Granulocyte Activation After Coronary Angioplasty in Humans

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To determine whether percutaneous transluminal coronary angioplasty (PTCA) would lead to neutrophil activation with subsequent discharge of proteolytic enzymes, like elastase, and oxygen free radicals, like superoxide anion, blood samples were taken from the coronary sinus and aorta in 14 patients with stable angina and one-vessel disease who underwent PTCA. Neutrophils were separated by means of the Ficoll-Hypaque system and were stimulated to detect release of elastase and generation of superoxide anion. Plasma levels of elastase were also measured by an immunoenzymatic method. PTCA was successful in all patients. Plasma elastase levels increased significantly at the end of the procedure compared with pre-PTCA values both in the coronary sinus (from 129.2±16.6 to 286.6±39.7 µg/l, p<0.005) and in the aorta (from 117.4±13.6 to 258.1±41.3 µg/l, p<0.005). On the other hand, superoxide anion released in the supernatants after neutrophil stimulation by phorbol-myristate-acetate decreased after PTCA in the coronary sinus (before PTCA, 60.1±7.1; after PTCA, 40.7±6.8 nmol 1×10⁷ granulocytes/ml/15 min, p<0.05), whereas a mild but not significant decrease was observed in the aorta (from 58.3±10.9 to 55.3±8.6 nmol 1×10⁷ granulocytes/ml/15 min, p=NS). Similarly, elastase release in the supernatants after neutrophil stimulation by Ca²⁺ ionophore A23187 decreased after PTCA in the coronary sinus (from 226.1±26.5 to 159.2±27.8 µg 4×10⁴ granulocytes/ml, p<0.05), whereas no change was observed in aorta (from 233.5±47.3 to 188.7±31.3 µg 4×10⁴ granulocytes/ml, p=NS). These results suggest that neutrophil activation occurs during PTCA and leads to higher plasma elastase levels and to a decreased ability of stimulated granulocytes to release their toxic compounds at the end of the procedure. Activation of neutrophils may have potential implications regarding the occurrence of the pathophysiological processes occurring after balloon angioplasty in humans. (Circulation 1990;82:140–146)

Circulating granulocytes defend the body against invading microbes by releasing a complex assortment of agents and toxins.¹ The potentially destructive armamentarium of neutrophils may also act as mediator of inflammatory tissue damage and may also play a critical role in the pathogenesis of vascular injury.² Proteolytic enzymes, like elastase, and reactive oxygen metabolites, like superoxide anion, discharged by activated neutrophils have direct cytotoxic effects and are able to destroy human endothelial cells.³ The purpose of this study was to ascertain whether neutrophil activation takes place after percutaneous transluminal coronary angioplasty (PTCA) in humans. We hypothesized that the release of toxic substances by granulocytes during PTCA may amplify the endothelial damage caused by balloon dilatation of the vessel. Moreover, neutrophils may potentiate the platelet activation⁴ that commonly occurs after PTCA as a result of arterial injury and that is thought to play an important role in the pathogenesis of restenosis.⁵ Accordingly, 14 patients with stable angina who underwent PTCA were studied to determine whether the procedure would lead to enhanced discharge of elastase and generation of superoxide anion by activated neutrophils. The results of this investigation, indicating an increased release of such toxic substances after PTCA, may be relevant to a better understanding of the pathophysiological processes that can take place after balloon angioplasty in humans.

Methods
Study Patients and PTCA Protocol

The study group consisted of 14 patients scheduled for elective one-vessel PTCA. All patients had stable

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exertional angina and a positive exercise stress test defined as the development of typical chest pain accompanied by ST segment depression of at least 1 mm at peak exercise. No patient had had a previous myocardial infarction. Patients with prior PTCA or bypass surgery were excluded from the study. The patients' medications, including aspirin (500 mg/day) and calcium channel blockers (diltiazem 120 mg t.i.d. or nifedipine 20 mg t.i.d.) were not discontinued on the day of the procedure.

