Plasma mediated neutrophil stimulation during coronary angioplasty: autocrine effect of platelet activating factor

Tomasz Siminiak, Daniel J O’Gorman, Manjit Shahi, David Hackett, Desmond J Sheridan

Abstract

Background—Polymorphonuclear neutrophils are involved in the development of myocardial injury during ischaemia and reperfusion. Coronary angioplasty has been shown to result in neutrophil activation. This may be a result of contact with ligands expressed by endothelial cells or response to soluble stimuli released from ischaemic tissue into the plasma or both.

Objective—To investigate plasma mediated neutrophil activation during angioplasty.

Methods and results—Plasma samples were collected from the coronary sinus, femoral artery, and femoral vein of 14 patients undergoing angioplasty, before and after the first balloon inflation and at the end of the procedure. Plasma samples were incubated with washed neutrophils isolated from healthy donors. Expression of the adhesion molecules CD18 integrin and L-selectin (Leu-8) was measured by flow cytometry, and superoxide anion production was measured by chemiluminescence. Plasma samples from the coronary sinus and femoral artery but not from the peripheral vein induced increased expression of neutrophil CD18 after balloon deflation. Modification of the expression of L-selectin was not noted. Production of superoxide anion by neutrophils was stimulated by plasma samples from the coronary sinus, but not by those from the femoral artery or vein. This plasma mediated neutrophil stimulation was prevented when the neutrophils were pretreated with platelet activating factor receptor antagonists BN52021 or BN50739. The platelet activating factor concentration detected in the coronary sinus was not higher than in control plasma.

Conclusion—Brief ischaemia during coronary angioplasty leads to the release of soluble stimuli capable of inducing neutrophil integrin expression and free oxygen radical production. Platelet activating factor may act as an autocrine neutrophil stimulus under these conditions.

Keywords: neutrophil stimulation; platelet activating factor; coronary angioplasty; ischaemia/reperfusion

Substantial clinical and experimental evidence has been obtained in recent years to support the involvement of neutrophils in the development of myocardial injury, particularly during ischaemia followed by reperfusion. Neutrophil accumulation in the area of myocardial necrosis initiates a cascade of events resulting in the elimination of injured myocytes and subsequent scar formation. However, neutrophil activation in ischaemic myocardium may also result in the destruction of injured myocytes which have the potential for recovery.

Activation of neutrophils during myocardial ischaemia/reperfusion may occur as a result of neutrophil contact with endothelial adhesion molecules expressed within ischaemic myocardium or in response to soluble stimuli released from ischaemic myocardium. Intravascular neutrophil activation results in the subsequent expression of surface adhesion molecules. Adhesion molecules expressed by activated neutrophils 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.

We have recently shown that plasma obtained from a peripheral vein in patients with acute myocardial infarction can induce chemotaxis, adherence, and superoxide anion production by neutrophils obtained from healthy control donors, suggesting release of neutrophilic stimuli from ischaemic myocardium into the peripheral circulation.

Several investigators have shown that ischaemia/reperfusion injury may be reduced by neutrophil depletion using a variety of methods including neutrophil antiserum, leukopak filters, or pharmacological interventions which modify neutrophil activation. Neutrophil induced biochemical damage of ischaemic myocardium may involve three mechanisms: (a) the release of free oxygen radicals, proteolytic enzymes, and leukotxin by activated neutrophils; (b) neutrophil activation in ischaemic myocardium results in their aggregation and adherence to endothelial cells with subsequent capillary plugging and impairment of coronary blood flow; and (c) vasoconstriction may result from leukotriene B4 release by activated neutrophils.

