Neutrophil Function in Ischemic Heart Disease

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Neutrophils contribute to the healing of and scar formation in myocardium after ischemic injury. Many recent studies indicate that neutrophils may be involved in the genesis and propagation of myocardial ischemia. To characterize neutrophil function in ischemic heart disease, neutrophil chemotaxis, leukotriene B4 (LTB4) generation, and elastase release in plasma were measured in 20 patients with stable angina, 17 patients with unstable angina or acute myocardial infarction (AMI), and 20 age-matched control subjects. Neutrophils from patients with stable angina exhibited markedly increased chemotactic activity and LTB4 generation as compared with the age-matched control subjects (p<0.01). Neutrophils of nine of 17 patients with unstable angina or AMI clumped spontaneously ex vivo and exhibited marked pseudopod formation and granule extrusion on electron microscopy. Subsequent chemotactic activity and LTB4 generation by neutrophils from these patients was less than in patients with stable angina, suggesting previous in vivo activation. Plasma levels of peptide BB, a product of fibrin degradation by human neutrophil elastase, were approximately 15-fold higher (p<0.001) in patients with unstable angina or AMI (588±171 pmol/l, mean±SEM) compared with those in patients with stable angina (37±25 pmol/l) or control subjects (40±22 pmol/l), confirming intense in vivo neutrophil activation. Our study shows enhanced neutrophil function in patients with ischemic heart disease. The increased neutrophil chemotactic activity and LTB4 generation may be markers of stable angina pectoris. Intense neutrophil activation in unstable angina or AMI, as manifested by morphologic changes in neutrophils and elastase release, may relate to ongoing in vivo cellular activation. *(Circulation 1989;79:549–556)*

Experimental studies in animals demonstrate that neutrophils play important roles in the pathophysiology of myocardial ischemia and infarction.1-5 Several epidemiologic studies in human subjects also indicate the relevance of leukocytes in ischemic heart disease. Elevation of leukocyte count in peripheral blood is associated with increased risk of acute myocardial infarction,6-10 its recurrence,11 and the incidence of ventricular fibrillation in the postinfarction period.12 Leukocyte count has also been shown to correlate with the extent of coronary artery disease observed at coronary angiography.13

After ischemic injury, neutrophils rapidly accumulate in the areas of injury.14 After migration into these areas, neutrophils discharge oxygen-free radicals,15 proteolytic enzymes, and arachidonic acid metabolites, such as leukotriene B4 (LTB4).2 Neutrophil activation is relevant in “reperfusion injury”16,17 and loss of coronary vasodilator reserve after reperfusion.18 Although peripheral neutrophil activation has been shown to correlate with the severity of ischemic heart disease in humans in one study,19 very little is known about the functional characteristics of neutrophils in patients with ischemic heart disease. In our study, we examine several aspects of neutrophil function to determine neutrophil activity in various forms of ischemic heart disease.

**Methods**

**Subjects**

All control subjects and patients were men. Twenty patients with angiographically documented coronary artery disease (CAD) and stable angina pectoris, 17 patients with unstable angina pectoris or acute myocardial infarction (AMI), and 20 control subjects participated in this study, which was approved by the
Institutional Review Boards of the University of Florida, College of Medicine, and the Veterans Administration Medical Center, Gainesville, Florida.

Patients with stable angina had typical exertional chest pain and angiographically documented CAD. Unstable angina was defined as an increase in the frequency or severity of chest pain or both in patients with previously documented CAD or new onset of symptoms suggestive of myocardial ischemia with subsequent angiographic demonstration of CAD unaccompanied by serum creatine kinase elevation or a new q wave in the electrocardiogram.AMI was defined as a clinical history suggestive of AMI and elevation in serum creatine kinase or development of a new q wave on the electrocardiogram. Normal control subjects were in good general health and free of any systemic illness. The mean age of patients with stable angina, patients with unstable angina or AMI, and control subjects was similar (mean, 58, 60, and 53 years, respectively). No attempt was made to alter the patients’ medications, and those taking nonsteroidal antiinflammatory drugs or steroids were excluded. Most patients with stable angina (n = 15) were not taking any drugs, whereas among patients with unstable angina or AMI, 14 were taking calcium blockers and four were taking β-blockers.