After premedication with diazepam and local anesthesia, a femoral artery sheath was placed by a single-wall entry technique. A 7F Gorlin catheter was introduced percutaneously under local anesthesia into an antecubital vein and advanced into the coronary sinus. Heparin (10,000 units) was given intravenously. Heparinized blood samples (20 ml) were then obtained from a Gorlin catheter in the coronary sinus and from the guiding catheter in the aorta at the beginning and at the end of the procedure. Angioplasty was performed with balloon dilation catheters ranging in diameter from 2.5 to 3.5 mm when inflated. Balloon sizes were chosen on the basis of estimates of the diameter of normal segments adjacent to the stenosis. Each balloon inflation was maintained for 45 to 90 seconds at a pressure ranging from 4 to 7 atm. Angioplasty was considered successful if a reduction in the severity of the obstruction to less than 50% diameter narrowing was obtained without important procedure-related complications. At the end of the procedure, blood samples from both the coronary sinus and the aorta were obtained again.

**Control Group**

The control group included 11 male patients who underwent coronary arteriography because of suspected ischemic heart disease. All complained of angina on exertion and had a positive exercise stress test. All patients were taking aspirin and calcium antagonists that were not suspended on the day of the procedure. Coronary arteriography was performed by the femoral approach. A 7F Gorlin catheter was introduced percutaneously under local anesthesia into an antecubital vein. Heparinized blood samples (20 ml) were simultaneously obtained from the coronary sinus and the aorta at the beginning and at the end of the procedure. White blood cells and neutrophil counts were determined before and after PTCA both in the coronary sinus and in the aorta.

**Separation of Granulocytes**

Peripheral blood leukocytes were obtained from the heparinized blood samples taken in the coronary sinus and in the aorta. Twenty milliliters of 6% dextran were added to blood samples that were immediately placed on ice. The polymorphonuclear leukocytes were purified by means of the Ficoll-Hypaque system. Contaminating erythrocytes in the granulocytes fraction were removed by lysis with 0.75% ammonium chloride solution containing 20 mM Tris HCl buffer (final pH 7.4) and 0.25% autologous plasma. The purity of the cell fractions was 92±2.3% granulocytes. All of these separation steps were performed at 4°C with cells maintained in buffer containing 150 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, and 1 mM KH2PO4 (pH 7.4) to minimize up-regulation of integrin glycoproteins to the cell surface. Cell viability was greater than 95% as measured by trypan blue exclusion or release of the cytoplasmic enzyme lactic dehydrogenase. Cells were suspended in phosphate buffer saline at 1×10^7/ml and evaluated within 30 minutes from completion of the separation procedure.

**Measurement of Superoxide Production**

The method used to measure superoxide production was that described by Babior et al. The spectrophotometric measurement is based on the capacity of superoxide to react with the iron contained in cytochrome c type VI (Sigma Chemical Co., St. Louis, Mo.) at rest and after stimulation with phorbol-myristate-acetate. This reaction takes place in both the presence and absence of superoxide dismutase, which transforms superoxide anion into hydrogen peroxide. Readings were obtained with a spectrophotometer (Perkin-Elmer, Norwalk, Conn.) at wavelengths of 550 and 468 nm. The results are expressed in nanomoles of superoxide anion 1×10^7 granulocytes/ml/15 min. Each experiment was performed in triplicate.

**Assay of Elastase**

Plasma levels of granulocytic elastase were measured with an immunoenzymatic method (Granulocyte Elastase kit from Merck Immunoassay, Darmstadt, FRG). Briefly, the plastic tube provided in the test kit is coated with antibodies specific to granulocytic elastase. The complex of the granulocyte elastase-α1-proteinase inhibitor present in the biological sample was bound to these antibodies by its granulocyte elastase component. Enzyme-labeled antibodies specific to the α1-proteinase inhibitor were then added. The enzyme used for labeling was alkaline phosphatase, and the substrate was 4-nitrophenyl phosphate. Substrate hydrolysis was stopped by adding sodium hydroxide solution (2 mol/l). The color intensity of the reaction mixture was measured in a photometer and was proportional to the concentration of the granulocyte elastase-α1-proteinase inhibitor in the biological sample. The granulocyte elastase concentration was determined from a calibration curve.