The injurious role of neutrophil activation during myocardial infarction has been extensively studied, however, the role of neutrophil activation during brief periods of myocardial ischaemia, and its underlying mechanisms, remains unclear. Single reports indicate neutrophil activation in the coronary sinus after ischaemia caused by balloon inflation during coronary angioplasty. These findings suggest that myocardial ischaemia alone, without tissue necrosis, is sufficient to stimulate...
neutrophils. Balloon dilatation induces arterial injury, and damage to endothelium can expose membrane receptors, for immunoglobulin and complement factors, which promote neutrophil activation.13 Thus, although previous studies have shown neutrophil activation after coronary angioplasty, it is not known whether this stimulation occurs as a result of (a) the expression of endothelial receptors or (b) the release of soluble substances capable of neutrophil activation from ischaemic myocardium. As we have shown release of soluble stimuli for neutrophil activation after myocardial infarction,4,14 it may also be expected to occur after ischaemia induced by coronary angioplasty. Furthermore, platelet activating factor, a potent stimulus to neutrophil activation, has been shown to be released from ischaemic myocardium and may contribute to plasma mediated neutrophil stimulation after myocardial ischaemia.15,16

The purpose of the current study was to investigate whether substances capable of neutrophil activation are released during coronary angioplasty and whether platelet activating factor participates in this neutrophil stimulation. Coronary angioplasty is a model for brief reversible ischaemia. In order to exclude the possibility of systemic neutrophil activation secondary to prolonged myocardial ischaemia or necrosis, or both, we have excluded patients with unstable angina or recent myocardial infarction.

**Patients and methods**

**Patients**

The study group included 14 patients undergoing angioplasty of the left anterior descending coronary artery. All patients had stable exertional angina and a significant stenosis of the proximal left anterior descending coronary artery. Patients with diabetes mellitus, renal failure, neoplastic conditions, or haematological diseases were excluded. No patient from the study group had suffered a previous myocardial infarction or undergone bypass surgery. Patients with acute congestive heart failure or significant tricuspid regurgitation were also excluded. The patients’ medications were continued on the day of the procedure. The medications were aspirin (150–300 mg/day in all patients), calcium channel blockers (seven patients), β blockers (six patients), and long acting nitrates (four patients).

The study protocol was approved by the regional ethics committee.

**Procedure**

After premedication with diazepam and local anaesthesia, an 8 French gauge sheath was placed in the femoral vein and a multipurpose catheter was advanced into the coronary sinus or, if not successful, as close to the sinus ostium as possible. An 8 French gauge sheath was placed in the femoral artery, the guiding catheter was introduced, and the coronary arteries were visualised. Isosorbide dinitrate (0.5 mg; Isoket, Schwarz Pharma, Chesham, Buckinghamshire) and 2000 units heparin were given by the intracoronary route and a further 8000 units were given intravenously. Blood samples were taken into EDTA containing tubes and after centrifugation plasma was stored at − 70°C until analysis. The initial samples were taken after administration of intracoronary drugs and immediately before balloon inflation. A second set of samples were taken immediately after the first balloon deflation, and a final set at the end of the procedure—that is, 5 min after last deflation. Samples were taken from the coronary sinus ostium, femoral artery, and femoral vein. Angioplasty was performed with balloons ranging from 2.5 to 4.0 mm when inflated, chosen on the basis of the estimated diameter of normal segments adjacent to the stenosis. The duration of the first balloon inflation was prolonged to 2 min, except in one patient in whom inflation was terminated after 90 s because of severe cardiac pain and pronounced ST segment elevation in the anterior leads. During the first inflation, 10 patients experienced moderate cardiac pain, 10 exhibited ST segment depression, and three had ST segment elevation in the anterior electrocardiogram leads. In total two to five inflations were performed in each patient to achieve a satisfactory result.

**Neutrophil preparation**

Peripheral blood neutrophils were isolated from EDTA samples obtained from healthy volunteers. Polymorphonuclear neutrophils were isolated by a single step centrifugation procedure on a Histopaque1119/1077 (Sigma-Aldrich, Gillingham, Dorset) gradient, which is a modification of the original method described by Boyum.17 After isolation neutrophils were washed twice in phosphate buffered saline (Flow Laboratories, Rickmansworth, Hertfordshire) suspended to 10⁷/ml in Hanks’ balanced salt solution (Sigma). Neutrophil suspensions were incubated with patient’s plasma samples or autologous plasma in a ratio 1:1 for 20 min at 37°C after which neutrophil function was evaluated. In a further series of experiments neutrophils were preincubated for 10 min with platelet activating factor receptor antagonists BN 50739 or BN 52021 (Institut Henri Beaufour, Paris) in final concentrations of 1 × 10⁻⁴ mol/l and 1 × 10⁻⁵ mol/l, respectively. These concentrations were proven sufficient to inhibit neutrophil activation by 10⁻⁴–10⁻⁵ mol/l platelet activating factor in vitro (data not shown). The in vitro effect of contrast medium used for visualisation of coronary arteries during angioplasty on neutrophil activation was evaluated by preincubation of the neutrophil suspension with the medium (Hexabrix320, May and Baker, Dagenham) diluted in autologous plasma to a final concentration of 10, 20, and 30%.

