STIMULATION OF NEUTROPHIL INTEGRIN EXPRESSION DURING CORONARY ARTERY BYPASS GRAFTING: COMPARISON OF CRYSTALLOID AND BLOOD CARDIOPLEGIC SOLUTIONS

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Objectives: This study was designed (1) to evaluate the influence of plasma obtained from patients undergoing coronary artery bypass grafting on L-selectin, CD11b, and CD18 expression on human neutrophils and (2) to determine the influence of the use of crystalloid or blood cardioplegia during bypass grafting on plasma-mediated expression of adhesion molecules on polymorphonuclear neutrophils.

Patients and methods: Patients undergoing coronary artery bypass grafting were divided into 2 groups to receive crystalloid or blood cardioplegic solutions. Peripheral vein, radial artery, and coronary sinus blood samples were drawn at aortic crossclamping, aortic crossclamp release, and 30 minutes after reperfusion. Human neutrophils were incubated with patients' plasma, and the expression of CD11b, CD18, and L-selectin was determined with flow cytometry.

Results: In patients receiving crystalloid cardioplegic solutions, plasma samples collected from the coronary sinus at aortic clamp release and 30 minutes thereafter induced significantly higher expression of neutrophil CD11b and CD18 than plasma samples obtained from a peripheral vein or artery at the same time points. The expression of L-selectin on polymorphonuclear neutrophils was significantly reduced with plasma obtained 30 minutes after reperfusion as compared with samples collected at aortic crossclamp release. In the group receiving blood cardioplegia, no significant differences in CD11b, CD18, or L-selectin expression were found.

Conclusions: (1) Ischemia/reperfusion after coronary artery bypass grafting is associated with the release of factors capable of neutrophil activation from myocardium into the circulating blood. (2) The release of soluble stimuli for neutrophils during bypass grafting may be modified by the cardioplegic solution. (J Thorac Cardiovasc Surg 2000;119:1270-7)

Activated polymorphonuclear neutrophils (PMNs) are known to play a pivotal role in the secondary inflammatory response to myocardial ischemia/reperfusion injury. When stimulated, neutrophils are able to release a number of harmful agents, such as proteolytic enzymes and oxygen-derived free radicals, which can damage surrounding cells and extracellular structures. In addition to the neutrophil-induced direct injury of parenchymal cells, chemotactic stimulation enhances PMN adhesion to endothelial cells and homotypic aggregation, thus leading to plugging of microvessels, reduction in coronary blood flow, and further extension of tissue injury. Substantial evidence has been derived from experimental studies to support an important role of neutrophils in injury after prolonged ischemia/reperfusion. Interventions aimed at systemic depletion of circulating neutrophils such as leukocyte filters, antineutrophil antibodies, or hydroxy urea resulted in significant reduction of myocardial infarct size in an animal model.[1-3] Similar results were obtained in experiments investigating anticomplement or antiadhesion approaches.[4-6]

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The noxious effects of neutrophils are thought to be specifically dependent on certain adhesion glycoproteins that are expressed on the surface of leukocytes, endothelial cells, and myocytes. L-selectin, a member of the selectin family, which is constitutively expressed on lymphocytes and neutrophils, is important for the first contact between PMNs and endothelial cells. Its activity is responsible for neutrophil margination and allows other adhesive mechanisms to operate. Activation of leukocytes results in rapid disappearance of the antigen from the cell membrane. Shed material—soluble form of L-selectin—can be found in normal serum. CD11b/CD18, or leukocyte adhesion receptor Mo1, belongs to the integrin family. It is a heterodimer composed of a β-chain, known as CD18, coupled to an α-chain (CD11b). This antigen is present in low concentration on the surface of unstimulated neutrophils, monocytes, and macrophages. On chemotactic stimulation, it becomes rapidly (within minutes) up-regulated. CD11b/CD18 seems to play a pivotal role in the process of neutrophil-mediated ischemia/reperfusion injury: It is responsible for PMN adherence to the endothelium and subsequent extravasation and accumulation at sites of inflammation; it is involved in neutrophil aggregation; and it modulates parenchymal cell toxicity due to neutrophil-derived free radical activity.[7,8]