In 13 patients, samples of granulocyte suspensions were also collected 6 minutes after stimulation with Ca^2+ A23187 at a 1×10^−3 M final concentration. These samples were centrifuged for 2 minutes at 11,000g/min in an Eppendorf centrifuge. The elastase assay was then performed on the resulting supernatants. The data are expressed in μg 4×10^6 granulocytes/ml.
Statistical Analysis

Values are expressed as mean±SEM. Comparisons between groups were made with the Wilcoxon signed-rank test. The correlation between percent changes in the various parameters of neutrophil function and total time of balloon inflation or total amount of contrast medium given during the procedure was accomplished by linear regression analysis.

Results

PTCA Procedure

The dilated vessel was the left anterior descending coronary artery in 12 patients and a dominant right coronary artery in two patients (Table 1). PTCA was successful in all 14 study patients; coronary stenosis was reduced from 73.5±3.1% to 25.5±3.3%. Mean inflation time was 180 seconds (range, 90–330 seconds). During inflation, 11 patients had chest pain associated with ST elevation, whereas one patient had only mild chest discomfort accompanied by slight ST depression, and two patients did not show any changes during balloon inflation. Total amount of dye given during the procedure was 153±6 ml. All procedures were free of complications with no electrocardiographic or enzymatic evidence of myocardial necrosis. No allergic reactions to radiographic contrast medium were observed.

Elastase and Superoxide Anion Release by Neutrophils

Neutrophil count did not change after PTCA both in the coronary sinus (pre-PTCA, 3,240±500/mm³; after PTCA, 3,840±500/mm³; p=NS) and in the aorta (pre-PTCA, 3,120±200/mm³; after PTCA, 4,040±400 mm³; p=NS). Plasma elastase increased both in the coronary sinus (from 129.2±16.6 to 4,040±400 mm³; p=NS) and in the aorta (from 3,840±500/mm³; p=NS) and in the aorta (from 3,120±200/mm³; p=NS) and in the aorta (from 3,120±200/mm³; p=NS). Plasma elastase increased both in the coronary sinus (from 129.2±16.6 to 286.6±39.7 µg/l, p<0.005) and in the aorta (from 117.4±13.6 to 258.1±41.3 µg/l, p<0.005) (Figure 1). On the contrary, elastase measured in the supernatant after stimulation by Ca²⁺ A23187 decreased in the coronary sinus (from 226.1±26.5 to 159.2±27.8 µg 4×10⁶ granulocytes/ml, p<0.05), whereas a mild but not significant decrease was observed in the aorta (from 233.5±47.3 to 188.7±31.3 µg 4×10⁶ granulocytes/ml, p=NS) (Figure 2). Data on superoxide anion production after stimulation with phorbol-myristate-acetate were obtained in 13 patients. Superoxide anion release in the coronary sinus significantly decreased (from 60.1±7.1 to 40.7±6.8 nmol 1×10⁷ granulocytes/ml/15 min, p<0.05), whereas no change was observed in the aorta (from 58.3±10.9 to 55.3±8.6 nmol 1×10⁷ granulocytes/ml/15 min, p=NS) (Figure 3). In the 11 patients who had chest pain associated with ST elevation, no significant correlation was found between the total duration of artery occlusion and the percent variations in plasma elastase, elastase released in the supernatant, and superoxide anion generation in the coronary sinus and aorta. Likewise, no correlation was found between such changes in neutrophil function and total amount of dye given during the procedure.

