesterol-loaded macrophage superoxide anion release, cytokines, and adhesion of monocytes to human endothelium were significantly increased in hyperlipidemic patients compared with controls. Furthermore, following cholesterol loading, there were no significant differences in monocyte versus cholesterol-loaded macrophage activity (P = .71). Also, CD14 and CD11b surface expression on monocytes was significantly increased in hyperlipidemic patients as compared with controls. The magnitude of change in the monocytes versus cholesterol-loaded macrophages was similar.

Conclusions.—From these studies, we can conclude that the monocyte, which is readily accessible, is an appropriate cell to study for modulation of proatherogenic activity, especially with regard to genomic and proteomic analyses/microarrays.

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Many data support the concept that atherosclerosis is an inflammatory process.1,2 The earliest event in atherogenesis appears to be endothelial cell dysfunction. Various noxious insults including hypertension, diabetes, smoking, dyslipidemia, and hyperhomocystinemia can result in endothelial cell dysfunction. After endothelial cell dysfunction, mononuclear cells, such as monocytes (MOs) and T lymphocytes, initially loosely attach to the endothelium, and thereafter adhere firmly to the endothelium and then diapedes into the subendothelial space. The rolling and tethering of leukocytes on the endothelium is orchestrated by adhesion molecules such as selectins (E-selectin, P-selectin), cell adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1), and integrins. Chemotaxis and entry of MOs into the subendothelial space are promoted by MO chemoattractant protein 1, interleukin 8 (IL-8), and a newly reported chemokine, fractalkine. Thereafter, macrophage colony-stimulating factor promotes the differentiation of MOs into macrophages. Macrophages incorporate lipids from oxidized low-density lipoprotein (LDL) via the scavenger receptor pathway (CD36, scavenger receptor A), becoming foam cells, the hallmark of the early fatty streak lesion. After the fatty streak lesion, smooth muscle cells migrate into the intima, proliferate, and form the fibrous cap. It is currently believed that lipid-laden macrophages, during the process of necrosis and apoptosis, release matrix metalloproteinases and other proteases, which cause a rent in the endothelium. Because the lipid-laden macrophage is enriched in tissue factor, this is released from the macrophage and comes in contact with the circulating platelets, resulting in thrombus formation and acute coronary syndromes (unstable angina and myocardial infarction).

Thus, the MO-macrophage is a pivotal cell present in all stages of atherogenesis, from the fatty streak lesion to the complicated lesion. Monocytes and macrophages, when appropriately stimulated, can produce many biologically active mediators that can influence virtually all aspects of atherogenesis, such as reactive oxygen species and proinflammatory cytokines such as IL-1 and tumor necrosis factor α (TNF) and tissue factor.

Recent exciting evidence of the pivotal role of MO-mac-
rophages in atherogenesis comes from studies of apolipoprotein E knockout mice (which develop fulminant atherosclerosis). In these mice, it has been shown that additional knockout of the gene for CCR2 (the receptor for MO chemotactic protein 1) significantly decreases lesion formation. Also, in osteopetrotic mice, it has been shown that knocking out the gene for macrophage colony-stimulating factor decreased lesion formation in apolipoprotein E knockout mice.

The importance of MO-macrophages in atherogenesis is further underscored by the preliminary findings of certain altered MO functions in hypercholesterolemia, a known risk factor for coronary artery disease. Hypercholesterolemia has been shown to be associated with an abnormally high number of morphologically abnormal MO-macrophages in the bloodstream. Mononuclear cells from hypercholesterolemic subjects with no clinical signs of vascular disease have an increased capacity for reactive oxygen species generation and greater adhesion to endothelium than normcholesterolemic subjects. However, while a few studies have studied reactive oxygen species generation and adhesion in hypercholesterolemic patients, there is a paucity of data on cytokine release in these patients. In addition, there is a lack of data comparing the proatherogenic activity of MOs and cholesterol-laden macrophages (CLMs), especially in combined hyperlipidemia. Thus, the MO-macrophage is a crucial cell in the genesis of the atherosclerotic lesion that is present during all stages of atherosclerosis. However, there is a paucity of data comparing circulating MOs to the CLMs (foam cells) with regard to their atherogenic properties, especially in subjects with established risk factors for coronary artery disease such as hyperlipidemia. The purpose of this study was to determine whether the circulating blood MO is representative of the CLM with regard to its proatherogenicity and to test whether these proatherogenic properties are increased in patients with established risk factors for atherosclerosis such as hyperlipidemia.