Neutrophil activation on ischemia/reperfusion may result from a direct contact with endothelial adhesion molecules expressed in the ischemic heart, or it can be mediated by certain stimuli released from myocardium to the circulating blood. In a previous study, plasma obtained from peripheral blood of patients with acute myocardial infarction had a potential to induce integrin expression and reactive oxygen species production by control PMNs, thus confirming the latter possibility.[9] The aim of this study was to investigate whether stimuli capable of neutrophil activation are released from ischemic and reperfused myocardium during coronary artery bypass grafting (CABG). We evaluated the expression of CD18, CD11b, and L-selectin on human neutrophils incubated with plasma obtained from patients undergoing routine CABG. In addition, we compared plasma-mediated stimulation of PMN adhesion molecule expression in patients receiving crystalloid and blood cardioplegic solutions during the CABG procedure.

Patients and methods

Patients studied. The study group consisted of 50 patients with coronary artery disease from a total of 950 patients qualified for CABG during a 1-year period on the basis of the routinely used clinical and angiographic criteria. So that we could avoid the possible effect of the maneuvers on the results, all patients included in the study group were operated on by the same team (n = 83). The exclusion criteria were a history of heart surgery, recent myocardial infarction (<3 months), diabetes mellitus, renal failure, the use of inotropic support or intra-aortic balloon pumping during the operation, and the existence of an inflammatory disease, factors which are known to affect PMN function. Patients undergoing CABG as a minimally invasive procedure and those being operated on without aortic crossclamping were also excluded. Thus, of the 83 patients, 33 patients were excluded. Ejection fraction estimated by echocardiography ranged from 28% to 68% (mean 68%). The bypass circuit period ranged from 51 to 182 minutes (mean 86 minutes), and the aortic clamping time was 17 to 64 minutes. During cardiopulmonary bypass (CPB), the hematocrit value was kept minimally at 22% to 25%, potassium at 5.5 to 6.0 mmol/L, and diuresis was maintained at 100 to 150 mL/h, with the use of furosemide as necessary.

The operation was performed with CPB at moderate hypothermia (30°-32°C). Patients were randomly assigned to 1 of 2 groups. In group A, myocardial preservation was achieved by antegrade infusion of cold crystalloid St Thomas' Hospital cardioplegic solution. In group B, antegrade blood cardioplegia according to Buckberg (blood and crystalloid solution in a 4:1 ratio) was used. After grafting, patients were rewarmed to 36°C and separated from CPB by the gradual reduction of venous return to the bypass circuit.

Finally, 50 patients (8 women and 42 men; 34-73 years, mean age 53 years) were enrolled in the study (25 in each group). No patient was excluded after randomization. No statistically significant differences in mean age, sex, left ventricular ejection fraction, CPB time, and myocardial ischemia time were found between the 2 study groups.

Informed consent to participate in the study, which was accepted by the local ethics committee, was obtained from each subject.

Patients' plasma samples. Peripheral venous blood samples were drawn into tubes containing heparin (5 IU/mL) before the installation of CPB. Other samples were collected from the basilic vein, the radial artery, and the coronary sinus directly before myocardial ischemia (aortic crossclamping), at the beginning of reperfusion (aortic clamp release), and 30 minutes after reperfusion. The samples were immediately centrifuged and the plasma was deeply frozen (−70°C) until analysis to enable simultaneous examination of all plasma samples.

