Cardiac release of chemoattractants after ischaemia induced by coronary balloon angioplasty

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Abstract

Objective—To investigate the release of chemoattractants after myocardial ischaemia during balloon angioplasty.

Design—Sampling of femoral arterial and coronary sinus blood before and immediately after the first balloon inflation during angioplasty. In a study group of 16 patients the balloon was kept expanded for two minutes, whereas in a control group of eight patients the first balloon inflation was brief (<10 s).

Main outcome measures—Chemotaxis of neutrophils from healthy donors towards patient plasma (Boyden chamber), superoxide anion production by normal neutrophils after incubation with patient plasma (cytochrome C reduction).

Results—In the study group, coronary sinus plasma after balloon deflation was more chemoattractive to normal neutrophils (median relative increase 24% (quartiles: 4%, 45%), $p = 0.008$) and induced a higher superoxide anion production in normal neutrophils (44% (10%, 97%), $p = 0.013$) than arterial plasma. Concomitantly, the degree of activation of patient neutrophils was increased in coronary sinus blood compared with arterial blood, as shown by an increased proportion of neutrophils reducing nitroblue tetrazolium (21% (9%, 38%), $p = 0.006$) and a decreased neutrophil filterability (-16% (-3% , -40%), $p = 0.003$) in coronary sinus blood. In the study group before balloon inflation and in the control group before and after balloon inflation differences between arterial and coronary sinus blood were not significant. Signs of ischaemia (lactate release, ST segment changes) were only detected in the study group.

Conclusion—After transient myocardial ischaemia during balloon angioplasty there is a local release of chemoattractants, associated with neutrophil activation.

(Br Heart J 1993;70:27-34)

gressive cell activation with discharge of various toxic compounds predominately derived from leucocytes.¹ Consistent with these concepts, changes in neutrophil function suggestive of cell activation have been found in peripheral and coronary sinus blood of patients with symptomatic coronary artery disease.^{8,9} The changes even occur in stable angina,^{8,9} when myocardial ischaemia is transient and brief. During percutaneous transluminal coronary angioplasty (PTCA), repetitive short periods of myocardial ischaemia were associated with local neutrophil activation, as indicated by increased neutrophil elastase concentrations in coronary sinus blood.¹⁰ Moreover, in peripheral arterial occlusive disease with intermittent claudication short periods of ischaemia cause local alterations of neutrophil function.¹¹

In coronary artery disease it is not known whether the changes in neutrophil function in stable angina or those found after PTCA are due to myocardial ischaemia itself or to processes occurring at the atherosclerotic plaque.^{8-10,12} The purpose of our study was to find whether transient coronary occlusion during PTCA induces a release of chemoattractants with neutrophil activation that can be related to the ischaemia.

Patients and methods

PATIENTS

The study group comprised 24 patients (18 men, six women, median age 56 (quartiles: 48,64)) with single vessel disease undergoing elective PTCA of a left anterior descending coronary artery (LAD) stenosis proximal to the first septal perforator. The stenoses had to show an area reduction of 70% or more on interactive computerised analysis (CRP GmbH, Konstanz, Germany) and had to be classified as type A.¹³ All patients had chronic stable angina of Canadian Cardiovascular Society functional class III.¹⁴ The indication for PTCA was based on a low threshold (work load ≤ 100 W) for exercise induced myocardial ischaemia on bicycle ergometry. Patients with detectable collateral blood supply to the distal LAD, previous Q wave infarction, or a history of non Q wave infarction as well as those with interfering non-cardiac diseases, such as inflammatory disorders, malignancy, or diabetes mellitus, were not eligible for the study.

The study was approved by the institutional ethics committee for human subjects. All patients gave written informed consent.

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Accepted for publication
2 December 1992

Recent experiments suggest that interactions between leucocytes, platelets, and endothelial cells may cause microvascular injury in myocardial ischaemia.¹⁻⁴ These cell to cell interactions involve an ongoing release of chemoattractants and cytokines⁵⁻⁷ and a pro-

STUDY PROTOCOL

During the study, patients were kept on aspirin (100 mg/day) and short acting oral nitrates, but the rest of their medication had been discontinued for at least five half lives. After premedication by 10 mg diazepam given orally and local anaesthesia, the coronary sinus was cannulated through the right internal jugular vein with a 7F multipurpose catheter. A 9F femoral artery sheath was inserted percutaneously under local anaesthesia and 10 000 IU of heparin were given intra-arterially. Balloon diameters when inflated ranged from 2.5 mm to 3.5 mm, depending on the size of the normal vessel segment adjacent to the stenosis. The guide wire of the balloon catheter was placed in the stenosis with the aid of fluoroscopy. Up to this point 25 ml to 65 ml of contrast medium (Solustrast, Byk-Gulden, Konstanz, Germany) had been used to manipulate the coronary sinus catheter and the balloon catheter.

