Acute Coronary Syndromes

Increased Circulating Monocyte Activation in Patients With Unstable Coronary Syndromes

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OBJECTIVES
The primary objective of this research was to assess the activation level of circulating monocytes in patients with unstable angina.

BACKGROUND
Markers of systemic inflammatory responses are increased in patients with unstable coronary syndromes, but the activation state and invasive capacity of circulating monocytes have not been directly assessed.

METHODS
Peripheral blood mononuclear cell (MC) activation in blood samples isolated from patients with stable and unstable coronary artery disease was measured in two studies. In study 1, a modified Boyden chamber assay was used to assess spontaneous cellular migration rates. In study 2, optical analysis of MC membrane fluidity was correlated with soluble CD14 (sCD14), a cellular activation marker.

RESULTS
Increased rates of spontaneous monocyte migration (p < 0.01) were detected in patients with unstable angina (UA) (Canadian Cardiovascular Society [CCS] angina class IV) on comparison to patients with acute myocardial infarction (MI), stable angina (CCS angina classes I to III) or normal donors. No significant increase in lymphocyte migration was detected in any patient category. Baseline MC membrane fluidity measurements and sCD14 levels in patients with CCS class IV angina were significantly increased on comparison with MCs from normal volunteers (p < 0.001). A concomitant reduction in the MC response to activation was detected (p < 0.05).

CONCLUSIONS
Using two complementary assays, activated monocytes with increased invasive capacity were detected in the circulation of patients with unstable angina. This is the first demonstration of increased monocyte invasive potential in unstable patients, raising the issue that systemic inflammation may both reflect and potentially drive plaque instability. (J Am Coll Cardiol 2001;38:1340–7) © 2001 by the American College of Cardiology

Monocytes are present in all stages of atherogenesis, potentiating inflammatory responses during early plaque development and initiating breakdown and rupture of the fibrous cap (1,2). The exposed lipid core of an unstable plaque is highly thrombogenic, producing acute vascular occlusions that result in myocardial infarction (MI) and unstable angina (UA). Increased adhesion and invasion of monocytes at sites of unstable plaque development have been demonstrated on histological sections in many prior reports (1–6).

Significant increases in circulating serum markers of monocyte (7–14) and T-cell (13–18) activation in patients with UA and MI have been detected. Although local monocyte activation inside the atherosclerotic plaque acts as a focus for inflammation, cellular activation that occurs while cells are still within the general circulation may also, theoretically, contribute to unstable plaque formation. Elevated levels of serum markers of inflammation (7,9,10), specifically C-reactive protein (CRP) (9,10,19,20), serum amyloid A (SAA) (20,21), CD11b/CD18 adhesion molecules (22,23), interleukin-1β (IL-1β), IL-6 (24), IL-8 (24,25) and macrophage chemotactic protein (MCP-1) (26), as well as increased levels of circulating leukocyte aggregates (11) and increased monocyte cellular (MCP-1, IL-1β and IL-8) messenger ribonucleic acid levels (18), have been reported. Whether this increase in circulating cell activation markers reflects an increase in the generalized activation state and invasive capacity of monocytes in the blood remains unproved.

We have measured activation of circulating peripheral blood mononuclear cells (MCs) in two independent studies. In the first study (study 1), the rate of spontaneous migration of MC isolated from peripheral blood samples was assessed as a marker for a generalized increase in cellular activation and invasive capacity in patients with varying...
degrees of coronary artery disease (CAD) (27). In the second study (study 2), the membrane fluidity of MCs was analyzed as a marker for cellular activation in patients with varying extents of CAD and correlated with levels of serum markers. Preceding the activation of cellular functions such as migration or tissue invasion, cells rearrange their surface membrane components. With this rearrangement, there is an associated change in membrane fluidity. Prior studies have demonstrated that membrane fluidity is a marker for cellular activation in platelets (28,29), endothelial cells (30), T cells (31) and epithelial cells (32), and altered membrane fluidity has also been reported to correlate well with clinical conditions such as diabetes (33) and Alzheimer’s disease (34).

**METHODS**

**Patient selection and stratification.** Patients over 18 years of age admitted for cardiac catheterization and capable of informed consent were considered eligible. The MC migration was assessed in 29 subjects (study 1) and membrane fluidity was assessed in 31 subjects (study 2). Mononuclear cells were also isolated from normal volunteers (14 for study 1 and 17 for study 2). Both study groups were stratified according to guidelines established by the Canadian Cardiovascular Society (CCS) for symptomatic angina (35). This investigation conforms to the principles outlined in the Declaration of Helsinki, and all studies were approved by the London Health Sciences Centre, University Hospital (London, Ontario, Canada) and the University of Alberta Hospitals (Edmonton, Alberta, Canada) ethical review boards.

