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Acute cardiac inflammatory responses to postischemic reperfusion during cardiopulmonary bypass[1]

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Abstract

Objectives: The investigation centers on whether there is a reperfusion-induced specific cardiac inflammatory reaction after bypass surgery. **Background:** Cardiopulmonary bypass (CPB) leads to systemic inflammation. Additionally, cardiac inflammation due to reperfusion could occur. Knowledge about nature and time course of this reaction might help to develop cardioprotective interventions. **Methods:** In 12 patients receiving coronary bypass grafts, arterial and coronary venous blood was obtained before onset of CPB, and 1, 5, 10, 25, 35 and 75 min after cardiac reperfusion. Plasma levels of IL6 and IL8 were measured by immunoassay. CD11b, CD41, and CD62 on blood cells were quantified by flow cytometry. Measurement of CD41, a platelet marker, on neutrophils and monocytes allowed detection of leukocyte–platelet microaggregates. **Results:** Transcardiac veno–arterial difference of IL6 rose in the 10th and 25th min of reperfusion (from 0 to 7 pg/ml; $p<0.05$), and after 75 min (15 pg/ml). IL8 did not change. CD11b on neutrophils (PMN) decreased transcardially to 95, 88 and 82% of the initial level in the 5th, 10th, and 75th min, respectively, suggesting sequestration of activated neutrophils. CD62 on platelets rose about 30% in the 75th min. Initially, leukocyte–platelet microaggregates were formed during coronary passage (+31% of the arterial level for PMN, +23% for monocytes). During reperfusion, coaggregates were retained (PMN: −1% and −7% in the 5th and 10th min, monocytes: −22%, −13% and −12% in the 1st, 5th and 10th min. **Conclusions:** During early reperfusion after aortic declamping, the coronary bed is already a source of proinflammatory stimuli and target for activated leukocytes, partly in conjunction with platelets. Mitigation of these phenomena might help to improve cardiac function after CPB especially in patients at risk.
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Keywords: Cytokines; Leukocytes; Platelets; Reperfusion

1. Introduction

Numerous experimental and clinical studies have demonstrated that cardiopulmonary bypass (CPB) leads to a systemic inflammatory response, characterized by activation of neutrophils [1] and platelets [2], formation of leukocyte–platelet coaggregates [3,4], and elevation of cytokine plasma levels [5]. The main reasons for this generalized inflammation are the contact of blood with artificial surfaces in components of the extracorporeal circulation apparatus [6,7], and the influence of priming solutions [7]. However, the possible role of the heart as a source of inflammatory stimuli and as a target of inflammation in the clinical situation of CPB is less well characterized. This could be relevant, because after aortic declamping global cardiac reperfusion occurs, and reperfusion, as such, represents a massive proinflammatory stimulus in the heart [8,9]. In turn, different features of inflammation depress cardiac performance: intracoronary retention and activation of neutrophils represents a main aspect of reperfusion injury [11], while some cytokines have direct negative inotropic effects [10]. Consequences of reperfusion injury, such as myocardial stunning and coronary low-reflow, might, thus, infringe the clinical

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benefit of interventions employing extracorporeal circulation and clamping of the aorta. Indeed, antiinflammatory medication [12] and removal of leukocytes from the circulation [13] seem to have beneficial effects on cardiovascular function after CPB.

Transcardiac inflammation has already been described in some clinical situations of reperfusion, particularly balloon angioplasty under stable [14] or post-infarction conditions [9]. There even seems to be a causal link between acute inflammatory responses during reperfusion (formation of leukocyte–platelet aggregates) and the occurrence of restenosis [14]. In the present study, we investigated inflammatory reactions in blood during coronary passage in patients undergoing coronary artery bypass grafting, both before extracorporeal circulation and after the 1st, 5th, 10th, 25th, 35th and 75th min of reperfusion after aortic declamping. In detail, we analyzed plasma levels of cytokines (IL6 and IL8) and activation markers on leukocytes (CD11b, a subunit of the adhesion molecule Mac-1) and platelets (CD41, a subunit of the GPIIb/IIIa receptor, and CD62, i.e. P-selectin), and determined the percentage of platelet-carrying leukocytes in systemic arterial and coronary venous blood. Furthermore, transcoronary differences of the soluble form of the adhesion molecule ICAM-1, and of leukocyte- and platelet-counts were measured.

2. Methods

2.1. Patients

12 patients, <75 years old, undergoing elective coronary artery bypass graft surgery and with an ejection fraction >55% entered the study, which was approved by our institutional ethics committee. The study was performed according to the latest amendment of the Declaration of Helsinki. All patients gave their written informed consent for inclusion in the study. Exclusion criteria were myocardial infarction within 14 days before the operation, inflammatory diseases or intake of immunosuppressants. The clinical characteristics of the study patients are summarized in Table 1.

2.2. Anesthesia and surgery

The patients were anaesthetized with either sufentanil+midazolam, sufentanil+propofol, or remifentanil+midazolam; no volatile anaesthetics were used. Oxygenation of the blood was performed with bubble oxygenators (Dideco, Mirandola, Italy). After initiation of extracorporeal circulation, the blood was cooled to 26–28°C (moderate hypothermia). A 1000-ml volume of cold Bretschneider solution (custodiol®) was used for antegrade cardioplegia.