**IMMUNOFLOW CYTOMETRY**

At the end of the incubation period equal numbers of cells were mixed with monoclonal antibodies to human cell surface receptors (CD18, CD11b, or Leu-8, Becton Dickinson, Oxford) together with 2% paraformaldehyde

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**Figure 1** Expression of neutrophil CD18 antigen after incubation with plasma samples obtained from the coronary sinus before (pre) and after (post) the first balloon inflation and at the end of coronary angioplasty. Cells were preincubated with saline (●) or platelet activating factor receptor antagonists BN50201 (●) or BN50739 (●). Values are expressed as mean (SEM) mean channel of fluorescence (MCF).

* P < 0.05 versus preinflation; ** P < 0.05 versus saline treated.
fixative solution in phosphate buffered saline for 30 min. Antibodies were conjugated with phycoerythrin or fluorescein. After three further washes cells were resuspended in Isoton (Becton Dickinson) for flow cytometry. Measurements were performed with a FACSScan (Becton Dickinson) equipped with an argon laser (488 nm) and three band pass 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, respectively; Becton Dickinson). Results are expressed as mean channel of fluorescence (relative units) calibrated by the equipment producer, as commonly used, and shown as the difference between specific and non-specific antibody binding.

SUPEROXIDE ANION PRODUCTION
Production of superoxide anion by neutrophils was measured using lucigenin amplified chemiluminescence according to a previously described method. Investigated or autologous plasma (80 μl) was placed in each separated well of a white microtitre plate (Dynech, Birlinghurst, W Sussex) and neutrophil suspension (80 μl) and lucigenin (100 μl) at a concentration of 10⁻⁴ mol/l in phosphate buffered saline were added. The measurements were performed in duplicate. Light emission was measured continuously for 60 min with the use of a microtitre plate based luminescence analyser (Amerlite, Kodak Clinical Diagnostics, Amersham, Buckinghamshire). The peak value detected under each well was recorded for statistical analysis. Results are expressed as a per cent of peak light emission of neutrophils incubated with autologous plasma.

PLATELET ACTIVATING FACTOR MEASUREMENT
Samples were extracted according to Lopez-Farre et al. Briefly, methanol was added to serum or stimulated neutrophils in a ratio of 1:5:1 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. Platelet activating factor was purified by fractionation on silica sample preparation cartridges. The dried samples were dissolved in 5 ml acidified chloroform (pH 5) and applied to activated 100 mg silica cartridges (ISOLUTE, Jones Chromatography, Hengoed). The columns were serially eluted with 5 ml chloroform: methanol (3:1), 5 ml chloroform: methanol (3:2), 5 ml chloroform: methanol (1:3) and platelet activating factor was eluted with 2 ml methanol: water (3:1) into polypropylene tubes. This was again dried by vacuum and stored at −70°C until analysis. Platelet activating factor was measured by a scintillation proximity radioimmuno assay (Amersham, Little Chalfont, Buckinghamshire). Samples were dissolved in assay buffer under the same conditions as standards.

STATISTICAL METHODS
Non-parametric statistics were used because our data were not normally distributed, as assessed by the Kolomogorov-Smirnov test. Evaluation was performed using the Friedman multiple comparison paired test. Data are given as mean (SEM).