Neutrophil preparation and flow cytometry. Neutrophils were isolated from peripheral blood samples obtained from healthy volunteers. PMNs were isolated by a single-step centrifugation procedure on Gradisol gradient (Polfa, Kutno), which is a modification of the method described by Böyum.[10] After centrifugation, neutrophils were washed twice and suspended in 10 mL of saline solution. Red blood cells were lysed with hemolytic buffer (0.828 g NH_4Cl and 0.1 g $KHCO_3$ dissolved in 100 mL of distilled water with 20 μL of 1N NaOH). The neutrophils were resuspended in Hanks solu-

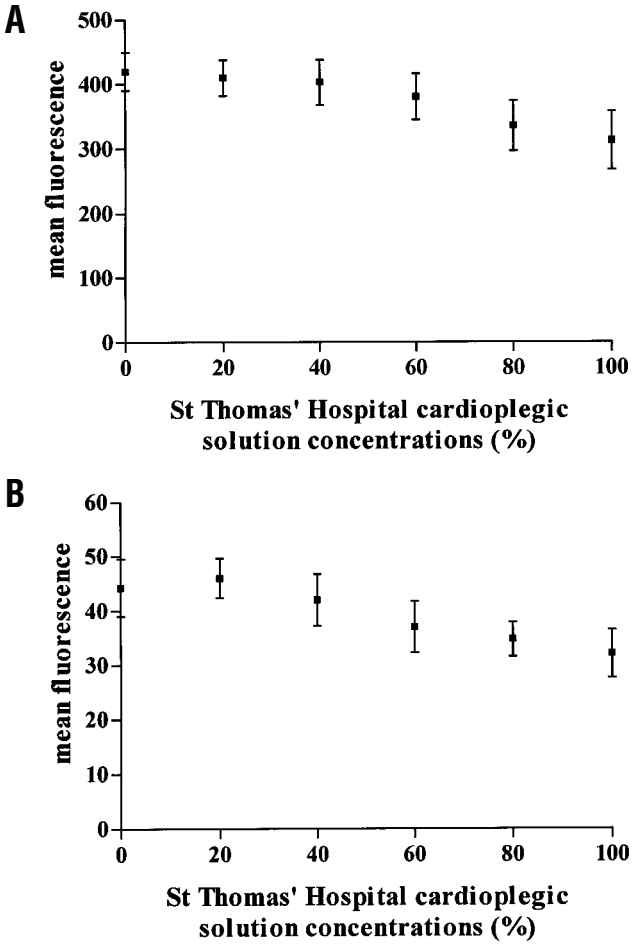

Fig 1. The in vitro effect of increasing concentrations of St Thomas' Hospital cardioplegic solution on CD11b **(A)** and CD18 **(B)** on neutrophils.

tion (Sigma Chemical Co, St Louis, Mo). PMNs were incubated with patients' plasma samples or St Thomas' Hospital cardioplegic solution for in vitro testing (15 minutes at 37°C), and the expression of CD18, CD11b, and L-selectin was determined by direct 2-color immunofluorescence staining of blood leukocytes and flow cytometry. Aliquots of neutrophil suspension were incubated for 30 minutes with fluorescein isothiocyanate- or phycoerythrin-conjugate murine monoclonal antibodies to human CD18, CD11b, or L-selectin (Becton Dickinson, Oxford, United Kingdom). The cells were washed 3 times with phosphate-buffered saline solution, resuspended in Isoton solution (Becton Dickinson, Franklin Lakes, NJ), and flow cytometry analysis was performed immediately. Flow cytometry was performed with a Becton Dickinson FACScan instrument equipped with an argon laser (488 nm) and 3 bandpass filters. Ten thousand events were acquired from each sample. A gate was drawn around the granulocyte population identified by the characteristic forward and side scatter profile. The neutrophils that expressed levels of fluorescence for an individual adhesion molecule that exceeded nonspecific background fluorescence were considered to be positively labeled for that molecule. The level of surface expression for each adhesion molecule was assessed as the mean fluorescence intensity of staining for that molecule.

Statistical analysis. Since the data were not normally distributed, as assessed by the Kolgomorov-Smirnov test, statistical analysis was performed by the nonparametric repeated-measures analysis of variance within groups and with the Mann-Whitney test for comparisons between groups. Data are shown as means ± SD.

Results

The in vitro influence of St Thomas' Hospital cardioplegic solution on β-integrin expression. Incubation of neutrophils with St Thomas' Hospital cardioplegic solution in in vitro conditions resulted in a significant decrease in the expression of CD11b and CD18 on these cells (Fig 1).