None of the patients or control subjects had smoked within 2 hours of blood collection. Blood was collected from patients with unstable angina or AMI within 1 hour of chest pain.

Blood Collection and Separation of Neutrophils

Peripheral venous blood was collected in heparin (10 units/ml) for neutrophil isolation and in acid-citrate-dextrose for measurement of elastase activity. The heparinized blood was layered over MonoPoly Resolving Medium (Flow Laboratories) and centrifuged at 500g for 30 minutes at 24°C. Red blood cells were lysed by briefly (15 seconds) suspending the neutrophil-rich layer in hypotonic saline solution followed by addition of hypertonic saline. The neutrophil-rich layer was then removed and washed in Hank’s Buffered Salt Solution (HBSS) without Ca2+ and Mg2+, as described previously.20 Neutrophil suspension (1 × 107 cells/ml) consistently contained more than 98% neutrophils, and their viability as determined by Trypan blue exclusion was more than 95%.

Neutrophil Chemotaxis

Directed chemotaxis to the peptide formyl-methionyl-leucyl-phenylalanine (f-MLP) was measured in a chemotaxis chamber (Neuro Probe, Cabin John, Maryland) as previously described.20,21 The number of neutrophils in the upper chamber was kept constant at 1 × 105. The lower chamber contained f-MLP (0, 10−8, and 10−7 M). Polycarbonate filters (5.0 μm pore, Neucleopore, Pleasanton, California) separated the upper and lower compartments of chambers, which were incubated in humidified air with 5% CO2 for 30 minutes at 37°C. The filters were fixed in methanol and stained with Hemacolor stain. In each experiment, all measurements were made in triplicate, and the number of cells migrated counted in five separate high-power fields (×400) at each concentration of f-MLP.20,21

Neutrophil LTB4 Generation

To determine LTB4 generation, neutrophils (1 × 107/ml) were incubated with calcium ionophore A23187 (10 μM) at 37°C for 15 minutes in a shaking water bath. The reaction was terminated by placing the tube in ice for 5 minutes and subsequently adding cold methanol (0.5 ml). Suspensions were centrifuged at 25,000g for 5 minutes at 4°C and the supernate analyzed for LTB4 by radioimmunoassay (RIA) in duplicate using supplies from New England Nuclear, Boston, Massachusetts.20–22 Previous experiments in which LTB4 was quantitated with reverse-phase high-pressure liquid chromatography demonstrated good correlation with the values obtained at RIA (r=0.91). Neutrophil LTB4, measured by RIA was inhibited with lipooxygenase inhibitor U60,257 by 78%.20 In the present study, therefore, only RIA values are presented.

Neutrophil Elastase Release

A vasoactive peptide (Arg-Pro-Ala-Pro-Pro-Ile-Ser-Gly-Gly-Tyr-Arg-Ala), produced by the action of elastase on fibrin(ogen) and corresponding to amino acids 30–43 of the fibrinogen Bβ chain, was synthesized; antibodies were raised in rabbits; and an RIA was developed.23,24 The antibody obtained exhibited both high specificity and high sensitivity to peptides released by neutrophil elastase degradation of the Bβ chain of fibrin(ogen).

Peptides released by plasmin, trypsin, chymotrypsin, collagen, thrombin, or pancreatic elastase degradation do not react with the antibodies. The cross-reaction with fibrinogen is extremely low (less than 2%). The only cross-reaction of importance is against a larger peptide fragment, a precursor to Bβ 30–43, released by degradation of fibrin(ogen) by neutrophil elastase. This fragment is very stable in plasma and is in fact responsible for most (or all) of the immunoreactivity in human plasma.

The detection limit of this RIA is 30–50 pmol/l. Normal human plasma concentration of this peptide varies between 0 and 120 pmol/l.23,24 The individuals performing the measurement of peptide Bβ in plasma (T.G.P.S. and R.W.) were kept blinded as to the clinical diagnosis of patients.

