Plasma-mediated neutrophil activation during acute myocardial infarction: role of platelet-activating factor

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(Received 19 July 1994/20 March 1995; accepted 13 April 1995)

INTRODUCTION

In recent years the importance of polymorphonuclear neutrophils (PMNs) in the inflammatory response to myocardial injury has been recognized. PMN activation is particularly prominent during reperfusion following a period of ischaemia. Neutrophil accumulation in areas of myocardial necrosis is beneficial through the initiation of a cascade of events leading to the elimination of dead myocytes and the strengthening of the myocardial wall by scar formation. However, PMN activation in the ischaemic myocardium may also result in the destruction of injured myocytes that had the potential to recover (reviewed in [1, 2]).

Intravascular neutrophil activation results in the expression of surface adhesion molecules. Adhesion molecules expressed by activated PMNs belong to the integrin family, of which the main molecule is CD11b/CD18, and the selectin family, the most important of which is L-selectin [3, 4]. PMN activation in ischaemic myocardium results in their aggregation and adherence to endothelial cells with subsequent capillary plugging and impairment of coronary perfusion. Vasoconstriction may also result from leukotriene B₄ release from activated PMNs. Activated PMNs also give rise to biochemical damage in the ischaemic myocardium through the release of oxygen free radicals, proteolytic enzymes and leukotoxin. Several investigators have shown a reduction in ischaemia–reperfusion injury following neutrophil depletion using a variety of methods, including neutrophil antisera, leucocyte filters or pharmacological interventions which modify PMN activation [1, 2].

Despite recent advances in our understanding of PMN activation within ischaemic myocardium and its injurious role during myocardial infarction, the mechanisms underlying neutrophil activation remain unclear. Activation of PMNs during myocardial ischaemia–reperfusion may occur as a result of PMN contact with endothelial adhesion molecules.
expressed within ischaemic myocardium or in response to the release of soluble stimuli. Thus, although previous studies have shown PMN activation after myocardial infarction, it is not known whether this stimulation occurs as a result of expression of endothelial receptors or via release of neutrophil-oriented soluble stimuli from ischaemic myocardium. We have found that peripheral plasma obtained from patients with acute myocardial infarction can induce chemotaxis, adherence and superoxide anion production by PMNs obtained from healthy donors [5–7], suggesting systemic release of neutrophilic stimuli from ischaemic myocardium. Furthermore platelet-activating factor (PAF), a potent stimulus of PMN activation [S-lo], released into the circulation during acute myocardial infarction can induce chemotaxis, adherence and superoxide anion production by PMNs obtained from patients with acute myocardial infarction (AMI) and whether PAF represents one of these stimuli.

MATERIALS AND METHODS

Subjects studied

The study group included 14 consecutive patients with acute myocardial infarction. This group comprised 11 males and three females with an age range of 49–78 (mean 62) years. Exclusion criteria included bacterial and viral infections, renal failure, diabetes mellitus, haematological disease and cardiogenic shock. Patients were included in the study on the basis of a typical history, characteristic changes on the electrocardiogram and elevated plasma levels of creatine kinase, lactate dehydrogenase and aspartate transaminase. Peak creatine kinase concentrations ranged from 352 to 4190 (mean 1924) i.u./l. The infarcted areas, as assessed by electrocardiographic changes, comprised four inferoposterior, four inferior, two anterior, one anteroseptal, two anterolateral and one posterior, of which three were subendocardial. Ten patients received treatment with streptokinase (1.5 million units in 1 h), while all 14 received aspirin, intravenous heparin and intravenous glyceryl trinitrate. Peripheral venous blood EDTA (final concentration 1.5 mg/ml) samples were taken on admission, being 1–12 h after the onset of pain (mean 5.2 h), at 6 h thereafter and on days 1, 2, 5 and 7. The samples were centrifuged immediately and the plasma frozen (−70°C) until analysis.

The study protocol was accepted by the regional ethics committee, and each subject gave their informed consent.