**MC isolation.** For patients, blood samples were taken from the femoral arterial sheath during cardiac catheterization before injection of contrast or additional medication. Blood was taken up into ethylenediamine tetra-acetic acid containing Vacutainer tubes (Beckton Dickinson and Col-laborative Biomedical Products, Bedford, Massachusetts). Blood samples from normal volunteers were drawn via antecubital venous puncture. All blood samples were diluted 1:1 in phosphate-buffered saline (PBS; 8.4 mmol/l Na₂HPO₄, 1.9 mmol/l NaH₂PO₄, 0.15 mol/l NaCl, pH 7.4) and layered over Ficoll–Paque Plus (Pharmacia Biotech, Upplands, Sweden). For MC migration assays, gradients were centrifuged in a Beckman GS-15R centrifuge (400 g, 35 min, 18°C) (36). Cells were resuspended in Dulbecco’s minimal essential medium (DMEM, Gibco BRL, Burlington, Ontario) and used immediately for cell migration assays. Cell viability >95% was demonstrated by trypan blue exclusion. For membrane fluidity assays, cells were spun in a Sorvall laboratory centrifuge (880 g, 25 min, room temperature), and MCs were collected from the interface, and washed three times in PBS (720 g, 10 min, 4°C). A 1-ml, platelet-free serum sample was collected and stored at −80°C for soluble CD14 (sCD14) measurements. Isolated MCs were stained with Wright’s stain (Sigma, Oakville, Ontario) and enumerated by hemocytometer.

**Cell migration assay.** Circulating MCs from normal donors and patients with CAD were used to develop a two-chamber mononuclear migration assay, based on the original Boyden assay (36,37). Polycarbonate membranes in transwell units (8-μm pores; Costar, Atlanta, Georgia) were coated with a 1:50 dilution of Matrigel:DMEM for 1 h in 5% CO₂ at 37°C. Unbound Matrigel:DMEM was aspirated and filters rinsed with PBS. A 1:50 dilution provided a nonobstructing <0.1-mm-thick extracellular matrix protein coating on the upper surface of the filter and the inside margins of the pores as determined by electron microscopy (not shown), stimulating connective tissue-induced cellular migration through the membrane pores and onto the underside of the filter. With lower dilutions of Matrigel, monocytes did not penetrate the protein barrier. The same lot of Matrigel was used for all assays to avoid variability in Matrigel preparations. Next, 3 × 10⁵ MCs in unsupplemented DMEM were added to the upper walls (36). Chambers were incubated for 2 h at 37°C, 5% CO₂. Media and cells were then aspirated from the upper well, and nonmigrating, adherent cells on the upper surface of the membrane were removed by scraping the upper surface of the filter with PBS rinsing.

Migrating MCs on the lower surface of the filter were fixed for 15 min in 3% glutaraldehyde (Polysciences, Warrington, Pennsylvania), incubated for 3 min in 0.5% Triton X-100 (ICN Biochemicals, Aurora, Ohio) (36). The MC identification criteria included 12- to 20-μm diameter, a horseshoe-shaped, acentric, lighter staining nucleus (as compared with lymphocytes), and a nucleus-to-cyttoplasm ratio >1.0 (Fig. 1A). Lymphocytes were smaller (<12 μm) oval-shaped cells with small, darker-staining nuclei and scant cytoplasm. The numbers of migrating monocytes and lymphocytes were counted in three high-power fields for each filter. Triplicate samples (filters) were assessed for each cell isolate. To determine optimum incubation time, MCs

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**Abbreviations and Acronyms**

ANOVA = analysis of variance  
BSA = bovine serum albumin  
CAD = coronary artery disease  
CCS = Canadian Cardiovascular Society  
CRP = C-reactive protein  
DMEM = Dulbecco’s minimal essential medium  
fMLP = formyl-methionyl-leucyl-phenylalanine  
IFID = ischemic heart disease  
IL = interleukin  
LPS = lipopolysaccharides  
MC = mononuclear cell  
MCP-1 = macrophage chemoattractant protein-1  
MI = myocardial infarction  
OA = optical analysis  
PBS = phosphate-buffered saline  
PMA = phorbol-12-myristate-13-acetate  
SAA = serum amyloid A protein  
sCD14 = soluble CD14  
UA = unstable angina
from donors with stable (3 x 10^5 MCs) were loaded in the upper well and MC migration assessed from 5 min to 4 h. Based on this preliminary experiment, subsequent migration assays were run for 2 h.