Immediately after positioning the guide wire for the balloon catheter, blood samples were drawn simultaneously from the coronary sinus and from the femoral artery for one minute. Without any further application of contrast medium, the balloon was placed in the stenosis and inflated. In the study group of 16 patients the balloon was kept fully expanded for two minutes. In the control group of eight patients, however, the balloon was only briefly inflated until full expansion was achieved (<10 s). Immediately after deflation of the balloon, a second set of blood samples was drawn simultaneously from the coronary sinus and from the femoral artery for one minute. The blood samples were put on ice and processed immediately.

EFFECT OF PATIENT PLASMA ON NORMAL NEUTROPHILS

Normal neutrophils were taken from healthy donors. Preparation of neutrophils by density gradient separation was performed at 4°C.^{15 16} Blood samples (50 ml) were anticoagulated with CPDA (Na-citrate, phosphate buffer, dextrose, adenine; Fa Greiner, Nürtingen, Germany) and were mixed with 15 ml of 3% dextran in normal saline (w/v, MW 266 000 g/mol). After sedimentation the supernatant was collected. Residual erythrocytes were lysed in 15 ml ice cold distilled water, and after 25 seconds, isotonicity was reconstituted by addition of 5 ml 3.6% NaCl. Leucocytes were resuspended in phosphate buffered saline (PBS) and were layered on a two step density gradient made up of Histopaque 1077 on top of Histopaque 1119 (Sigma, Deisenhofen, Germany). After centrifugation at 800 *g* for 20 minutes the lower white cell band was harvested, washed twice, and resuspended in PBS. The yield ranged between 40% and 60% of the neutrophils in whole blood. Ninety seven per cent of the cells collected were neutrophils with more than 95% viable cells (trypan blue exclusion).

Chemoattractiveness of patient plasma was assessed by the method of Boyden.^{17 18} In essence, neutrophils from one single sample

of a normal donor were exposed to various plasma samples of a single patient. Each experiment was performed in duplicate. A 48 well microchemotaxis chamber¹⁸ (Neuro-Probe, distributed by Nuclepore, Tübingen, Germany) was used with polycarbonate filters (3.0 µm pores, Nuclepore GmbH) to separate the upper and lower compartments. With the lower compartments containing plasma samples diluted by an equal volume of PBS, the mounted microchemotaxis chambers were preincubated at 37°C in humidified air with 5% CO₂ for 30 minutes. After filling the upper compartments with control neutrophils in PBS (5 × 10⁵ cells/ml), the microchambers were kept under the same conditions for another 80 minutes. Thereafter, the filters were removed and the neutrophils at the side of the filter facing the upper compartment were wiped off. After air drying and staining with Wright's stain (Sigma, Deisenhofen, Germany) the number of cells migrated was counted in five high power fields (magnification × 400). The effect of patient plasma on chemokinesis of control neutrophils was assessed in additional experiments. In these experiments the neutrophils contained in the upper compartments were suspended in the same diluted plasma samples as were put into the lower compartments.

To assess the stimulation by patient plasma of superoxide anion production in control neutrophils, the cells were incubated with patient plasma at 37°C for 15 minutes. Then control neutrophils were resuspended in PBS and washed twice. Superoxide anion production was measured by the superoxide dismutase (SOD) inhibited reduction of cytochrome C as described by Babior *et al*.¹⁹ The standard reaction mixture contained 2.5 × 10⁵ neutrophils, 45 µmol/l cytochrome C (from horse, Sigma) with or without 1000 U/l SOD (specific activity 30 000 U/mg, Sigma), and enough Hank's balanced salt solution to make a final volume of 0.5 ml. The mixtures were incubated at 37°C for 15 minutes before the reaction was stopped by putting the vials on ice. After centrifugation at 4°C, the SOD inhibited reduction of cytochrome C was found in the supernatant by measuring absorbance at 550 nm ($E_{550\text{ nm}} = 2.11 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, Twinreader, Flow Laboratories, Meckenheim, Germany). Results are expressed as nmol superoxide anions / 5 × 10⁵ neutrophils / 15 min. All experiments were performed in triplicate.