PMN preparation

Peripheral blood neutrophils were isolated from EDTA samples obtained from healthy subjects. Polymorphonuclear neutrophils were isolated by a single-step centrifugation procedure on Histopaque 1119/1077 (Sigma) gradient, which is a modification of the original method described by Boyum [11]. After isolation, neutrophils were washed twice in phosphate-buffered saline (PBS, Flow Laboratories) suspended to 108 ml in Hanks' balanced salt solution (HBSS, Sigma). PMN suspensions were incubated with patients' plasma samples or autologous plasma in a ratio 1:1 for 20 min at 37°C, after which PMN function was evaluated. In a further series of experiments, PMNs were preincubated for 10 min with PAF receptor antagonists BN50739 or BN52021 (Institut Henri Beaufour, SA) at final concentrations of 10−6 mol/l and 10−5 mol/l respectively. These concentrations were proven to inhibit PMN activation by PAF in vitro: CD18 expression increased to 180%±15%, 210%±19% and 205%±20% of initial fluorescence after incubation with PAF in concentrations 10−6, 10−5 and 10−7 mol/l, respectively, and this effect was inhibited by BN50739 (98%±6%, 104%±8% and 101%±9%, respectively) and BN52021 (103%±5%, 106%±9% and 102%±7%, respectively). Each individual set of plasma samples was evaluated using PMNs from a single donor.

Immuno-flow cytometry

Cells were aliquoted at the end of the incubation period and mixed with monoclonal antibodies to human cell-surface receptors (CD18, CD11b or Leu-8, Becton Dickinson, Oxford, U.K.), together with 2% paraformaldehyde fixative solution in PBS for 30 min. Antibodies were phycoerythrin or fluorescein conjugated. After three further washes, cells were resuspended in Isoton (Becton Dickinson) for flow cytometry. Measurements were performed with a FACScan (Becton Dickinson) equipped with an argon laser (488 nm) and three bandpass filters. Data were analysed by gating the neutrophils' forward scatter versus side scatter. Non-specific protein binding was assessed with an isotype-matched immunoglobulin (IgG2b or IgG1, Becton Dickinson). Results were expressed as the difference in mean channel of fluorescence between specific and non-specific antibody binding.

Superoxide anion production

Superoxide anion production by neutrophils was measured using lucigenin-amplified chemiluminescence according to a previously described method [12]. An 80 μl aliquot of study or autologous plasma was placed in each separated well of a white microtitre plate (Dynatech) and 80 μl of isolated PMN suspension in HBSS and 100 μl of 10−4 mol/l lucigenin in PBS were added. The measurements were performed in duplicate. The light emission was measured continuously for 60 min with the use of a microtitre plate-based
luminescence analyser (Amerlite, Kodak Clinical Diagnostics). The peak value detected under each well was taken for statistical analysis. Results are expressed as per cent of peak light emission of PMNs incubated with autologous plasma.

**PAF measurements**

Samples were extracted essentially according to Lopez-Farre et al. [13]. Briefly, 1.5 sample volumes of methanol were added to serum or to stimulated neutrophils, to extract lipid into the organic phase. Chloroform and water were then added to give a final ratio of methanol–water–chloroform of 1:0.9:1. The lower, chloroform-rich, phase was aspirated and vacuum dried under nitrogen. PAF was then purified by fractionation on silica sample preparation cartridges. The dried samples were dissolved in 5 ml of acidified chloroform (pH 5) and applied to activated 100-mg Si cartridges (Isolute, Jones Chromatography, Hengoed, Mid Glamorgan, U.K.). The columns were serially eluted with 5 ml of chloroform–methanol (3:1), 5 ml of chloroform–methanol (3:2) and 5 ml of chloroform–methanol (1:3) and the PAF was eluted with 2 ml of methanol–water (3:1) into polypropylene tubes. This was again dried by vacuum under nitrogen, and stored at –70°C until analysis. PAF was measured by a scintillation proximity RIA (Amersham). Samples were dissolved in assay buffer under the same conditions as standards.

**Data analysis**

Since our data were not normally distributed, as assessed by the Kolmogorov–Smirnov test, statistical evaluation was performed by the non-parametric repeated measures ANOVA (Friedman multiple comparison paired test). Data are given as means ± SEM.

**RESULTS**

Incubation of PMNs with plasma samples obtained at the time of admission resulted in CD18 antigen expression at a level of 18.5 ± 2.4. Plasma obtained 6 h later increased CD18 expression (20.9 ± 2.1; P < 0.05 versus plasma taken on days 5 and 7), as did plasma taken on the next day (20.8 ± 2.7; P < 0.05) while incubation with plasma taken at days 5 and 7 decreased CD18 expression. Stimulation of CD18 antigen expression by samples taken 6 h and 1 day after admission was inhibited in the presence of BN52021 and BN50739 (Fig. 1).