Electron Microscopy

Isolated neutrophils were centrifuged into a button in a conical tube, fixed in 1% glutaraldehyde/4% formalin solution, postfixed in 1% osmium tetroxide, and then set in an agar button. Most of the agar was cut from around the fixed button, which was then dehydrated through graded alcohols and acetone before embedding in eponaraldite during over-
night polymerization. Sections were cut on an LKB-Huxley ultramicrotome, stained with 2% lead citrate and 1% saturated uranyl acetate, and viewed in a Zeiss 109 electron microscope. The individual performing the electron microscopy (W.H.D.) was kept unaware of the patients’ clinical state.

**Supplies**

HBSS was purchased from Flow Laboratories, McLean, Virginia. Bovine albumin, f-MLP, and ionophore A23187 were purchased from Sigma Chemical, St. Louis, Missouri.

**Statistical Analysis**

Because the data were similar in patients with unstable angina and those with AMI, all data in both subgroups were combined. All data from multiple measurements on neutrophil LTB4 generation and chemotaxis were averaged, and comparison between groups was made by analysis of variance.

Data on plasma B2 levels were determined to be distributed nonparametrically by Kolmogorov-Smirnov test. Kruskal-Wallis analysis was performed on the data in various groups, and Mann-Whitney test was used to determine differences between groups. A p value less than 0.05 was considered significant.

**Results**

Peripheral blood leukocyte counts in patients with stable angina (n=14) were 6,200±400/mm³ (mean±SEM), in patients with unstable angina or AMI 8,800±1,200/mm³ (n=12), and in the control subjects 6,400±800/mm³ (n=17). Number of neutrophils as percent of total leukocytes in peripheral blood was 65±4%, 70±4%, and 63±5%, respectively, in each group. Total leukocyte and neutrophil counts among the three groups were not significantly different. The yield of neutrophils from peripheral blood in the control subjects (n=20) and patients with stable angina (n=20) was 10.1±0.5×10⁶ and 10.0±0.5×10⁶ cells/ml, respectively (p=NS), but the yield was less in patients with unstable angina or AMI (6.9±0.6×10⁶ cells/ml, n=17, p<0.01) compared with normal subjects or patients with stable angina.

**Neutrophil Chemotaxis**

In all control subjects and patients with stable angina, a concentration-dependent increase in neutrophil migration in response to f-MLP was noted. Neutrophils from patients with stable angina exhibited markedly increased (p<0.01) spontaneous as well as f-MLP–stimulated chemotaxis (Figure 1). In contrast, there was a wide variation in neutrophil migration in patients with unstable angina or AMI—from no migration at all to markedly increased chemotactic response to f-MLP. As a group, however, spontaneous neutrophil migration in patients with unstable angina or AMI was increased compared with the normal subjects (229±17 vs. 124±21 cells/5 high power fields, p<0.01). However, neutrophil chemotaxis in response to f-MLP 10⁻⁸ M (335±31 vs. 251±48 cells) or f-MLP 10⁻⁷ M (438±36 vs. 316±71 cells) was not significantly increased. Furthermore, in two patients with unstable angina and one with AMI, there was no neutrophil migration; in these three patients there was evidence of intense spontaneous clumping of neutrophils as described subsequently.

**Neutrophil LTB4 Generation**

Neutrophil LTB4 generation in normal subjects was 30.0±3.8 ng/10⁷ cells. Neutrophil LTB4 generation in patients with stable angina was higher than in the control subjects (60.9±6.6 mg/10⁷ cells, p<0.01). On the other hand, neutrophil LTB4 generation in patients with unstable angina or AMI (35.7±4.7 ng/10⁷ cells) was similar to that in the control subjects and was significantly less than that in patients with stable angina (p<0.05) (Figure 2).

**Neutrophil Elastase Activity**

Plasma levels of peptide B2 were approximately 15-fold higher in patients with unstable angina or AMI (mean, 588±171 pmol/l) than in the normal
subjects (mean, 40±22 pmol/l; p<0.001) (Figure 3). However, in patients with stable angina, plasma levels of peptide Bβ (mean, 37±25 pmol/l) were similar to the normal subjects and much lower than in patients with unstable angina or AMI (p<0.01). As shown in Figure 3, peptide Bβ levels were measured only in 12 patients with unstable angina or AMI. Of these, six with the highest values revealed spontaneous ex vivo clumping.