FUNCTION TESTS IN PATIENT NEUTROPHILS

The proportion of activated neutrophils was assessed by the nitro blue tetrazolium test. This was performed on freshly drawn heparinised blood samples (20 U/ml) by a modification of the method of Park *et al*.²⁰ as described earlier.¹¹ Briefly, the blood specimens were incubated at 37°C for 15 minutes with an equal volume of an 0.1% solution of nitro blue tetrazolium (Sigma, Deisenhofen, Germany) in PBS (pH 7.2) and then kept at room temperature for another 15 minutes. Smears were prepared and stained by

Table 1 Baseline characteristics of the study population

	Study group (n = 16)	Control group (n = 8)	p Value
Age	54 (48, 64)	56 (51, 64)	0.67
M/F	13/3	5/3	0.62
Degree of stenosis (% area)	92 (85, 95)	90 (83, 94)	0.58
Inflation pressure (atm)	4.8 (4, 5)	4.5 (4.3, 5.3)	0.98
ST segment deviation (sum of V ₂ V ₄ V ₆ (mV)	0.5 (0.38, 0.68)	0 (0, 0)	0.001

Values are median (quartiles).

Wright’s stain (Sigma). The percentage of neutrophils containing formazan deposits of at least the size of a lobe of the nucleus was designated as a positive nitro blue tetrazolium score.

Passive neutrophil deformability was analysed by the method of Nash *et al.*²¹ In essence, after discarding platelet rich plasma, neutrophils were separated from ethylene diamine tetraacetate (EDTA) anticoagulated blood by means of a two step density gradient made up of Histopaque 1077 on top of Histopaque 1119. Purified neutrophils were resuspended in PBS containing 1 g/l EDTA and 5% autologous plasma, and the cell concentration was adjusted to 5 × 10⁵ neutrophils/ml. Neutrophil filtration was performed in a St George’s filtrometer (Carri-Med, Dorking, England) with polycarbonate membrane filters (Nuclepore, Tübingen, Germany), nominal pore diameter 8 µm. Filtration was carried out under constant pressure of 294 Pa (= 3 cm H₂O). With a microcomputer linked to the filtrometer (software by Carri-Med, Dorking, England) the relative filtration rate (with respect to buffer alone) after filtration of 1 ml of neutrophil suspension (the so called late relative filtration rate) was determined as a measure of neutrophil filterability.

OTHER METHODS

The cells were counted with a Coulter Counter, Model ZF (Coulter Electronics, Herts, England). Blood smears were examined by an experienced technician. Total neutrophils were evaluated by multiplying the white cell count by the differential neutrophil count. Lactate concentrations were measured enzymatically with a standard kit (Boehringer Mannheim, Germany).

STATISTICAL ANALYSIS

Results are reported as median (quartiles)

unless otherwise indicated. Differences between matched samples were tested by Wilcoxon’s matched pairs signed ranks test, and differences between the study group and the control group by the Mann-Whitney-Wilcoxon rank sum test. A p value <0.05 in the two tailed test was regarded as significant.

Results

BASELINE CHARACTERISTICS OF THE STUDY POPULATION

Table 1 shows the baseline characteristics of the study population. There were no significant differences between the control group and the study group in terms of age, sex, inflation pressure needed for full expansion, and severity of LAD stenosis before PTCA. In all patients PTCA was completed successfully and resulted in a median reduction in stenosis of 66% (54%, 81%).

In the study group, all patients experienced angina during PTCA that was accompanied by significant ST segment changes (table 1). Furthermore, immediately after balloon deflation lactate concentrations in coronary sinus blood were significantly higher than in arterial blood (table 2, fig 1B). Under resting conditions lactate concentrations did not differ significantly between coronary sinus blood and arterial blood (fig 1B).

In the control group, only one patient had angina during the first balloon inflation, but none of the patients showed significant ST segment changes (table 1). Also, lactate concentrations in coronary sinus blood did not significantly increase after balloon inflation (table 2, fig 1A).