**MATERIALS AND METHODS**

**Subjects**

This study was approved by the institutional review board and all subjects gave informed consent. Subjects were included with no exclusion criteria other than age, race, or socioeconomic status. Control subjects were recruited if they fulfilled the following criteria: normolipidemic (total cholesterol and total triglycerides < 200 mg/dL and LDL-cholesterol < 100 mg/dL on at least 2 occasions), without elevated levels of lipoprotein(a) and homocysteine; non-smokers; nondiabetics; not on antioxidant supplementation; no chronic disorders; no bleeding diathesis; normal complete blood count and renal and liver function; alcohol intake less than 1 ounce/day; not taking hypolipidemic drugs, thyroid drugs, non-steroidal anti-inflammatory drugs, oral contraceptives, or anti-coagulants; and no previous or family history of coronary artery disease. Female subjects were studied in the follicular phase of the menstrual cycle.

Hyperlipidemic subjects were recruited with the same inclusion criteria, except that they had an LDL-cholesterol of greater than 160 mg/dL on 2 occasions, without any secondary causes, while combined hyperlipidemic subjects were selected if their plasma triglyceride was greater than 200 mg/dL and LDL-cholesterol was greater than 160 mg/dL on 2 occasions 1 week apart. None of these subjects had initiated therapeutic lifestyle changes and/or lipid-lowering therapy.

**Methods**

Complete blood count and plasma lipid and lipoprotein profile were assayed as routine tests in the Clinical Pathology Laboratory. Mononuclear cells were isolated from fasting heparinized samples of 120 mL of venous blood by Ficoll-Hypaque centrifugation. Leukocyte count was performed on a Coulter counter (Beckman Coulter, Indianapolis, Ind) and then cells were plated (5 x 10³ cells) in 6-well Primaria plates in RPMI 1640 medium. Incubation was performed at 37°C for 2 hours in 5% CO₂/95% air, after which nonadherent cells were removed after washing 3 times with phenol red–free RPMI 1640 medium. These cells were then split into 2 sets. One set of MOs was used to assess MO function on the day of isolation. The second set of MOs was incubated in media for 9 days, differentiated into macrophages, and then loaded with acetyl LDL as described below. All reagents used to assay MO function were tested for endotoxin contamination by the Limulus endotoxin assay. The viability of the MOs was checked by Trypan blue exclusion. Lipo polysaccharide (LPS), a known activator of MOs, was used to activate MOs (1–10 µg/mL). All tests of MO function on day 1 were performed following LPS activation.

For obtaining macrophages, MOs (approximately 2 x 10⁶ cells per mL) were cultured for 7 days at 37°C in a 5% CO₂ incubator in the presence of RPMI supplemented with 20% autologous serum to differentiate into MO-macrophages. Human LDL (1.019–1.063 g/mL) was prepared from pooled plasma from normal volunteers frozen at −80°C, by a 2-step rapid sequential flotation centrifugation as described previously. Low-density lipoprotein was acetylated with repeated additions of acetic anhydride as reported previously. Following incubation of macrophages for 24 hours with acetyl LDL (100 µg/mL), the macrophages were washed 3 times with RPMI 1640 medium. Total and free cholesterol content of macrophages following lipid loading was performed by gas chromatography of trimethylsilyl ether derivatives as described previously, and esterified cholesterol was calculated by gravimetric analysis of free from total cholesterol. Monocyte function was studied on day 1, and macrophage function was studied on day 11 with cholesterol loading.

Superoxide anion release in LPS-activated MOs (10 µg/mL) and in CLMs was measured by the superoxide dismutase inhibitable reduction of ferricytochrome C. The release of IL-1β and TNF-α was measured in LPS-activated (1 µg/mL) MOs and CLMs using a highly sensitive human immunoassay as reported previously. Monocyte/macrophage adhesion to human aortic endothelial cells was studied using a fluorescent labeling method as reported previously. Surface expression of CD14, CD11b, very late antigen 4 (VLA-4), and CD40 on MOs was assessed by flow cytometry using fluorochrome conjugated antibodies and appropriate isotype controls as described previously.

**Statistical Analysis**

Statistical analyses were performed with the aid of the General Clinical Research Center biostatistician using SAS statistical software (SAS, Cary, NC). Comparison of indices of MO and macrophage function was made with paired t tests or nonparametric (Wilcoxon signed rank) tests if data were skewed. Spearman or Pearson correlation coefficients were calculated to compare mediator activity between MO and CLM.

**RESULTS**

Baseline characteristics of controls and hyperlipidemic subjects are shown in Table 1. There were no significant differences in age or body mass index. However, as expected, subjects with hyperlipidemia had significantly elevated levels of total cholesterol, LDL cholesterol, and triglycerides and lower high-density lipoprotein levels compared with matched controls.