**Immunohistochemical identification of migrating monocytes.** Morphologic identification of monocytes was confirmed by immunohistologic staining for CD14 and by independent blinded assessment by a pathologist (P.N.N.). Filters were fixed in 0.1% glutaraldehyde in PBS (30 min) and subsequently incubated with 0.3% H_2O_2 in methanol (30 min). Filters were then washed three times in PBS for 5 min, blocked for 15 min in 10% normal horse serum in PBS and incubated at 4°C overnight with mouse monoclonal anti-human CD14 antibody (Caltag Laboratories, Burlingame, California), diluted 1/100 in 0.1% bovine serum albumin (BSA) in PBS and washed three times in 0.1% BSA in PBS. Anti-isotypic, anti-rabbit IgG antibodies (Sigma, Oakville, Ontario) diluted 1/100 in 0.1% BSA in PBS and primary antibody-deficient samples served as negative controls. Filters were then incubated with biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, California), diluted 1/50 for 1 h at room temperature and washed in PBS; this was followed by avidin-biotin complex amplification (Vectastain Elite, Vector Laboratories, Burlingame, California) (36) for 1 h at room temperature. Colors were developed with liquid 3-3’-diaminobenzidine reagent (substrate). Membranes were then counterstained for 30 s in Gill’s No. 2 Hematoxylin.
Solution (Sigma, Oakville, Ontario), rinsed and permanently mounted on glass slides in Permount for light microscopy.

**Optical analysis (OA) of cellular membrane fluidity.** For OA of membrane fluidity (38) the ratio of pyrene excimer (493 nm) to pyrene monomer (393 nm) fluorescence emission intensity \( \frac{I_{\text{exc}}}{I_{\text{mon}}} \) after 325 nm excitation (Liconix He/Cd laser, Santa Clara, California) was measured using an Ocean Optics S2000 spectrophotometer attached to a Power MacIntosh 9500/200 computer equipped with SpectraWare analysis software (Fig. 2). The rate of excimer formation is a diffusion-controlled process proportional to the pyrene molecular collision rate in cellular membranes (28,32,38–40). The MCs were labeled with 4 mmol/l pyrene dissolved in pure ethanol (Sigma, Missussauga, Ontario) for 20 min at 37°C, with gentle shaking, and were then washed twice to remove residual pyrene label. The optimal time for loading pyrene (20 min) into purified MCs was determined by time course (data not shown). A control sample lacking MCs was also treated with pyrene, washed as for the cell samples and tested for nonspecific pyrene excimer formation in solution.

**Monocyte stimulation.** To ascertain whether increased migration reflected an increased level of activation of circulating MCs, the cells were pretreated with lipopolysaccharide (LPS) for 30 min using blood from five normal donors and two patients with unstable CAD (41) and cell migration assessed as described. For functional response analyses using the membrane fluidity assay, MCs were treated with either saline control or activated with 2 U/ml human thrombin,
100 ng/ml phorbol-12-myristate-13-acetate, or 10^{-7} \text{ mol/l formyl-methionyl-leucyl-phenylalanine (fMLP)} (activators obtained from Sigma, Oakville, Ontario).

**sCD14 measurements.** The sCD14 was measured in serum samples collected for membrane fluidity assay by commercial sandwich-type ELISA (IBL, Hamburg, Germany), using an oligoclonal capture antibody, followed by an enzyme-tagged monoclonal detection antibody.

**Statistics.** Cellular migration (study 1) and membrane fluidity (study 2) in each CCS category were compared by Fisher-protected least significant difference multiple comparison (analysis of variance [ANOVA]). Mean migrating cell counts were used for statistical analysis. Potential effects of cardiac medications were assessed by ANOVA. Differences were considered significant at p < 0.05.

**RESULTS**

**Analysis of MC migration in patients.** A 10-fold increase in monocyte migration (Fig. 1) was detected in patients with UA (CCS angina class IV) on comparison with normal donors and a 3-fold increase in cell migration was detected when compared to patients with stable angina (p < 0.05) (Fig. 1B). No differences were found, however, between monocyte migration rates for cells isolated from normal donors and patients with stable angina (p = 0.497). Cells obtained from patients with acute MI did not differ significantly in migration pattern from the other groups. A twofold increase in cell migration rates was detected after LPS stimulation of MCs from normal donors (Fig. 1C); however, no increase was detected for cells from patients with UA. Using the saline control as a baseline to calculate relative migrating cell counts, significant differences were detected with 10 ng and 100 ng LPS (p < 0.001) (Fig. 1C). No significant differences were detected for acute MI or stable angina patients. No significant differences in lymphocyte migration rate was seen in CSS class I, II, III or IV angina or acute MI patients (Fig. 1B). Negative controls consisting of an irrelevant rabbit, anti-isotypic IgG antibody control and primary antibody deficient incubation did not stain positively. Lymphocytic cells did not stain with anti-CD14 antibody.

