Patterns of changes in neutrophil adhesion molecules during normothermic cardiopulmonary bypass

A clinical study

Abstract. The adhesion of activated neutrophils to endothelial cells is a key feature of the inflammatory response to cardiopulmonary bypass (CPB) because it "unlocks" a cascade of cytotoxic events. This adhesion is made possible by the sequential involvement of two sets of neutrophil cell surface receptors: L-selectin and $\beta_2$ integrins (CD11a/CD18; CD11b/CD18; CD11c/CD18). We have assessed the changes in the expression of these adhesion molecules in ten patients who underwent various open-heart procedures with the use of "warm" (33.4°C-37°C) CPB. Arterial blood samples were obtained before, during and after bypass and processed for immunofluorescent flow cytometric analysis. CD11a expression remained unchanged throughout the study period. Conversely, CD11b drastically increased early after the onset of bypass (at 15 min of bypass: 172±17 [mean fluorescence (arbitrary units), mean±SEM] versus 63±13 before bypass, $P<0.02$) and was still markedly elevated 30 min after the end of bypass (160±38, $P<0.05$ versus the pre-bypass value). CD11c expression underwent a similar upregulation (at 15 min of bypass: 54±5 versus 34±5 at baseline, $P<0.01$). L-selectin expression did not change significantly during the period of observation. Put together, these results suggest that CPB is associated with an increased adhesive potential of neutrophils, which enhances their binding to the vascular endothelium and thereby initiates tissue damage through the release of cytotoxic mediators from adherent cells. Manipulation of integrin expression could therefore represent an effective means of alleviating the component of bypass-induced inflammatory tissue damage which is more specifically neutrophil-mediated. [Eur J Cardio-thorac Surg (1996) 10:279-283]

Key words. Neutrophils · Adhesion molecules · Cardiopulmonary bypass

Introduction

The adhesion of activated neutrophils to endothelial cells triggers a cascade of cytotoxic events and is therefore considered a key feature of the inflammatory response to cardiopulmonary bypass (CPB) that underlies the occasional development of postoperative organ dysfunction [6, 9, 20]. This adhesion process sequentially involves two sets of neutrophil adhesion molecules [29]: L-selectin and $\beta_2$ integrins. L-selectin initiates neutrophil adhesive interactions by mediating a "weak" binding of unactivated neutrophils to endothelial cells. This binding becomes subsequently "strong" as integrins upregulated on the surface of activated neutrophils interact with their specific endothelial ligands.

Until now, few studies have assessed the changes of expression of these neutrophil adhesion molecules during clinical CPB, and all of them have concentrated on a single integrin (CD11b/CD18, also named CR3) [8, 10]. The
The present study was therefore designed to characterize more extensively the changes that involve the three $\beta_2$ integrins as well as L-selectin during CPB in human beings.

**Patients and methods**

**Study population**

Ten patients were entered into this research protocol, which was approved by our institutional ad hoc committee. There were eight men and two women ranging in age from 44 to 71 years (mean: $60 \pm 3$ years). Five patients had coronary artery bypass surgery, four patients had an aortic valve replacement and one patient underwent resection of a left atrial myxoma.

All patients were given a balanced anesthetic, including fentanyl, flunitrazepam and pancuronium. Cardiopulmonary bypass was established between the two venae cavae and the ascending aorta. Cardiopulmonary bypass equipment consisted of a roller pump, a membrane oxygenator and an on-line arterial filter. The extracorporeal circuit was primed with 1 500 ml of crystalloid solution in all patients. Bypass was run at a flow rate of 2.2 l/min per m$^2$ and mean arterial pressures were maintained in the range of 60–70 mmHg. Core temperature, as assessed by a nasopharyngeal probe, was allowed to drift to an average of 33.4 °C. Myocardial protection was provided by continuous retrograde warm blood cardioplegia which was delivered by our previously described low-dilution technique [18]. Administration of heparin before cannulation and its subsequent reversal with protamine sulfate were accomplished in a standard fashion. The duration of CPB varied from 37 to 120 min (mean: 84.7) and the aortic cross-clamping time ranged from 20 to 96 min (mean: $55 \pm 6$).

Blood samples were collected shortly after anesthetic induction, at 5, 10 and 15 min of bypass and 30 min after the end of bypass. Pre- and post-bypass samples were drawn from the radial artery line; during bypass, samples were obtained from the arterial limb of the oxygenator. All samples were kept on ice for a short period until flow cytometric analysis.