Following lipid loading, there was a significant increase in cholesterol ester content of macrophages from hyper-
lipidemic subjects compared with controls (controls: 304 ± 45 μg/mg cell protein; hyperlipidemic patients: 366 ± 37 μg/mg cell protein; P = .02). The effect of differentiation on MO-macrophage proatherogenic activity was tested in the day 1 MOs and in differentiated macrophages of the same subjects on day 11 after cholesterol loading (CLM), and the results are depicted in Table 2. As shown, MO superoxide anion release; levels of the proatherogenic cytokines IL-1, TNF, and IL-6; and adhesion of MOs to human endothelium were significantly increased in patients with hyperlipidemia compared with controls. Furthermore, CLMs had similar levels of superoxide, IL-1, TNF, and adhesion and significantly increased IL-6 compared with MOs and had similar percent increases in hyperlipidemic subjects compared with controls (percent increase in MO for superoxide, 78% vs 72% for CLM; percent increase in MO for TNF; 49% vs 62% for CLM; percent increase in MO for IL-6, 30% vs 22% for CLM). Following cholesterol loading of macrophages, there were no significant differences in mediator activity compared with MO from the same subjects, as denoted by significant correlation coefficients and P values for superoxide anion release (r = 0.78, P < .001), IL-1 release (r = 0.64, P = .02), TNF release (r = 0.69, P = .02), IL-6 release (r = 0.78, P = .004), and MO-endothelial cell adhesion (r = 0.71, P < .001).

Finally, we also examined the effect of MO differentiation into macrophages on the expression of CD14, a MO marker, expression of adhesion molecules CD11b and VLA-4, and surface expression of CD150 in controls and hyperlipidemic patients. CD14 and CD11b surface expression on MOs was significantly increased in hyperlipidemic patients as compared with controls. As expected, there was a significant decrease in CD14 expression in the day 11 macrophage as compared with the day 1 MO in both controls and patients with hyperlipidemia. The magnitude of change in the MO versus the CLM was similar, especially for CD11b and CD40 (Table 3; CD11b: r = 0.632, P = .04, CD40: r = 0.556, P = .04, CD14: r = −0.33, P = .04; VLA-4: r = 0.321, P = .63).

COMMENT

Inflammation is pivotal in atherosclerosis. Invasión del vaso sanguíneo por leucocitos, principalmente macrófagos derivados de células MOs, es una de las primeras etapas en la arterioesclerosis. Infiltrando macrófagos progresivamente acumulan modificaciones lipídicas, resultando en la formación de células de grasa. La producción de proinflamatorios citocinas propaga la respuesta inflamatoria en un autoanárquico y paracéntrico. Mientras que el macrofago se presenta en todas las etapas de aterosclerosis, en los estudios clínicos en humanos la actividad biológica derivada del flujo periférico MO. Por lo tanto, en este artículo, comparamos las actividades proatherogénicas del flujo periférico MO a las de un CLM. Reportamos aquí que MOs de sujetos hiperohiperlipidémicos son más proatherogénicos que aquellos de sujetos sanos. Cuando estos MOs son derivados de macrofagos y cargados con colesterol, la producción de citocinas y adhesión se incrementa significativamente.

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### Table 1. Subject Characteristics and Plasma Lipid and Lipoprotein Profile*

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 16)</th>
<th>Hyperlipidemic Patients (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>36 ± 14</td>
<td>39 ± 18</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 ± 6</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>153 ± 44</td>
<td>279 ± 103†</td>
</tr>
<tr>
<td>Total triglycerides, mg/dl</td>
<td>77 ± 37</td>
<td>279 ± 109†</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
<td>89 ± 23</td>
<td>176 ± 100‡</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
<td>49 ± 20</td>
<td>34 ± 19†</td>
</tr>
</tbody>
</table>

* BMI indicates body mass index; LDL, low-density lipoprotein; and HDL, high-density lipoprotein. All values are presented as mean ± SD.

† P < .001 compared with controls.
‡ P = .03 compared with controls.

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### Table 2. Time Course of Superoxide Release, Cytokines, and Adhesion*

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Hyperlipidemic Patients</th>
<th>Percent Increase From Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide anion, nmol/min/mg protein</td>
<td>MO 0.22 ± 0.14</td>
<td>0.39 ± 0.19†</td>
<td>78</td>
</tr>
<tr>
<td>IL-1, ng/mg protein</td>
<td>MO 0.21 ± 0.09</td>
<td>0.37 ± 0.08†</td>
<td>72</td>
</tr>
<tr>
<td>TNF, pg/mg protein</td>
<td>MO 1.2 ± 0.3</td>
<td>1.97 ± 0.48†</td>
<td>64</td>
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<tr>
<td>IL-6, pg/mg protein</td>
<td>MO 84.5 ± 23.4</td>
<td>127 ± 58.1†</td>
<td>49</td>
</tr>
<tr>
<td>Adhesion, %</td>
<td>MO 28 ± 14</td>
<td>49 ± 21§</td>
<td>75</td>
</tr>
</tbody>
</table>

MO indicates day 1 lipopolysaccharide-activated monocyte; CLM, day 11 cholesterol-loaded macrophage; IL, interleukin; and TNF, tumor necrosis factor. All values are presented as mean ± SD.