Plasma-mediated changes in the expression of CD11b. The expression of CD11b by PMNs (Fig 2) stimulated with plasma obtained before the installation of CPB did not differ between the 2 study groups. In patients receiving crystalloid cardioplegic solution, the CD11b expression of neutrophils induced by plasma obtained from the coronary sinus at aortic crossclamp release was significantly higher ($P < .05$) than that mediated by plasma obtained from the radial artery and basilic vein at the same time (371.1 ± 5.5 vs 318.4 ± 79.5 and 333.0 ± 170.5 mean fluorescence, respectively). Expression of CD11b by neutrophils incubated with plasma collected from the coronary sinus at 30 minutes of reperfusion was significantly higher than at aortic crossclamping and aortic crossclamp release (437.1 ± 155.5 vs 293.3 ± 76 and 371.1 ± 95.5 mean fluorescence, respectively). However, in patients receiving blood cardioplegia, no significant increase in the PMN CD11b expression was observed when cells were incubated with plasma taken at the beginning of reperfusion. Thirty minutes thereafter, plasma-mediated stimulation in the coronary sinus was significantly lower in these patients, as compared with subjects receiving crystalloid cardioplegia (374.5 ± 113 vs 437.1 ± 155.5, $P < .05$).

Plasma-mediated changes in the expression of CD18. The patterns of changes in CD18 expression (Fig 3) in the group receiving crystalloid cardioplegic solution paralleled those described for CD11b. CD18 expression by PMNs incubated with plasma collected from the coronary sinus at aortic crossclamp release was significantly higher than that induced by plasma

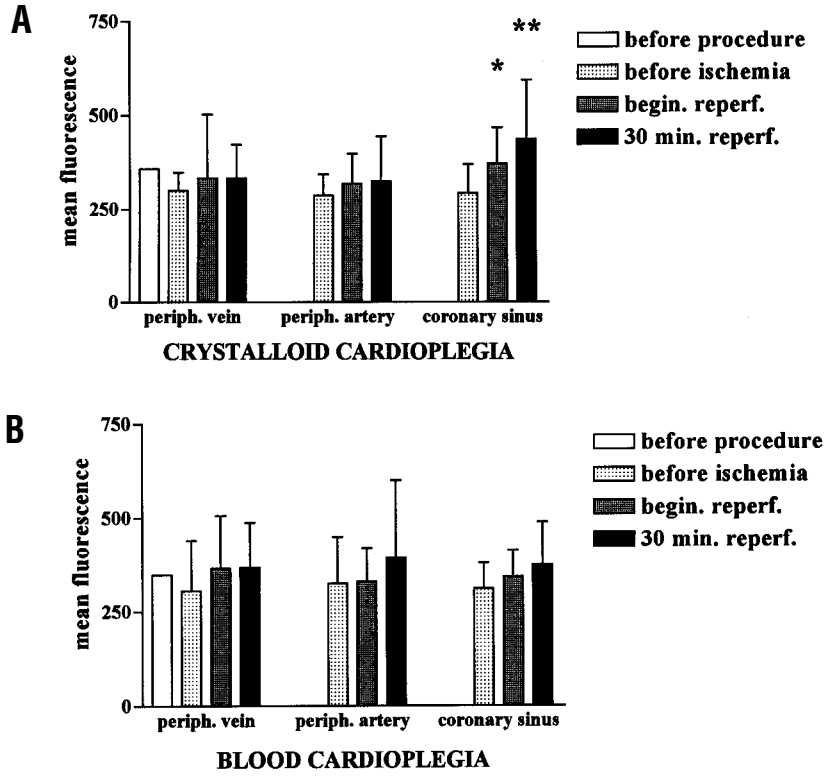

Fig 2. The effect of plasma obtained during CABG from patients receiving crystalloid **(A)** or blood **(B)** cardioplegic solutions on CD11b expression of neutrophils. Plasma samples were taken from peripheral vein, peripheral artery, and coronary sinus before myocardial ischemia (at aortic crossclamping), at the beginning of reperfusion (at aortic clamp release), and 30 minutes after reperfusion. Mean values ± SD. $^{*}P < .05$ versus peripheral vein and artery. $^{**}P < .05$ versus before ischemia and at the beginning of reperfusion.