Results
CD18 antigen expression of control neutrophils incubated with plasma taken from the coronary sinus of patients before balloon inflation was 41.2 (6.6) (relative fluorescence units; mean channel of fluorescence) and significantly increased when incubated with plasma taken after the first balloon deflation (49.9 (7.9); P < 0.05) but the difference was not significant when incubated with plasma taken the end of angioplasty procedure (45.0 (7.1)) (fig 1). Similarly, a significant increase in CD18 expression was observed after exposure of neutrophils to samples taken from the femoral artery after balloon deflation (62.6 (10.8)) compared with arterial samples taken before inflation (54.2 (9.7); P < 0.05) but not to samples taken 5 min after last balloon deflation (54.6 (8.7)) (fig 2).

Neutrophil integrin expression after incubation with samples obtained from femoral vein before angioplasty was 43.2 (5.1) and was not significantly changed after the first balloon deflation (46.2 (8.1)) or at the end of the procedure (45.1 (7.2)), (fig 3).

Pretreatment of neutrophils with the platelet activating factor receptor blocker BN52021 inhibited stimulation of CD18 expression by coronary sinus plasma after balloon deflation (43.0 (5.3); P < 0.05 v untreated neutrophils) and this effect was also observed with BN50739 (41.9 (4.9); P < 0.05 v untreated neutrophils) (fig 1). Similarly, stimulation of CD18 expression by arterial plasma taken after balloon deflation was not observed in the presence of BN50739 (55.5 (5.8); P < 0.05 v untreated) or BN50739 (52.8 (6.1); P < 0.05).

The expression of L-selectin was not affected by incubation with plasma obtained after balloon deflation from the coronary sinus (before inflation 12.8 (4.0) v 10.5 (3.2) post-deflation), femoral artery (11.9 (4.2) v 11.2 (4.4), or femoral vein (13.4 (6.1) v 11.8 (4.9)).

Superoxide anion production by neutrophils (expressed as per cent of production after incubation with autologous plasma) after incubation with plasma from the coronary sinus before balloon inflation was 92.1 (5.8)% and increased after the first balloon deflation to 121.2 (6.1)% (P < 0.05) but was not increased when incubated with plasma taken at the end of the angioplasty procedure (94.4 (6.2)% (fig 4). Plasma mediated stimulation of neutrophil superoxide anion production was reduced in the presence of BN52021 (101.9 (2.9)%; P < 0.05 v untreated) or BN50739 (103.9 (3.8)%; P < 0.05). No significant difference in superoxide production was observed when neutrophils were incubated with plasma taken from the femoral artery.

Figure 2 Expression of neutrophil CD18 antigen after incubation with plasma samples obtained from femoral artery before (pre) and after (post) the first balloon inflation and at the end of coronary angioplasty. Cells were preactivated with saline ( ■ ) or platelet activating factor receptor antagonists BN50201 ( ■ ) or BN50739 ( □ ). Values are expressed as mean (SEM) of fluorescence (MCF). ★ P < 0.05 versus preinflation; ★★ P < 0.05 versus saline treated.
artery (101·2 (5-8), 108·7 (4-8), and 104·2 (5-1)% for samples taken before and after the first balloon inflation and at the end of the procedure, respectively) or femoral vein (98·2 (4-9), 97·3 (5-1), and 101·0 (6-9)%).

In vitro incubation of neutrophils with contrast medium diluted to concentrations of 10, 20 and 30% in autologous plasma did not alter CD18 expression (44·1 (5-1), 43·8 (4-2), and 44·0 (6-1), respectively), L-selectin expression (12·1 (4-2), 11·9 (5-1), and 10·9 (3-8)), or superoxide anion production (102·3 (2-1), 101·2 (2-0), and 100·9 (2-9)%).

Concentrations of platelet activating factor in the coronary sinus plasma samples taken after the first balloon inflation were not higher than in samples taken before angioplasty (post-deflation 3·5 (0-5) v 3·1 (0-4) ng/ml before inflation) or in venous plasma from healthy controls (3·0 (0-4) ng/ml).

Discussion
The pathophysiological role of activated neutrophils in the development of myocardial injury during ischaemia/reperfusion is related to their adherence to endothelial cells with subsequent coronary capillary plugging and reduction in coronary blood flow, as well as to the release of highly reactive oxygen species which exert a cytotoxic effect on injured myocytes, which would otherwise have the potential to recover.