Neutrophil Morphology

Neutrophils from nine of 17 patients with unstable angina or AMI demonstrated spontaneous clumping. Electron microscopy revealed many pseudopods at the cell borders and multiple cytoplasmic vacuoles. There was evidence of granular extrusion suggesting cellular activation. The cells often adhered at the ends of the projections without distinguishable junctional activity (Figure 4). In contrast spontaneous clumping of neutrophils was not observed in any patient with stable angina or control subject.

and neutrophils did not spontaneously clump in any subject. Neutrophil shape was generally circular with no pseudopod formation, evidence of granular extrusion, or intercellular adherence (Figure 5).

Discussion

Previous studies have indicated that activation of coagulation cascade and platelets participates in coronary thrombosis, and thereby the evolution and propagation of acute myocardial ischemia.25,26 Adherence of monocytes to the atherosclerosis-prone arteries in experimental animals27 and of mast cells at the site of coronary spasm in humans28 also suggests that these cells may be involved in the pathogenesis of atherosclerosis and myocardial ischemia. Ischemic myocardium generates large amounts of thromboxane and leukotrienes, which could increase coronary vascular tone.29 Platelets stimulate neutrophil chemotaxis and LTB4 generation,21 and neutrophil-derived oxygen-free radicals and peptido-leukotrienes cause platelet aggregation.30–32 These observations suggest that cell-cell interactions may underlie the evolution of myocardial ischemia.

The present study shows that neutrophils of patients with documented CAD and stable angina have increased chemotactic activity (spontaneous as well as in response to f-MLP) and enhanced potency to generate LTB4, although their morphology is not different from neutrophils from normal subjects. Also, there is no evidence for ongoing in vivo neutrophil activation, as measured by peptide Bβ levels in the plasma. The increased functional potential of neutrophils may be a marker of stable CAD, and could have important roles in the manifestations of coronary atherosclerosis. The increase in neutrophil functional state may result in platelet activation and subsequent thrombosis in coronary arteries with atherosclerosis.30 Additionally, after coronary reperfusion, neutrophil hyperactivity may lead to extension of infarct size, stunned myocardium, and loss of coronary vasodilator reserve.18–18 These effects may be related to mechanical obstruction of coronary microvasculature and biochemical denaturation of endothelium-derived relaxing factor by superoxide radicals.33 Lastly, neutrophils, via release of LTB4, may increase microvascular permeability and attract other circulating neutrophils to the site of tissue injury.34

In contrast, neutrophils from patients with unstable angina or AMI were found to have undergone in vivo activation, manifested by severalfold increase in plasma Bβ, which is an index of neutrophil elastase release.23,24 Spontaneous ex vivo clumping of neutrophils in some patients also indicates state of hyperactivity. The yield of neutrophils from these patients was also lower than in control subjects or patients with stable angina, perhaps due to spontaneous clumping. The morphologic changes in neutrophils (i.e., pseudopod formation and granule extrusion) also suggest intense activation. Spontaneous migratory activity of these neutrophils was
also increased, but the stimulated activity (in response to f-MLP) was often decreased particularly in instances where the yield of cells was low and clumping of cells was visually evident. In light of previous in vivo activation, it was not surprising that the chemotactic response of these neutrophils and their potential to generate LTB₄ were not increased upon further stimulation. It is also likely that active neutrophils were preferentially trapped in the coronary circulation, and less active neutrophils in peripheral circulation had normal or subnormal functional characteristics. It can be speculated that spontaneous trapping of neutrophils in coronary circulation may be detrimental by occluding small arterioles and inducing endothelial injury via release of free oxygen radicals.