The expression of CD11b by neutrophils exposed to plasma obtained from the patients with AMI at the time of admission was 223 ± 27. Plasma taken 6 h later (279 ± 28; P < 0.05 versus plasma taken at days 5–7) and on the next day (272 ± 26; P < 0.05 versus plasma taken at days 5–7) increased CD11b antigen expression, whereas plasma samples obtained on days 5 and 7 after the onset of symptoms did not. Plasma-mediated stimulation of neutrophil CD11b expression was inhibited in the presence of BN52021 or BN50739 (Fig. 2).

The expression of L-selectin by PMNs incubated with plasma obtained from the patients at the time of admission was 3.38 ± 0.41. Significantly higher expression of L-selectin was observed when PMNs were incubated with plasma obtained on the fifth (4.23 ± 0.65) and seventh (4.81 ± 1.1) days after admission (Fig. 3). Plasma-mediated neutrophil L-selectin shedding was not affected by cell pretreatment with BN52021 or BN50739.

Superoxide anion production by neutrophils exposed to plasma collected at the time of admission measured by chemiluminescence was 168 ± 14 (relative light units) and was increased by incuba-
FIG. 3. Expression of neutrophil L-selectin after exposure to plasma taken from patients with acute myocardial infarction at the time of admission and 6 h and 1, 2, 5 and 7 days later. Expressed as mean channel of fluorescence (MCF). Means ± SEM. *P < 0.05 versus days 5-7.

FIG. 4. Neutrophil superoxide anion production after exposure to plasma taken from patients with acute myocardial infarction at the time of admission and 6 h and 1, 2, 5 and 7 days later. Cells were pretreated with saline (•) or PAF receptor blockers BN52021 (□) or BN50739 (■). Expressed as per cent of peak light emission of PMNs incubated with autologous plasma. Means ± SEM. *P < 0.05 versus days 5-7. **P < 0.05 versus untreated cells.

DISCUSSION

Three possible mechanisms explain the pathophysiological role of activated PMNs in the myocardial damage that occurs during reperfusion following ischaemia. Adherence to endothelial cells with subsequent coronary capillary plugging reduces coronary blood flow, leukotriene-mediated vasoconstriction may further limit myocardial perfusion and release of highly reactive oxygen species may exert a cytotoxic effect on reversibly injured ischaemic myocytes [1].

Several studies have confirmed the involvement of the β2-integrin family in the increased PMN adherence to endothelium during myocardial ischaemia. In patients with unstable angina, neutrophils taken from the coronary sinus showed increased expression of the CD11b/CD18 complex compared with PMNs taken from the aorta, indicating a transcardiac increase in PMN adhesion molecules as a result of myocardial ischaemia [14, 15]. In addition, the accumulation of PMNs in the myocardium after experimental ischaemia–reperfusion is inhibited by the administration of F(ab)2 fragments of a monoclonal antibody against the CD18 chain [6, 7], and also by antibodies which recognize the CD11b z-subunit [16]. A significant increase in coronary blood flow was observed in an experimental ischaemia–reperfusion model after administration of antibodies that blocked CD18 [17] or CD11b subunits [18]. Thus, the expression of PMN adhesion molecules may be involved in capillary plugging and contribute to the no-reflow phenomenon.

Activated PMNs also express L-selectin, a member of the selectin family of glycoproteins which interacts with sialyl Lewis antigen expressed by activated endothelial cells, and probably with other, unidentified endothelial counter-receptors [4]. L-selectin is thought to play a role in the migration of PMNs along the endothelium. Recent studies have shown that L-selectin is involved in PMN extravasation and their subsequent accumulation in ischaemic myocardium [4]. Administration of a monoclonal antibody that recognizes L-selectin attenuated myocardial necrosis and decreased neutrophil accumulation in ischaemic myocardium in a feline model of experimental ischaemia–reperfusion [19]. Antibody against neutrophilic L-selectin also protected against the loss of endothelium-dependent relaxation observed in coronary artery rings isolated from cats subjected to myocardial ischaemia and reperfusion [20]. Furthermore, blockade of P-selectin also reduces PMN adhesion to endothelium during reperfusion of ischaemic myocardium [20, 21].