Control Group

All patients had significant coronary artery disease, involving two-vessel in four patients and one-vessel in seven patients. Mean values of plasma elastase were similar at the beginning and end of coronary arteriography in the coronary sinus (before, 104.5±9.8; after, 117.4±20.6 µg/l, p=NS) and aorta (before, 102.2±10.1; after, 107.3±9.9 µg/l, p=NS). No difference was also found in mean values of elastase released in the supernatant after stimulation by Ca²⁺
A23187 before and after coronary arteriography in the coronary sinus (before, 291±74; after, 283±28 μg 4×10^6 granulocytes/ml, p=NS) and aorta (before, 276±47; after, 232±70 μg 4×10^6 granulocytes/ml, p=NS) and in the superoxide anion production in the coronary sinus (before, 67.3±6; after, 71.5±7 nmol 1×10^7 granulocytes/ml/15 min, p=NS) and aorta (before, 71.8±8; after, 73.4±6 nmol 1×10^7 granulocytes/ml/15 min, p=NS). Total quantity of dye was 119.8±5.3 ml, which was significantly less than the amount given in patients undergoing PTCA (p<0.05). The procedure did not give rise to any complications.

Discussion

Although it is known that neutrophils play an important role in the pathophysiology of myocardial ischemia and infarction, data on neutrophil function in patients with coronary artery disease are scanty. Mehta et al.14 recently studied 37 patients with ischemic heart disease and 20 age-matched control subjects by measuring plasma levels of peptide Bβ, an index of neutrophil elastase release, and leukotriene B4 generation by peripheral blood neutrophils. The investigators found that patients with stable angina had an increased neutrophil function as shown by the enhanced potential to generate leukotriene B4 during stimulation by calcium ionophore, whereas patients with unstable angina or recent myocardial infarction had undergone in vivo neutrophil activation as demonstrated by increased plasma levels of peptide Bβ and by the lower ability of stimulated neutrophils to generate leukotriene B4. Because
activation of neutrophils results in release of a variety of inflammatory mediators, including granular constituents.\textsuperscript{1,15} Large quantities of granulocyte proteases, such as elastase, can be detected in plasma.\textsuperscript{16} On the other hand, in light of previous in vivo activation, isolated neutrophils may respond to further ex vivo stimulation by releasing lesser quantities of their toxic substances.\textsuperscript{14}

Our data show that after PTCA plasma levels of elastase increase, whereas stimulated neutrophils release lesser quantities of both elastase and superoxide anion. These results may be secondary to activation of neutrophils resulting in a release of proteolytic enzymes and oxygen free radicals during the procedure and in a decreased ability of stimulated neutrophils to discharge their toxic compounds at the end of PTCA. The finding that neutrophil count did not change significantly at the end of the procedure excludes the possibility that these data would represent release of a large number of neutrophils into the circulation leading to an elevated "baseline" elastase. Moreover, the data obtained in 11 control patients showing no change in elastase and superoxide anion generation after coronary arteriography indicate that activation of neutrophils was not dependent on some technical factors related to the procedure, like femoral artery puncture and coronary artery or coronary sinus catheterization. Although the total amount of contrast medium given to patients undergoing PTCA was greater than in patients undergoing coronary arteriography, it seems unlikely that such a small difference could account for the wide changes observed in neutrophil function after PTCA. Moreover, no relation was found between the total amount of dye given during the procedure and the percent changes in plasma elastase, elastase released in the supernatant, and superoxide anion generation in the coronary sinus and aorta. Of note, most of the changes observed in neutrophil function after PTCA were seen in the coronary sinus and not in the aorta. This finding may be explained by activation of neutrophils passing through the coronary circulation so that neutrophils obtained from the coronary sinus are "exhausted," thus releasing lower quantities of proteolytic enzymes or oxygen radicals. Alternatively, it can be hypothesized that a functionally competent pool of neutrophils is trapped in the coronary tree during PTCA, leaving in the circulation a less-functional population. A similar phenomenon occurs shortly after the initiation of hemodialysis and cardiopulmonary bypass when functionally competent granulocytes aggregate in the pulmonary vascular bed, whereas a less-active population of neutrophils remains in the circulation because of its inability to aggregate.\textsuperscript{17,18} This possibility however seems unlikely, because the latter conditions are associated with neutropenia, whereas no change in neutrophil count was observed in our patients. In this study, we did not investigate other neutrophil functions, such as cellular adherence or expression of membrane receptors. Changes in such neutrophil functions would have provided more compelling evidence that granulocyte activation had occurred during PTCA. However, elastase release occurs when neutrophils are triggered by foreign or host antigens, leading to partial degranulation of these leukocytes.\textsuperscript{1,16} Moreover, increase in plasma elastase has been considered as evidence of granulocyte activation in patients with ischemic heart disease.\textsuperscript{14}