obtained from the radial artery at the same time (51.4 ± 8 vs 45.3 ± 8 mean fluorescence, $P < .05$). Expression of CD18 induced by plasma collected from the coronary sinus 30 minutes after reperfusion was significantly higher than CD18 expressions mediated by plasma samples obtained from the radial artery at the same time and from the coronary sinus at aortic clamping and aortic clamp release (56.9 ± 9 vs 49.6 ± 11, 37.1 ± 7.5 and 51.4 ± 8 mean fluorescence, respectively; $P < .05$). In 25 patients receiving blood cardioplegic solution, no significant differences were observed when PMNs were incubated with plasma samples obtained from the coronary sinus, peripheral vein, or peripheral artery at aortic clamping, at aortic clamp release, or 30 minutes after reperfusion.

Plasma-mediated changes in the expression of L-selectin. In patients receiving crystalloid cardioplegic solution, L-selectin expression by PMNs (Fig 4) incubated with plasma drawn from the coronary sinus 30 minutes after reperfusion was significantly lower than at aortic clamp release (11.8 ± 7.5 vs 17.3 ± 8 mean fluorescence, $P < .05$). In the blood cardioplegia group, no significant differences in mean fluorescence values for L-selectin were found.

Discussion

Much attention has recently been focused on the role of PMNs in complications after ischemia/reperfusion during CABG. Activation of neutrophils during cardiac surgery with CPB is a well-known phenomenon. Activated leukocytes seem to play a pivotal role in inflammatory reactions initiated by CPB[11] that may lead to organ dysfunction.[12] Moreover, oxygen-derived free radicals released by activated PMNs may contribute to myocardial stunning often observed after cardiac operations.[13] The clinical relevance of leukocyte activation during CABG has recently been demonstrated in a few studies investigating the use of leukocyte-depleted cardioplegic solution or antiadhesion strategies. It has been shown that leukocyte depletion as an