**Membrane fluidity assay.** Generalized MC activation states were also assessed by membrane fluidity assay for normal individuals and patients with CAD. Figure 2A illustrates a typical pyrene spectrum before and after treatment with the activator thrombin. Significant increases in baseline membrane fluidity (I_{exc}/I_{mon}) were detected in cells obtained from patients with UA (CCS angina class IV) when compared to cells from normal individuals or patients with stable angina (p < 0.01 and p < 0.001, respectively; Fig. 2B). Membrane fluidity values for MCs isolated from UA patients were also distinguished readily from cells taken from patients with acute MI (p < 0.05; Fig. 2B). No significant correlation was observed between I_{exc}/I_{mon} ratio values and the last time recorded for chest pain for patients with stable or unstable angina (p = 0.5436). However, there was a trend toward an increase in membrane fluidity; for monocytes taken from a patient with pain within 24 h of admission, membrane fluidity was 1.271 ± 0.429 and for patients with pain more than 24 h prior to monocyte isolation membrane fluidity was 0.984 ± 0.185.

Treatment of MCs obtained from normal individuals with thrombin produced a statistically significant (p < 0.05) increase in membrane fluidity (I_{exc}/I_{mon}) corresponding to the same profile observed in untreated cells obtained from patients with UA (Fig. 2A, C). In addition, application of thrombin to cells obtained from UA patients did not result in any additional change in membrane fluidity. Both PMA and fMLP significantly reduced I_{exc}/I_{mon} in MCs from normal donors (by 45% and 29% compared to untreated MCs; p < 0.05), whereas changes in membrane fluidity in cells from patients with documented ischemic heart disease (IHD) were statistically significant only in the case of fMLP (a reduction of 35%; p < 0.05). Very little difference (15%; NS) was found in the baseline membrane fluidity value between patients with IHD and normal blood donors.

**sCD14 measurements.** A significant increase (p < 0.01) in levels of circulating sCD14 in the blood samples from patients with type IV UA was detected when compared to normal individuals and patients with acute MI (p < 0.01). Figure 3 demonstrates a 41% higher level of sCD14 in plasma obtained from patients with UA when compared to normal individuals and 48% higher levels than individuals with acute MI (5.9 ± 0.6 g/ml vs. 3.5 ± 0.26 g/ml and 3.1 ± 0.2 g/ml, respectively). Soluble CD14 levels in stable
angina patients showed a trend toward an increase that did not reach statistical significance \((p = 0.066)\).

**Analysis of monocyte activation and cardiovascular medications.** Potential medication-related effects on mononuclear cell migration and membrane fluidity were assessed and analyzed by ANOVA. Both monocyte and lymphocyte cell migrations did not correlate with any of the cardiovascular medications used \((p \geq 0.313)\), nor did membrane fluidity values \((p \geq 0.075)\), indicating that the observed changes in cell migration and membrane fluidity are not related to the use of cardiovascular medication in unstable coronary syndrome. The medications assessed were acetylsalicylic acid, other antiplatelet drugs (ticlopidine, clopidogrel, and abciximab), nitroglycerin, heparin and derivatives, beta-andrenergic receptor blockers, angiotensin-converting enzyme inhibitors, HMG-CoA reductase inhibitors (statins), calcium channel blockers and parasympatholytics.

**DISCUSSION**

We have assessed circulating MC activation in the peripheral blood of patients with UA using an assay of cellular invasive capacity in Boyden chambers and an OA assay of membrane fluidity. An increased baseline level of monocyte activation was detected in the circulation of patients with UA, but not in patients with stable angina or acute MI. These assays allow direct evaluation of mononuclear cell function by measuring the migratory response of cells exposed to matrix proteins in the absence of additional chemotactic factors. Only previously activated cells are expected to migrate in this assay. The significantly increased migration rates of monocytes from patients with UA support an increase in monocyte inflammatory responses during plaque destabilization and UA development, complementing serum-based markers. We postulate that the increased rate of migration might be produced by “priming” of circulating monocytes by a chronic inflammatory state initiated at sites of plaque instability. These results support our hypothesis that cells isolated from patients with UA are functionally altered and already activated in the peripheral circulation, making them refractory to further stimulation (Figs. 1 and 2).