PMN activation after AMI may be the result of a direct cell contact with endothelial adhesion molecules and/or stimulation by soluble neutrophilic stimuli released from ischaemic myocardium. The results of this study indicate that, during the first 48 h after myocardial infarction, soluble stimuli capable of stimulating PMN superoxide anion production, integrin expression and L-selectin shedding are released to peripheral blood. Both PMN integrin expression and superoxide anion production were significantly increased after incubation with plasma obtained from patients within the first 48 h after the onset of symptoms and was lower when cells were...
incubated with plasma taken on the following days. These results confirm the results of previous reports of PMN activation induced by plasma obtained from patients with MI [5–7] and provides evidence of increased adhesion molecule expression (integrins) and shedding (L-selectin) during this process. The involvement of selectins in PMN extravasation in ischaemic myocardium was recently shown [19–21]. Although the exact role of L-selectin expressed by PMNs is unclear, it is thought to be involved in PMN rolling along the endothelium and/or to act as a homing receptor [4]. The current results indicating L-selectin shedding upon activation in ischaemic myocardium is consistent with its role as a homing receptor.

In the present study we have observed plasma-mediated stimulation of the expression of CD18 and CD11b antigens in patients with AMI. It may therefore be speculated that soluble stimuli released from infarcted tissue activate neutrophils which adhere to endothelium via integrins and subsequently extravasate. We have not measured the expression of CD11a or CD11c antigens and therefore cannot be sure what proportion of the increase in CD18 expression might be in conjunction with CD11a/CD18 or CD11c/CD18 integrins rather than CD11b/CD18.

The nature of the neutrophilic stimuli released during AMI is uncertain. One potential candidate is PAF. PAF is a potent stimulus for PMN activation [10] and is released by ischaemic endothelial cells, resulting in the activation of PMNs, causing greater adherence to the endothelium [9]. Conflicting results have been reported on the release of PAF into the coronary sinus after brief myocardial ischaemia. In one study the level of PAF was shown to be increased in the great cardiac vein during coronary angioplasty [22], whereas others have not detected elevated PAF concentrations in the coronary sinus after PTCA-induced ischaemia or after rapid atrial pacing in patients with ischaemic heart disease, despite evidence of ischaemia as defined by lactate extraction [23]. Similarly, conflicting results have been reported concerning the role of PAF-induced PMN activation during ischaemia–reperfusion. Some reports indicate that neutrophil-dependent tissue injury during myocardial ischaemia is, in part, related to PMN activation by PAF [24], whereas in other studies administration of a PAF antagonist had no effect on PMN accumulation in ischaemic–reperfusion injury [25]. In this study an increase in PAF levels in samples capable of producing PMN activation could not be demonstrated, suggesting that PAF released from ischaemic myocardium may not be directly involved in plasma-mediated PMN stimulation, or alternatively the concentrations needed to cause activation are below the sensitivity of the assay. Recent studies suggest that PAF is an important mediator of PMN–platelet co-operation in ischaemic myocardium [26]. Mechanical and electrical dysfunction caused by platelet infusion in isolated hearts during ischaemia–reperfusion was significantly worsened in the presence of PMNs, and this effect was prevented by treatment with a PAF receptor antagonist [26].

Neutrophils are a rich source of platelet-activating factor [27], and PAF released by activated PMN may also act as an autocrine stimulus. Exposure of PMNs to certain stimuli, for example endothelin, results in PAF production by PMNs and subsequent autocrine stimulation. This explains why cell pretreatment with PAF receptor blockers inhibits PAF-mediated PMN self-stimulation [8]. In the present study, PMN pretreatment with both PAF receptor blockers BN52021 and BN50739 decreased plasma-mediated neutrophil stimulation after AMI in the absence of elevated plasma levels of PAF, suggesting that PAF is acting as an autocrine PMN stimulant.

In conclusion, incubation of washed PMNs from healthy donors with plasma from patients with acute myocardial infarction caused PMN activation with increased expression of integrin adhesion molecules, shedding of L-selectin, and increased superoxide production, effects which are inhibited by PAF antagonism. These findings suggest that PMN activation may occur in the peripheral circulation as a result of the release of soluble stimuli from the heart after acute myocardial infarction, with possible involvement of PAF as an autocrine stimulus.

ACKNOWLEDGMENTS

T.S. was a recipient of a European Society for Cardiology Research Fellowship. The work was supported in part by the British Heart Foundation.

REFERENCES