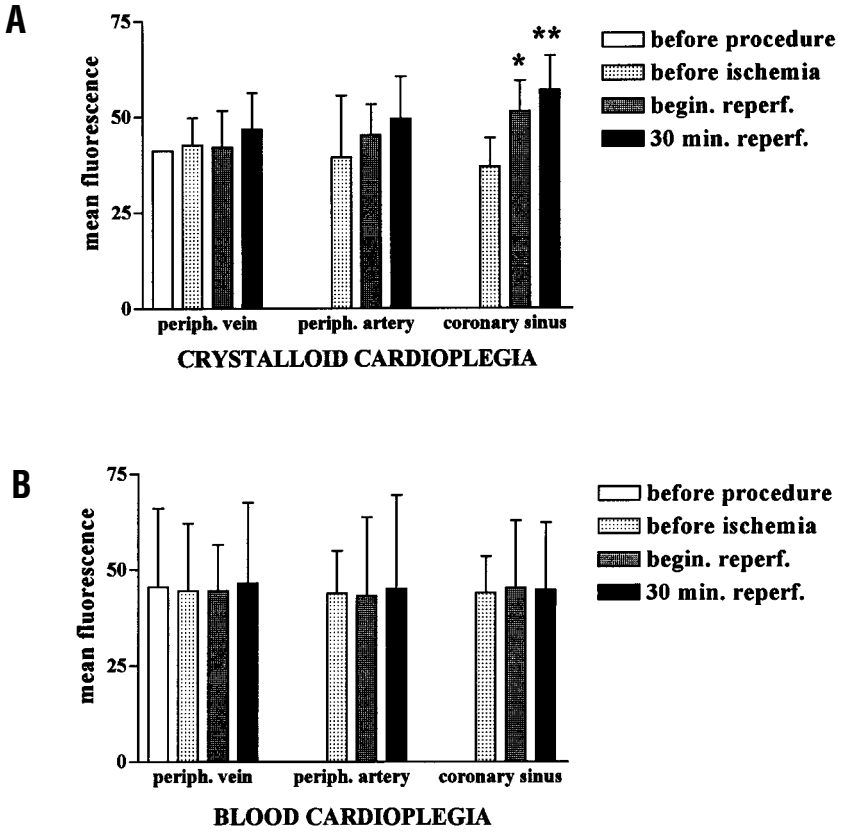

Fig 3. The effect of plasma obtained during CABG from patients receiving crystalloid **(A)** or blood **(B)** cardioplegic solutions on CD18 expression of neutrophils. Plasma samples were taken from peripheral vein, peripheral artery, and coronary sinus before myocardial ischemia (at aortic crossclamping), at the beginning of reperfusion (at aortic clamp release), and 30 minutes after reperfusion. Mean values ± SD. $^{*}P < .05$ versus peripheral artery. $^{**}P < .05$ versus before ischemia and at the beginning of reperfusion.

adjunct to terminal blood cardioplegia was associated with better clinical outcome in patients with left ventricular hypertrophy.[14] Similarly, the inhibition of leukocyte-endothelial interaction by monoclonal antibodies against leukocyte adhesion molecules seems to have a beneficial effect on myocardial function after heart surgery both in animals[15-17] and in human beings.[18]

As intercellular adhesion is a critical step in the sequence of events leading to granulocyte-dependent tissue injury, the changes in neutrophil expression of adhesion molecules during cardiac operations have been extensively studied. In the majority of studies the β-integrin expression was found to be up-regulated during CPB. Increased expression of CD11b/CD18 on neutrophils was observed in isolated CPB circuits[19,20] and in animal models,[21,22] as well as in human beings.[23-25] However, in these studies the mechanisms leading to PMN activation remained unclear. The enhanced β-integrin expression could be related not only to inflammatory stimuli released in ischemic tissues but also to interactions with endothelial adhesion molecules or to direct contact of the blood with synthetic surfaces of the CPB circuit. The present study has clearly demonstrated that ischemia and reperfusion during CABG is associated with release of stimuli enhancing integrin expression by PMNs. In neutrophils obtained from healthy volunteers and incubated with plasma collected from the coronary sinus at the beginning of reperfusion (aortic clamp release) and at 30 minutes of reperfusion, expression of both CD11b and CD18 was significantly higher than that observed at the time of aortic crossclamping. Moreover, CD11b and CD18 expression was enhanced exclusively by the plasma from coronary sinus. As St Thomas' Hospital solution in vitro inhibited both CD11b and CD18