A striking and novel observation in this study is the increase in neutrophil elastase release. In some recent reviews, the contributory role of neutrophils in the propagation of myocardial injury after coronary occlusion has been emphasized. Neutrophils become activated in myocardial injury in response to a variety of chemoattractants released from the myocardium and platelets. Activation of neutrophils results in release of 5-lipoxygenase metabolites of arachidonic acid, principally LTB₄, oxygen-derived free radicals, and granular constituents, such as elastase. Elastase release can cause digestion of basement membranes and increase endothelial adhesiveness, permeability, and tissue edema. Decrease in number or function of neutrophils has been shown to have a salutary effect on the extent of myocardial injury in animals.

It is noteworthy that the highest elastase levels in plasma were associated with evidence of spontaneous clumping in patients with unstable angina or AMI, suggesting a correlation between in vivo and ex vivo activation. However, plasma Bβ levels were not uniformly elevated in all patients with unstable angina or AMI, and the spontaneous clumping of neutrophils was not observed in about half the patients with unstable angina or AMI. Intake of known and unknown pharmacologic agents by different patients, vagaries in the extent of disease and the pathologic state of the myocardium and coronary arteries, and the difficulty in precisely assessing the clinical state may have been the cause or causes of the variability. The CAD risk factors, such as smoking, diabetes mellitus, and hypertension, were widely prevalent in our study population, and no attempt was made to control for these variables. Furthermore, we assessed neutrophil chemotaxis in response only to f-MLP and assessed LTB₄ generation in response only to ionophore...
A23187 in this preliminary study. It is not known if similar neutrophil functional characteristics would be evident if other stimuli were used. Lastly, we made no attempt to correlate neutrophil activity in our patients with their clinical state and plasma creatine kinase levels, because of the relatively small number of subjects. Nonetheless, it is quite obvious that neutrophils are activated in patients with acute myocardial ischemia, and neutrophil functional capability is increased in patients with stable CAD, which may have its impact during acute myocardial ischemia.

The basis of enhanced neutrophil function in patients with stable CAD may relate to complement activation. Craddock et al. noted that membrane interactions and complement-mediated neutrophil activation result in enhanced leukocyte adherence, diminution of microvascular flow, and endothelial injury. Furthermore, cholesterol and atheroma lipids have been shown to be capable of activating complement. Smoking and gout are also associated with increased neutrophil function. Recently, Worthen et al. have shown that small concentrations of platelet activating factor (PAF, 10^{-9} M) enhance human neutrophil superoxide production, and small concentrations of lipopolysaccharide (100 ng/ml) increase neutrophil PAF production. We previously showed that platelets stimulate human neutrophil chemotaxis, LTB, generation, and free-oxygen radical release, and leukocyte-derived peptido-leukotrienes stimulate platelets “primed” with epinephrine. The “hyperactivity” of neutrophils in vitro in patients with stable CAD demonstrated in this study may also relate to “priming” of cells in response to PAF, hyperactive platelets, or some other stimuli. Regardless of the precise stimulus, enhanced neutrophil activity in stable CAD may provide a pathophysiological milieu for the progression of stable CAD to unstable myocardial ischemia. It is conceivable that patients who develop acute myocardial ischemia have abnormal leukocyte function before the onset of acute event. Neutrophil-mediated endothelial damage may allow alterations in vasomotor tone. Furthermore, enhanced neutrophil activity may result in neutrophil plugging and subsequent decreases in myocardial compliance characteristic with concomitant loss of myocardial function.

In summary, this study demonstrates qualitative and quantitative changes in neutrophil function that may have important implications in the development and consequences of CAD.

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References


34. Lewis RE, Granger HJ: Neutrophil dependent mediation of microvascular permeability. Fed Proc 1986;45:109–113


40. Craddock PR, Hammerschmidt D, Moldow CF, Yamada O, Jacob HS: Granulocyte aggregation as a manifestation of membrane interactions and complement: Possible role in leukocyte margination, microvascular occlusion and endothelial damage. Semin Hematol 1979;16:140–147

41. Hammerschmidt DE, Greenberg CS, Yamada O, Craddock PR, Jacob HS: Cholesterol and atheroma lipids activate complement and stimulate granulocytes: A possible mecha-

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