**Flow cytometric analysis**

Aliquots of 25 $\mu$l were immediately stained with saturating concentrations of mouse antihuman monoclonal antibodies to CD 11 a (IOT 16), CD 11 b (Leu 15), CD 11 c (LeuM5), and L-selectin (TQ 1) for 20 min at 4 °C. These antibodies are conjugated to phycoerythrin except for IOT 16 which is conjugated instead to fluorescein isothiocyanate. After two washes in phosphate-buffered saline (PBS), erythrocytes were lysed with FACS lysing solution for 10 min, and after centrifugation and removal of the supernatant, washed once with PBS and resuspended in a 1% solution of formaldehyde in PBS. Samples were then kept at 4 °C in the dark until analysis.

Flow cytometric analysis of cellular fluorescence was performed on a FACSscan cytometer. Green and red amplifier gains were calibrated with fluorescent beads before each experiment to check that relative fluorescence values were comparable between experiments. A total of 5000 events were recorded from each sample and analyzed with Lysis II analysis software. Neutrophils were gated on the basis of forward and orthogonal light scatter, and fluorescence was measured on a log scale. Data are expressed as the arithmetic mean of linear fluorescence values which correspond to the average degree of expression of the relevant epitope on the cell surface.

**Statistical analysis**

Comparisons were performed using one-way analysis of variance followed by Scheffé's test for multiple comparisons. Significance was set at the 0.05 level. All results are expressed as the mean ± standard error of the mean.

**Results**

The results are summarized in Fig. 1. CD 11 a expression remained unchanged throughout the study period. This contrasted with the sharp and early increase in CD 11 b and CD 11 c expression which peaked 15 min after the onset of bypass. Thirty minutes after the end of bypass, the levels of these two integrins were still elevated, as compared with baseline values, although the difference attained statistical significance only for CD 11 b. L-selectin expression did not change significantly over the period of observation. Thus, the mean fluorescence generated by the L-selectin-specific antibody was $886 \pm 52, 942 \pm 88$ and $926 \pm 77$ before bypass, 15 min after the onset of bypass and 30 min following its termination, respectively.

Furthermore, analysis of post-bypass samples demonstrated, in five patients, the emergence of a second population of cells with low fluorescence for CD 11 b and/or CD 11 c reflecting a weak expression of the corresponding epitopes (Fig. 2).
Discussion

Mechanisms of neutrophil adhesion during inflammatory states

Adhesion of activated neutrophils to endothelial cells is currently thought to play a pivotal role in the pathophysiology of bypass-induced inflammatory tissue damage [6, 9, 20]. This adhesion seems to occur as a two-step process [29]. The first step is characterized by the interaction between L-selectin, an adhesion molecule which is constitutively expressed on the surface of unactivated neutrophils, and its endothelial counter receptors. This interaction, described as rolling, is not strong enough to overcome the shear stress exerted by flowing blood but it allows a prolonged interaction time between neutrophils and activating stimuli, thereby setting the stage for the subsequent integrin-mediated adhesion strengthening. Namely, the second step is characterized by the firm attachment of the now activated neutrophils to endothelial cells. This binding involves another set of neutrophil adhesion molecules, termed β integrins. These integrins consist of three heterodimeric glycoproteins which share a common β subunit (CD18) and have distinct α subunits (CD11a, CD11b, CD11c) [28]. Whereas CD11a/CD18 is constitutively expressed at high levels, CD11b/CD18 and CD11c/CD18 are upregulated by different inflammatory mediators, in particular the complement cleavage product C5a, leukotriene B4 (LTB4), platelet activating factor and interleukin-8, all of which have been shown to be released during clinical CPB [5, 10, 12]. The activation-dependent upregulation of integrins allows neutrophil sticking through interactions between these cell surface adherence receptors and their endothelial ligands, and, in particular, between CD11b/CD18 and the intercellular adhesion molecule-1 (ICAM-1) [28].

The attachment of neutrophils to endothelial cells triggers the release of neutrophil-derived cytotoxic compounds. Thus, CD11b/CD18-dependent adhesion of neutrophils has been shown to mediate both oxygen-free radical production [24] and release of proteolytic enzymes from neutrophil granules [23]. These experimental data are supported by the clinical observation of a temporal relationship between CD11b/CD18 expression and a rise in plasma levels of elastase [8, 10]. The phenomenon may then feature an auto-amplification loop in that activated neutrophils also release metabolites of arachidonic acid, in particular the potent chemoattractant LTB4 [10], which promote the further recruitment and adhesion of inflammatory white cells at the sites of tissue injury [2, 17].