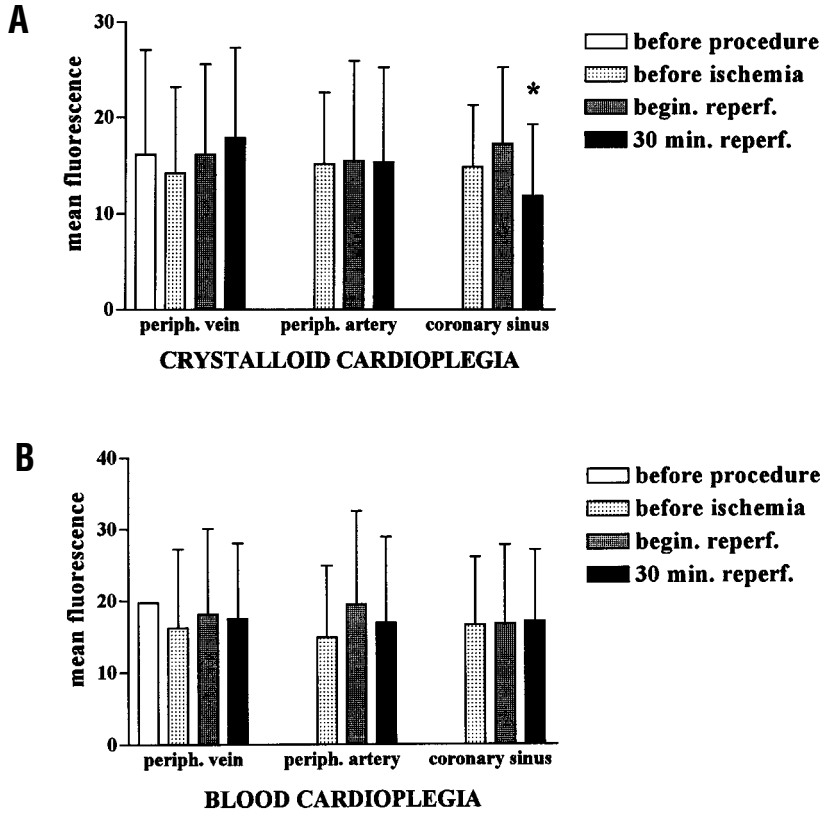

Fig 4. The effect of plasma obtained during CABG from patients receiving crystalloid **(A)** or blood **(B)** cardioplegic solution on L-selectin expression of neutrophils. Plasma samples were taken from peripheral vein, peripheral artery, and coronary sinus before myocardial ischemia (at aortic crossclamping), at the beginning of reperfusion (at aortic clamp release), and 30 minutes after reperfusion. Mean values ± SD. $^{*}P < .05$ versus at the beginning of reperfusion.

expression by PMNs, the stimulatory effect of the plasma obtained from the coronary sinus probably resulted from local ischemia and reperfusion. Furthermore, the difference between arterial and coronary sinus samples suggests that the effect was independent of the possible influence of extracorporeal circuit. The CD11b expression in the presence of plasma collected from a peripheral vein or artery at any time point was even somewhat lower than the value obtained before the procedure, which was probably an effect of the dilution of plasma in the CPB circuit. The observed plasma-mediated upregulation of surface β-integrin expression on neutrophils corresponds with previous findings suggesting that factors capable of stimulating of PMN adhesion are released from the myocardium after ischemia and reperfusion during CABG.[26] In contrast to changes in the β-integrin expression, in vitro activation of neutrophils results in rapid decrease in surface expression of L-selectin, probably due to shedding.[27] This pattern was found in our study, in which L-selectin expression on PMNs incubated with plasma collected from the coronary sinus of patients receiving crystalloid cardioplegic solution at 30 minutes of reperfusion was significantly lower than at aortic clamp release.

The interesting question arises of what could be the soluble stimuli responsible for neutrophil activation during heart surgery. Of the mediators that have been implicated in reperfusion-induced leukocyte activation, platelet-activating factor, leukotriene B4, complement fragments, tumor necrosis factor, and endothelins have received the greatest attention.[28] A previous study demonstrated that endothelin-1 was involved in stimulation of neutrophil adherence during CABG.[26] It was also reported that release of platelet-activating factor resulted in neutrophil activation after ischemia and reperfusion during acute myocardial infarction and coronary angioplasty.[9,29] Nevertheless, it is possible that during ischemia and reperfusion numerous media-

tors are released and their orchestrated action is involved in systemic PMN activation. Experimental design used in the present study enabled us to evaluate the total effect of those mediators that were of clinical relevance.

No significant changes in CD11b, CD18, and L-selectin expression were observed in the group receiving blood cardioplegic solution. At this stage of investigation, the exact mechanism responsible for the differences between groups receiving crystalloid and blood cardioplegic solutions is unclear. It was previously shown that the use of blood cardioplegia may reduce the release of proinflammatory cytokines in patients undergoing cardiac surgery.[30] The possible influence of other factors, including changes in osmolarity, cannot be ruled out. The difference in β-integrins and L-selectin surface expression in both study groups indicates that cardioplegia itself may influence the release of neutrophil-oriented stimuli from ischemic myocardium and thus modify neutrophil activation during CABG. This finding may be of clinical relevance and requires further investigation.

Conclusions

1. Ischemia and reperfusion after CABG results in the release of factors capable of PMN stimulation from the myocardium to the circulating blood.

2. The release of soluble stimuli for PMNs during CABG may be modified by the cardioplegic solution.

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