EFFECT OF PLASMA ON NORMAL NEUTROPHILS

In the study group, plasma from coronary sinus blood after balloon deflation was more chemoattractive than plasma from simultaneously taken arterial blood (table 2) and plasma from coronary sinus blood before balloon inflation (median difference: 5.1 (−0.2, 14.6) cells vision field; p = 0.016) (fig 2B). Concomitant significant changes in plasma induced chemokinesis, however, could not be found (not shown). Plasma stimulated superoxide anion production by normal neutrophils showed similar changes as plasma chemoattractiveness (fig 3B). After balloon deflation, plasma from coronary sinus blood

Table 2 Differences in arterial and coronary sinus blood immediately after balloon deflation in the study and control groups

	Study group		Control group		p Value
	D	D _{rel} (%)	D	D _{rel} (%)	
Lactate (mmol/l)	0.61 (0.28, 0.84)	80 (33, 98)	−0.11 (−0.35, 0.02)	−10 (−26, 1)	0.001
Plasma chemoattract (cells/vision field)	8.8 (0.1, 13.9)	24 (4, 45)	−1.5 (−6.9, 7.3)	−4 (−21, 16)	0.030
Stim SOP (nmol/5 × 10 ⁵ cells/15 min)	0.98 (0.32, 2.31)	44 (10,97)	0.27 (−0.43, 0.71)	27 (−16, 34)	0.030
PMN (1/nl)	0.13 (−0.06, 0.19)	3 (−1, 5)	0.04 (−0.06, 0.17)	1 (−1, 4)	0.65
NBT score (%)	2.1 (−0.1, 3.1)	21 (1, 38)	−0.4 (−1.9, 0.6)	−3 (−17, 6)	0.013
LFR	−0.05 (−0.01, −0.18)	−16 (−3, −40)	0.02 (−0.02, 0.07)	6 (−4, 17)	0.012

D, median absolute arterial and coronary sinus blood difference (quartiles); D_{rel}, median relative arterial and coronary sinus blood difference (quartiles); p values for the differences between the study group and the control group by two tailed test; lactate, serum lactate concentration; plasma chemoattract, chemoattractiveness of patient plasma to control neutrophils; stim SOP, superoxide anion production in control neutrophils stimulated by patient plasma; PMN, patient neutrophil count; NBT score, nitro blue tetrazolium score of patient neutrophils; LFR, late relative filtration rate of patient neutrophils.

Figure 1 Plot of femoral arterial (FA) and coronary sinus (CS) blood differences in lactate concentration before and immediately after the first balloon inflation (A) in the control group (brief balloon inflation) and (B) in the study group (balloon inflation for two minutes). *p* Values by two tailed test.

stimulated a higher superoxide anion production than plasma from simultaneously taken arterial blood (table 2) and plasma from coronary sinus blood before balloon inflation (median difference: 1.28 (0.19, 2.73) nmol/5 × 10⁵ cells/15 min; *p* = 0.004). Neither plasma stimulated superoxide anion production nor plasma chemoattractiveness showed any significant arterial and venous blood differences under resting conditions or changes in arterial blood after balloon deflation.

In the control group, there were no significant differences between arterial and coronary sinus blood in the effect of plasma on normal neutrophils (figs 2A and 3A). Neither did normal neutrophils respond differently to plasma from the coronary sinus before and

after balloon inflation (figs 2(A) and 3(A)). Differences in the effects of patient plasma from arterial and coronary sinus blood on control neutrophils immediately after balloon deflation were significantly greater in the study group compared with the controls (table 2).

FUNCTIONAL CHANGES IN NEUTROPHILS
Neutrophil counts did not show any significant differences between arterial and coronary sinus blood in either group (table 2).

In the study group, immediately after balloon deflation coronary sinus blood contained a higher proportion of nitro blue tetrazolium positive neutrophils than coronary sinus

Figure 2 Plot of FA and CS blood differences in chemoattractiveness of plasma to control neutrophils before and immediately after the first balloon inflation (A) in the control group (brief balloon inflation) and (B) in the study group, (balloon inflation for two minutes). Abbreviations and statistics as for fig 1.