It is possible that the arterial lesion caused by balloon dilation of the vessel would represent the stimulus causing neutrophil activation during PTCA. Endothelial injury alters the physiological interaction between leukocytes and the vessel wall increasing the adhesion of polymorphonuclear granulocytes to

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Plots of individual and mean±SEM of superoxide anion released in the coronary sinus and aorta before and after percutaneous transluminal coronary angioplasty by neutrophils stimulated by phorbol-myristate-acetate. Values are expressed in nmol 1X10\textsuperscript{7} granulocytes/ml/15 min.}
\end{figure}
endothelial cells.\textsuperscript{19–21} Damaged endothelium leads to the exposure of membrane receptors for immunoglobulin and complement fragments that promote neutrophil adherence. An additional explanation to our findings could be that neutrophil activation is secondary to myocardial ischemia occurring during balloon inflation. Neutrophil activation may be triggered by 15 minutes of ischemia followed by reperfusion.\textsuperscript{22} It is not known, however, whether repeated, albeit much shorter, bouts of ischemia followed by restoration of flow, as it occurs during PTCA, can induce the same effects on neutrophil function. In our patients, no correlation was found between the total duration of coronary occlusion and the percent changes in plasma elastase, elastase production, and superoxide anion release by granulocytes after PTCA. Electrocardiographic changes during coronary occlusion, however, are approximate markers of transient myocardial ischemia. In our study, we did not measure metabolic alterations or indexes of mechanical dysfunction induced by myocardial ischemia, and we did not attempt to prevent the occurrence of ischemia by distal coronary perfusion during balloon occlusion. Further studies are therefore needed to clarify the role of transient myocardial ischemia in triggering neutrophil activation during PTCA.

Potential Implications

Balloon dilation induces arterial injury that is mild in the area where the balloon has contacted the arterial wall, but it is deep in the region of a split or tear. In the dilated region, vascular injury with endothelial denudation and exposition of subendothelial structures and collagen fibrils triggers the immediate deposition and aggregation of platelets.\textsuperscript{5} Although the mechanisms of restenosis are not completely understood, they appear to be related to the hemorrhheological response to the therapeutic injury induced by the balloon on the vessel.\textsuperscript{23} The neutrophil activation that follows PTCA, through the release of proteolytic enzymes and the generation of oxygen free radicals, may aggravate the endothelial damage and further stimulate platelets, thus having a potential bearing on the subsequent development of restenosis. Oxygen radicals have also been implicated in the pathogenesis of stunned myocardium\textsuperscript{24–26} and the persistent postischemic myocardial dysfunction despite restoration of blood flow. Engler and Covell\textsuperscript{22} recently reported that marked reduction in the number of circulating granulocytes by leukopak filtration significantly enhances recovery of postischemic myocardial function in a canine model and concluded that activated granulocytes are a direct cause of stunned myocardium. Of note, Wijns et al\textsuperscript{27} found that coronary occlusion during PTCA was associated with profound alterations in diastolic function that persisted after restoration of myocardial blood flow. Activation of neutrophils and release of oxygen radicals may account for these still unexplained observations in patients undergoing balloon angioplasty.

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