The adhesion of neutrophils to endothelial cells requires the expression of adhesion molecules on the endothelial cell and neutrophil. Neutrophils constitutively express the \( \beta_2 \) integrin family of adhesion molecules. These glycoproteins contain a common \( \beta \) chain (CD18) and a distinct \( \alpha \) subunit (CD11a, CD11b, CD11c), and react with intercellular adhesion molecules-1 and -2 expressed by endothelial cells (reviewed in reference 2). Furthermore, neutrophils express L-selectin, a member of the selectin family of glycoproteins, which interacts with sialyl Lewis \( ^{a} \) antigen expressed by activated endothelial cells, and probably with other, unidentified endothelial counter receptors.

Several investigators have confirmed the involvement of the \( \beta_2 \) integrin family in increased neutrophil adherence to endothelium during myocardial ischaemia. Increased expression of the CD11b/CD18 complex was shown in neutrophils taken from the coronary sinus of patients with unstable angina compared with that of neutrophils taken from the aorta, indicating a transcardiac increase of neutrophil adherence molecules as a result of myocardial ischaemia.\(^{12,13} \) In addition, administration of P(\( \alpha \)-antithrombin) fragments of a monoclonal antibody against the CD18 chain inhibited neutrophil accumulation in the myocardium after experimental infarction followed by reperfusion,\(^{14} \) as did antibodies that recognise the CD11b \( \alpha \) subunit.\(^{15} \) A significant increase in coronary blood flow was observed in an experimental ischaemia/reperfusion model after administration of antibodies that blocked CD18 or CD11b subunits.\(^{15} \) Thus, the expression of neutrophil adhesion molecules may be involved in capillary plugging and contribute to the no reflow phenomenon. Preliminary studies have indicated that L-selectin, which is thought to have a role in neutrophil “rolling along” the endothelium, is involved in the extravasation and subsequent accumulation of neutrophils in ischaemic myocardium.\(^{16} \) Administration of a monoclonal antibody that recognises L-selectin attenuated myocardial necrosis and decreased neutrophil accumulation in ischaemic myocardium in a feline model of experimental ischaemia/reperfusion.\(^{17} \) Furthermore, antibody against neutrophilic L-selectin protected against the loss of endothelium dependent relaxation observed in coronary artery rings isolated from cats subjected to myocardial ischaemia and reperfusion. The contribution of P-selectin complexes, present on activated endothelium, to neutrophil adhesion has been suggested from studies utilising a monoclonal antibody to P-selectin.\(^{18,19} \)

This study provides direct evidence that brief periods of myocardial ischaemia during coronary angioplasty result in the release of soluble factors capable of stimulating neutrophil expression of CD18, a component of the integrin family of adhesion molecules. These can be detected in samples from the coronary sinus and femoral artery. Neutrophil activation has previously been shown to occur as a result of coronary angioplasty.\(^{20-28} \) Increased plasma levels of neutrophil elastase have been detected in the coronary sinus after angioplasty.\(^{20,26} \) Neutrophils released into the coronary sinus after balloon deflation were shown to be activated, as assessed by increased nitro-blue tetrazolium reduction and decreased filterability.\(^{21} \) It has been suggested that neutrophil activation during coronary angioplasty may be an effect of the contrast medium.\(^{18} \) However, we have not observed any significant modification of neutrophil activation after incubation of the cells with contrast medium at a concentration of 10-30% in autologous plasma. Furthermore, the first set of samples in each patient was taken after coronary artery visualisation and intracoronary drug administration, immediately before balloon inflation, and thus included possible effects due to drugs or contrast media.