Figure 3 Plot of FA and CS blood differences in plasma stimulated superoxide anion production by control neutrophils before and immediately after the first balloon inflation (A) in the control group (brief balloon inflation) and (B) in the study group (balloon inflation for two minutes). Abbreviations and statistics as for fig 1.

blood before balloon inflation (median difference: 3.2% (1.6%, 4.1%), $p = 0.001$, fig 4B). Furthermore, after balloon deflation filterability of neutrophils taken from coronary sinus blood was significantly lower than that before balloon inflation (median difference: -0.095 (-0.013 , -0.185), $p = 0.004$, fig 5B). Similar changes in the arterial blood were not found. Thus significant differences between arterial and coronary sinus blood nitroblue tetrazolium score (fig 4B) and in late relative filtration rate of neutrophils (fig 5B) were found immediately after balloon deflation (table 2).

In the control group, comparison of neutrophil data before and after balloon inflation did not show any differences; nor were there

any significant differences between arterial and coronary sinus blood before or after balloon inflation (figs 4A and 5A, table 2).

Differences between arterial and coronary sinus blood in nitro blue tetrazolium score and in late relative filtration rate of neutrophils immediately after balloon deflation were significantly more pronounced in the study group compared with the control group (table 2).

Discussion

Our present study shows for the first time that the heart, transiently rendered ischaemic by coronary artery occlusion during PTCA, releases chemoattractants for neutrophils.

Figure 4 Plot of FA and CS blood differences in nitro blue tetrazolium (NBT) score before and immediately after the first balloon inflation (A) in the control group (brief balloon inflation) and (B) in the study group (balloon inflation for two minutes). Abbreviations and statistics as for fig 1.

Figure 5 Plot of FA and CS blood differences in late relative filtration rate before and immediately after the first balloon inflation (A) in the control group (brief balloon inflation) and (B) in the study group (balloon inflation for two minutes). Abbreviations and statistics as for fig 1.

This was shown by increased chemotaxis of normal neutrophils towards plasma from the coronary sinus blood of the ischaemic heart. The potential of these chemoattractants for stimulating neutrophils was indicated by an enhanced superoxide anion production in normal neutrophils after preincubation with plasma from the coronary sinus blood of the ischaemic heart. Similar neutrophil activation, as found in normal neutrophils after preincubation with patient plasma, was found in the patients' native neutrophils immediately after transient myocardial ischaemia by balloon inflation, the coronary sinus blood contained more activated neutrophils with reduced deformability than did the arterial blood.

Cardiac release of chemoattractants with concomitant neutrophil activation was only found, if substantial myocardial ischaemia—as shown by myocardial lactate release and ST segment changes—was induced by balloon inflation. If, however, balloon inflation was brief (<10 s) and did not cause any significant myocardial lactate release or ST segment changes, no significant alterations in neutrophil function and plasma chemoattractiveness were found.

It is known from animal experiments^{22 23} as well as from studies in patients with peripheral arterial occlusive disease¹¹ that ischaemia below the threshold of irreversible cell damage may alter neutrophil function. In coronary artery disease, however, the changes in neutrophil function, as yet, could not be related to myocardial ischaemia.^{8–10} In a previous study on granulocyte activation after PTCA, interpretation of the data concerning the role of ischaemia was hampered by multiple non-standardised balloon inflations, by varying amounts of contrast media given, and by the lack of a control group or verification of myocardial ischaemia by metabolic alterations.¹⁰ In our study, an identical protocol

was applied to the control group and to the study group except for the duration of balloon inflation. This resulted in a significant difference in the extent of myocardial ischaemia between the two groups. With respect to plaque fissuring and endothelial tearing during balloon inflation, no substantial differences between the two groups are to be anticipated, as in both groups full balloon expansion was achieved with essentially the same inflation pressures. The present study, therefore, strongly suggests that the local release of chemoattractants with concomitant neutrophil activation is caused by myocardial ischaemia.

METHODOLOGICAL CONSIDERATIONS

Changes in coronary sinus blood must be assumed to reflect only part of the changes in the ischaemic area of the heart, as the coronary sinus receives only about 50% of its blood from the LAD region.^{24 25} Moreover, the plasma specimens taken for analysis may contain only part of the mediators generated in myocardial ischaemia. Despite immediate cooling and processing, some of the mediators with short biological half lives, in particular adenosine²⁶ and arachidonic acid metabolites,²⁷ are not effective under the given experimental set up. The most likely mediators of the increase found in chemotactic activity are, therefore, more stable compounds, such as cytokines (for example, interleukins 6 and 8^{28 29}) complement fractions,³⁰ thrombin,³¹ and platelet activating factor.³² Further studies with receptor antagonists may enable the mediators of the neutrophil responses to plasma from ischaemic myocardium to be identified.