† P = .03 compared to controls.
‡ There were no significant differences between MO and CLM for all the parameters except IL-6 (P = .04).
§ P < .001 compared with controls.

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### Table 3. Surface Expression of Adhesion Molecules*

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Hyperlipidemic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>MO 89 ± 33</td>
<td>115 ± 46†</td>
</tr>
<tr>
<td>CD11b</td>
<td>MO 48 ± 12</td>
<td>104 ± 37§</td>
</tr>
<tr>
<td>CD40</td>
<td>MO 48 ± 12</td>
<td>67 ± 13§</td>
</tr>
<tr>
<td>VLA-4</td>
<td>MO 49 ± 12</td>
<td>44 ± 21§</td>
</tr>
</tbody>
</table>

* CD indicates cluster of differentiation; MO, day 1 lipopolysaccharide-activated monocyte; CLM, day 11 cholesterol-loaded macrophage; and VLA-4, very late antigen 4. Data are expressed as mean ± SD in mean fluorescence units per 10,000 cells.

† P = .02 compared with controls.
‡ P = .04 compared with MO (day 1).
§ P < .001 compared with controls.
retain their increased proatherogenicity. Thus, we show that the MO is indeed representative of the classic CLM, using cells from both healthy controls as well as hyperlipidemic patients.

In MOs and CLMs, we report increased proatherogenic activity with a similar fold increase in hyperlipidemic patients compared with controls with regard to superoxide, cytokine release, and adhesion to endothelium. Previously, in diet-induced hypercholesterolemia in rats, Rogers et al observed hypercholesterolemia-induced augmentation of macrophage function. In comparison with macrophages from non-hypercholesterolemic animals, macrophages from hypercholesterolemic animals were 50% to 80% more adhesive to Bovine aortic endothelial cells and vascular smooth muscle cells. Although the production of superoxide was found to be the same for both normal and hypercholesterolemic macrophages, 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 MOs and intimal macrophages. Subsequently, Fan et al have also shown that, compared with macrophages from normal rats, macrophages from hypercholesterolemic rats revealed a higher rate of adherence to endothelial cells.

Stragliotto et al investigated some functions of MOs from 20 type IIa hypercholesterolemic and 5 homozygous familial hypercholesterolemic patients. Monocytes from the type IIa hypercholesterolemic patients released increased leukotriene B4, which was 40% to 60% of the normal value (P < .05 for all). In support of these data, Ghanim et al have reported that mononuclear cells are in a proinflammatory state in obesity.

The CD40-40L system has been shown to be upregulated in hypercholesterolemia. Here, we report that CD40 surface expression in hyperlipidemic patients is increased in both the MO as well as the CLM to the same degree, pointing to the importance of studying this system in MO in different conditions. In addition, we show increased activity of CD11b and VLA-4 in both MOs and CLMs of hypercholesterolemic patients compared with controls. This may be responsible for the increased adhesion of MOs from hypercholesterolemic patients to endothelium. Previously, Bath et al showed that MOs from hypercholesterolemic patients, as opposed to controls, were more adhesive to porcine aortic endothelial monolayers (P < 0.05 vs 1.17 ± 0.06). The patients’ MO total surface expression of the adhesion glycoprotein CD11b/CD18 (37.5 ± 7.1 vs 36.0 ± 7.1) was increased. The data suggest that circulating MOs are also functionally different in hypercholesterolemia.

In conclusion, we have convincingly shown that the MO is a valid representative of the CLM, or foam cell, with regard to proatherogenic activity, as evidenced by similar increases in superoxide, cytokine release, adhesion molecules, and adhesion to endothelium in hyperlipidemic patients compared with controls. The reported in vitro findings do not account for other cells, such as smooth muscle cells in an atheromatous plaque, that can also take up lipid and become foam cells; these cells will be studied in the future. Also, the studies of CLM in vitro do not exactly mimic the foam cell in vivo. However, from these studies, we can conclude that the MO, which is readily accessible, is an appropriate cell to study for modulation of proatherogenic activity, especially with regard to genomic and proteomic analyses/microarrays.

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References