Neutrophil activation after coronary angioplasty may be the result of direct cell contact with endothelial adhesion molecules or stimulation by soluble neutrophilic stimuli released from ischaemic myocardium, or both. The results from our current study provide evidence for plasma mediated neutrophil stimulation during angioplasty. Immediately after the first balloon deflation plasma samples obtained from the coronary sinus and the femoral artery contained factors capable of inducing CD18 antigen expression on the surface of washed neutrophils from healthy donors and coronary sinus plasma was capable of stimulating superoxide anion production by donor neutrophils. This effect has not been observed at the end of the procedure suggesting a rapid washout of soluble factors from reper-
fused myocardium. These findings are consistent with a previous report by Neumann et al.,23 who detected release of neutrophilic chemottractants into the coronary sinus, which were also capable of stimulating the release of neutrophil superoxide anion as measured by a reduction in cytochrome c. Intracardiac activation of neutrophil respiratory bursts was indicated by observations that neutrophils obtained from the coronary sinus released less superoxide anion in response to phospholipid myristate-acetate stimulation.10

Deep entry into the coronary sinus was not possible in all our patients, resulting in some contamination with systemic venous blood. There was no increase in neutrophil activating stimuli in samples taken from the femoral vein (fig 3), implying that the increase in activity in the coronary sinus has been underestimated.

The nature of the neutrophilic stimuli released during coronary angioplasty is uncertain. In the present study, pretreatment of donor neutrophils with platelet activating factor receptor blockers BN52021 and BN50739 decreased plasma mediated stimulation. Platelet activating factor is a potent stimulus for neutrophil activation10 and has been shown to be released by ischaemic endothelial cells resulting in greater adherence of the neutrophils to the endothelium.15 Conflicting results have been reported concerning the role of platelet activating factor as a neutrophil stimulus during ischaemia/reperfusion. Some reports indicate that neutrophil dependent tissue injury during myocardial ischaemia is, in part, related to activation by platelet activating factor,29 whereas in other studies administration of a platelet activating factor antagonist had no effect on neutrophil accumulation after ischaemia/reperfusion injury.30 Similarly, conflicting results have been reported on the release of platelet activating factor into the coronary sinus of non- myocardial ischaemia. The level of platelet activating factor has been shown to be increased in the great cardiac vein during coronary angioplasty,31 whereas others have not detected increased concentrations of platelet activating factor in the coronary sinus during angioplasty nor after rapid atrial pacing in patients with ischaemic heart disease despite evidence of ischaemia defined by lactate extraction.32 In this study platelet activating factor concentrations in samples capable of activating neutrophils were not increased during coronary angioplasty, suggesting that platelet activating factor released from ischaemic myocardium is not directly involved in plasma mediated neutrophil stimulation.

Neutrophils themselves are a rich source of platelet activating factor12 and recent studies suggest that platelet activating factor is an important mediator of neutrophil–platelet cooperation in ischaemic myocardium.33 Mechanical and electrical dysfunction caused by platelet infusion in isolated hearts during ischaemia/reperfusion was significantly worsened in the presence of neutrophils, and this effect was prevented by treatment with a platelet activating factor receptor antagonist.35

Platelet activating factor released by activated neutrophils may also act as an autocrine stimulus. Exposure of neutrophils to certain stimuli, for example endothelin, results in platelet activating factor production by neutrophils and subsequent autocrine platelet stimulation.34 This explains why cell pretreatment with platelet activating factor receptor blockers inhibits platelet activating factor mediated neutrophil self-stimulation.14 In the present study the finding that platelet activating factor receptor blockade inhibited plasma mediated neutrophil stimulation after angioplasty in the absence of increased plasma levels of platelet activating factor suggests that it acts as an autocrine neutrophil stimulus—that is soluble stimuli released from ischaemic myocardium during angioplasty activate neutrophils, which subsequently release platelet activating factor inducing further self-stimulation of neutrophils.

The pathophysiological importance and clinical relevance of neutrophil activation during myocardial infarction are accepted. The importance of neutrophil activation during brief ischaemia without necrosis, however, remains unclear. Neutrophil activation during brief ischaemia results in the release of oxygen free radicals and could contribute to myocardial stunning,35 although recent data have failed to support the role of neutrophil activation in the decrease of contractile function in stunned myocardium.36 Pronounced alterations in diastolic function are also associated with coronary angioplasty.37 It can therefore be speculated that activated neutrophils may contribute to this process. The present findings support the concept of plasma mediated neutrophil stimulation during coronary angioplasty and confirm the usefulness of angioplasty as a model of brief myocardial ischaemia in man. Future research on the role of autocrine platelet activating factor activation may infer a pharmacological approach to minimise neutrophil mediated myocardial injury during ischaemia.

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