Adherent neutrophils finally emigrate into the interstitial compartment where they can directly damage parenchymal cells as they come into direct contact with them [7, 31]. The cytotoxic effects of neutrophil-derived inflammatory mediators are further facilitated by the fact that they are released in a local microenvironment into which their circulating inhibitors are expected to have limited access [4]. These considerations clearly show that neutrophil-endothelial cell adhesion may be a critical determinant of the bypass-induced neutrophil-mediated tissue injury. If this injury is to be prevented clinically by appropriate therapeutic strategies, an accurate understanding of the molecular mechanisms that regulate the neutrophil adhesive process in human beings is first required. This was the purpose of the present study.

Interpretation of results

The major finding of the present study is that neutrophils of patients exposed to CPB undergo a marked upregulation of the CD11b integrin. This result is consistent with
the observations previously made both in an in vitro model of simulated extracorporeal circulation [21] and during clinical bypass [8, 10]. Whereas the increased expression of CD11b shortly after the onset of CPB parallels the time course of complement activation [8, 10], there is some evidence that the sustained increase in CD11b expression seen 30 min after the end of bypass could be more dependent on stimulation by LTB4 [10]. Likewise, CD11c expression increased over time, which is not unexpected since this integrin shares with CD11b the ability of being upregulated by inflammatory stimuli. The common post-bypass finding of a second population of cells with low fluorescence for CD11b and CD11c is consistent with the neutrophilia occurring over this time frame and which is partly due to a release of young cells, known to express low levels of CD11b/CD18 [11], from the bone marrow [22]. Finally, CD11a expression remained unchanged throughout the study period. A similar observation has previously been reported with the use of an ex vivo mock CPB circuit [21] and is probably due to the fact that CD11a primarily mediates adhesion of lymphocytes [1] and of unactivated neutrophils [26].

L-selectin expression did not change significantly throughout the study period. This observation is at variance with the laboratory findings that upregulation of CD11b coincides with a downregulation of L-selectin of similar magnitude [11, 13, 27]. A first possible explanation for this discrepancy is that shedding of L-selectin has actually been reported only in neutrophils that had extravasated [13] and were therefore, by definition, not accessible to our flow cytometry analytic technique. Alternatively, L-selectin might really be spared in vivo, which, together with the increased surface expression of CD11b, would enhance the adhesive potential of neutrophils and more specifically promote their pulmonary sequestration when lungs are reperfused at initially low flow rates, because the correspondingly low wall shear stresses could then facilitate L-selectin-mediated neutrophil rolling [2] and subsequent CD11b/CD18-mediated firm adhesion [14].

As previously mentioned, it is clear that the changes in the expression of neutrophil adhesion molecules reported in this study are those involving circulating cells. That these data may, however, have some relevance to adherent cells is suggested by two lines of evidence: (1) The increase in circulating levels of ICAM-1 during clinical bypass [8, 19], which most likely reflects an upregulation, at the endothelial level, of one of the major ligands for some neutrophil integrins [28], and (2) The limitation of tissue damage by monoclonal antibodies that block either CD11b/CD18 [25] or L-selectin [16] mediated neutrophil adhesion to the vascular endothelium.

Clinical implications

Although all our patients had an uneventful postoperative course, the small size of this population together with the roughness of the clinical endpoints used for assessing patient outcome do not allow the potential clinical relevance of our biological findings to be ignored. We acknowledge, however, in keeping with our previous data showing that temperature affects the magnitude of the inflammatory response to bypass [19], that a more profound degree of systemic cooling can delay the peak expression of neutrophil adhesion molecules [15]. Assuming that prevention of neutrophil-endothelial cell interactions would reduce post-bypass organ dysfunction, and in particular lung injury, our data identify neutrophil adhesion molecules as elective targets for therapeutic interventions that could be implemented at the time of surgery. This approach is further supported by the experimental demonstration of improved postoperative preservation of heart and lungs following administration of monoclonal antibodies against β2 integrins [3] or of new anti-inflammatory drugs (leumecdins) that inhibit CD11b/CD18 upregulation [9, 30].

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References