In previous studies,^{8–10} interpretation of changes in neutrophil function was hampered by possible artefacts occurring during the separation procedure. In our study, increased neutrophil function found after transient

myocardial ischaemia during PTCA is primarily based on the examination of unseparated neutrophils from freshly drawn samples from the nitro blue tetrazolium test. As neutrophil activation leads to a loss of passive deformability,³³⁻³⁵ filtrometry was used as an additional index for the degree of neutrophil activation. Filtrometry can be performed with anticoagulation by EDTA. At the expense of a potential underestimation of the in vivo changes, the use of EDTA minimises spontaneous activation and neutrophil aggregation during separation.³⁶ Neutrophil filtration is, therefore, hardly affected by separation artefacts.³⁶

In the experiments on the effect of patient plasma on normal neutrophils, separation induced modifications of neutrophil function cannot contribute to the variations within individual patients. This was assured by the use of control neutrophils from the same separation procedure for each specimen of one patient. At least part of the variation between individual patients in the effect of patient plasma on control neutrophils, however, must be attributed to the experimental design, as the control neutrophils were taken from different donors for different patients. For this reason, no attempt was made to correlate the extent of stimulation of control neutrophils by patient plasma with the degree of activation in patient neutrophils or with the extent of release of myocardial lactate.

It is not impossible that neutrophil function and mediator release is affected by the treatment with aspirin³⁷ or the exposure of blood cells to the foreign material introduced by the balloon. The changes associated with ischaemia however, cannot be attributed to these effects, as they did not differ between the control and the study group and were effective both under resting conditions and after balloon deflation.

PATHOPHYSIOLOGICAL CONSIDERATIONS

Release of chemoattractants and neutrophil activation immediately after myocardial ischaemia caused by balloon inflation could be the consequence of an interaction between blood cells and endothelial cells.⁴⁻⁶ This interaction is facilitated by the prolonged contact between blood cells and endothelial cells under low (or zero) shear stresses and by an accumulation of inflammatory mediators that are neither washed off nor counterbalanced by inflowing endogenous inhibitors.⁴⁻⁶ Potential mediators of a mutual modification of cell function between blood cells and endothelial cells include platelet activating factor^{32,38} tumour necrosis factor,^{39,40} interleukins 6 and 8,^{28,41} and vasoactive arachidonic acid metabolites.^{4,42,43} Furthermore, complement 1q fixation may occur at activated adherent neutrophils resulting in the formation of chemotactic complement fractions.^{7,30}

Consistent with these concepts, cardiac release of vasoactive substances has been shown in patients with coronary artery disease.⁴⁴ Our study shows, for the first time, a

release of chemoattractants with concomitant neutrophil activation in the ischaemic human heart during PTCA. Endothelial cell blood cell interactions with mutual cell activation may, therefore, be assumed to start early during myocardial ischaemia.

The local leucocyte activation may cause progressive cytotoxicity resulting in microvascular injury and even tissue damage.¹ The findings of our study may, therefore, help to explain the delayed functional recovery after PTCA, as indicated by: (a) delayed recovery to normal of coronary vascular reserve,⁴⁵ (b) prolonged diastolic dysfunction,⁴⁶ and (c) delayed resolution of exercise induced abnormalities on thallium-201 and glutamate-N-13 scintigraphy.^{47,48} Moreover, priming of various neutrophil and endothelial cell functions by mediator release may contribute to the cumulative detrimental effects of repetitive ischaemic episodes on coronary vasomotor reserve,⁴⁹ myocardial function,⁵⁰ and viability.⁵¹

In summary, our study shows a local release of chemoattractants with concomitant neutrophil activation after coronary occlusion for two minutes during PTCA, which is not found after brief (<10 s), but full balloon inflation. These findings may be explained by endothelial cell blood cell interactions with production of mediators, starting in the early course of myocardial ischaemia. The early changes in neutrophil function during myocardial ischaemia may contribute to the ischaemic injury.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 320), Bonn-Bad Godesberg, Germany. We thank Mrs Anita M Neumann for editorial help.

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