**Optical analysis.** Significantly increased baseline membrane fluidity in MC from UA patients was consistent with altered baseline activation states. The lack of changes in membrane fluidity upon activation treatment in MCs from UA patients, but not normal volunteers, acute MI or stable angina patients, further supports an altered baseline cellular activation (Fig. 2). Changes in membrane fluidity (either an increase or a decrease) are associated with cellular activation in response to effector functions as the cell consolidates its surface components, rearranges its cytoskeleton, and activates signal transduction pathways. Exposure to large, multifunctional proteins such as thrombin can also result in an increase in membrane fluidity \((39,40)\). Increased activation may be represented by a need for increased cell mobility and migration. Although several cell types have been demonstrated to alter membrane fluidity in response to activation, ours is the first report of application of a membrane fluidity assay for analysis of circulating human MCs. Untreated cells obtained from patients with UA had membrane fluidity profiles very similar to thrombin-treated cells obtained from normal individuals, and they were not altered after thrombin treatment, again indicating that these cells were already maximally activated (Fig. 2).

**Elevated markers of inflammation in patients with unstable angina.** It is notable that the observed differences in migratory capacity and membrane fluidity were confined to patients with UA and not patients with acute MI. Increases in inflammatory markers in patients with UA when compared to acute MI have been previously reported \((12,42)\). In 1994, Jude et al. \((12)\) found that patients with UA exhibited higher levels of tissue factor expression in MCs than did patients with stable angina, whereas no difference in this parameter was found for patients with acute MI and stable angina. In 1999, Liuzzo et al. \((42)\) observed consistently higher levels of CRP and SAA in patients with UA when compared to patients with totally un heralded MI. Further, significant differences between the IL-6 levels in blood plasma of patients with UA and acute MI have been reported by Manten et al. \((43)\). These studies suggest that these two clinical presentations may be related to different pathogenic components of acute coronary occlusion and are consistent with our findings.

**Elevated sCD14 in UA patients.** Patients with UA in this study had detectable increases in plasma CD14 \((sCD14)\) levels, a serum marker for activated monocytes, supporting increased MC activation. A membrane-anchored glycoprotein, CD14 is involved in adhesion to the endothelium and also the receptor for LPS \((44,45)\). Stimulation of CD14 (either by LPS or released \(sCD14\) molecules) results in the secretion of tumor necrosis factor, interferon and other proinflammatory cytokines \((44,45)\). One study has demonstrated elevated \(sCD14\) in patients with noninfectious, inflammatory diseases such as rheumatoid vasculitis and systemic lupus erythematosus, reflecting a generalized activation of circulating monocytes \((44,45)\). A genetic polymorphism in the CD14 promoter region has also been identified as a possible risk factor for MI \((46–48)\). This is the first genetic factor, barring conventional factors, associated with MI risk, linked to monocyte function, and might be an early clinical marker for progression to plaque instability. Unstable plaque development is a dynamic process whereby inflammatory and immune system cells become activated and further contribute to the pathogenetic sequelae. Our results support the conclusion that it is not the critical mass of atherosclerotic disease, but rather the extent of plaque instability with invading activated macrophages \((49)\), that is necessary to induce a systemic increase in the level of activation of circulating monocytes. Assays designed to evaluate the state of circulating monocyte activation may prove beneficial in preventative diagnoses.
Study limitations. T-CELL ACTIVATION IN UA PATIENTS. The present study does not rule out lymphocyte activation in addition to monocyte function. Our results are consistent with a limited T-lymphocyte activation profile, as has been reported for TH1-T-cells (50). The presence of enhanced MC invasion without concomitant accelerated lymphocyte migration in the Boyden chamber assay would, however, argue that the observed alterations in membrane fluidity reflect MC activation. This study also does not conclusively distinguish, but instead raises, the following questions: 1) Are monocytes activated secondary to the presence of unstable plaque? 2) Conversely, do activated monocytes contribute to the development of UA? It is probable that both processes are present and equally contribute to instability.

Conclusions. In these studies we have: 1) evaluated the migration rates of MCs isolated from the peripheral circulating blood, and 2) assessed the membrane fluidity of MCs as a marker of cellular activation in patients with differing extents of CAD. In patients with UA, but not in patients with stable angina or acute MI, increased spontaneous migration of monocytes and MC membrane fluidity were detected. Patients with UA also demonstrated increased levels of the monocyte-specific activation marker sCD14. Taken together, these results support the hypothesis that activated monocytes are present and detectable in the circulating blood of patients with UA, and they are likely associated with an upregulated inflammatory state initiated at sites of plaque rupture. Furthermore, these experiments provide a novel diagnostic tool for exploring cellular activation directly.

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