Table 1
Clinical characteristics and pre-medication of the study patients[a]

Age	64±2 years
Sex	3 women, 9 men
Ejection fraction	67±3%
Duration of ischemia (min)	52±5
Time to reperfusion of grafts (min)	26±2
Number of distal anastomoses	3.1±0.2
Number of proximal anastomoses	1.8±0.1
Diabetes	3
Aspirin	12
Nitrates	11
Beta-blockers	10
Diuretics	3
ACE-inhibitors	5

[a] Numerical values are means±S.E.M.

2.3. Blood samples

Blood samples (3 ml each) were drawn simultaneously from the cannulated radial artery and from the coronary sinus catheter: before administration of heparin, after the 1st, 5th and 10th min after release of the aortic cross clamp, as well as after release of the Lambert–Kay clamp (about 25th min of reperfusion), after application of protamine (about 35th min), and after 75 min of reperfusion. A multi-purpose catheter with a 5-cm tip (70° angle) was used for blood sampling. The dead space of the catheter being about 1 ml, the first 5 ml of every sample were discarded. The correct transcutaneous emplacement of the coronary sinus catheter was monitored by observing the pressure curve of the catheter and by measuring oxygen saturation of the blood samples. The pressure curves had to show a venous pattern throughout, and oxygen saturation had to be in the range of 25–40% for inclusion of the samples in the study.

2.4. Immunoassays

Immediately after sampling, an aliquot of the blood (2 ml) was stored on ice until centrifugation at 2000 *g* for 10 min to obtain platelet poor plasma. This was stored at −20°C until final processing. Concentrations of IL6 and IL8 were determined by sandwich type immunoassays (Endogen). In both cases standards covered a range from 0 to 1000 pg/ml.

Soluble ICAM-1 was also measured by ELISA (T Cell Diagnostics). In this case samples had to be diluted 1:100 to reach the optimum sensitivity of the assay (0.3 ng/ml).

Due to continuous hemodilution during the surgical intervention, all measured concentrations had to be corrected to the hematocrit of the arterial pre-heparin sample to ensure their comparability. Coronary veno–arterial differences of IL6, IL8 and ICAM-1 were calculated for each time point.

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2.5. Flow cytometry

For flow cytometry, 100-μl aliquots of the blood samples were immediately mixed with 1 ml FACS lysing solution (Becton Dickinson). This ensured simultaneous fixation of leukocytes and lysis of red blood cells. For analysis of platelets, 1 ml Cellfix (Becton Dickinson) was employed instead.

Leukocytes were pelleted for 5 min at 200 *g*, washed with Cellwash (Becton Dickinson), pelleted again, and then double-stained with phycoerythrin (PE) conjugated anti-CD11b antibodies (Exalpha) and with FITC labelled anti-CD41 antibodies (Serotec). The measurement of the platelet marker CD41 on leukocytes served to determine the percentage of neutrophils and monocytes carrying platelets. After incubation with the antibodies for 15 min in the dark, cells were washed, centrifuged and resuspended in Cellwash, prior to measurement.

For the analysis of platelets the fixed blood suspension was centrifuged at 1200 *g* for 5 min. The supernatant was discarded and the pellet resuspended in 1 ml Cellwash. A 50-μl volume of this suspension was incubated with antibodies against CD41 (FITC labelled, Serotec) and CD62 (PE conjugated, Harlan Seralab) for 15 min in the dark. Cells were then centrifuged at 1200 *g* for 5 min, washed with Cellwash, centrifuged again, finally resuspended in 500 μl Cellwash, and measured.

Flow cytometry was performed with a FACScan and Lysys II software (Becton Dickinson). Ten thousand events were acquired for each sample. Neutrophils, monocytes and platelets were discriminated by their characteristic pattern of forward and sideward scatter, and separately analysed after setting appropriate gates. The mean fluorescence intensity was taken as a measure of antibody binding. Reproducibility was ensured by regular calibration of the flow cytometer. The non-specific background was quantified by measurement of the fluorescence intensity of samples labelled with non-binding isotype matched antibodies (mouse IgG1-PE from SBA, Birmingham, USA, and mouse IgG2a-FITC from Exalpha), and subsequently subtracted.

The considerable variability of both leukocyte and platelet markers in the study population, even under basal conditions, made it necessary to normalize fluorescence intensities. The pre-interventional arterial value of each individual — termed basal in the following — was set as 100% for every flow cytometric parameter. All changes are therefore given in percentages.

2.6. Statistical analysis

The given veno–arterial differences are means of individually calculated differences, and not differences of mean values. All results are expressed as means±S.E.M. ANOVA for repeated measurements was applied for the comparison of values at different time points with the basal value. A difference was considered as statistically significant if $p<0.05$.

3. Results

3.1. Cytokine measurements

The arterial levels of IL6 rose continuously, from <0.1 pg/ml before the intervention, to 110 pg/ml after 75 min of reperfusion (Fig. 1A). Under basal conditions, there was no transcardiac concentration difference of IL6. In the 10th and 25th min of reperfusion, however, significant veno–arterial differences of IL6 (7 pg/ml) were to be detected. This increase of IL-6 concentration rose further to about 15 pg/ml in the 75th min of reperfusion (Fig. 1B).

The arterial content of IL8 was 8 pg/ml pre-operatively, and rose continuously until the 35th min of reperfusion (50 pg/ml), i.e. up to the application of protamine. Arterial IL8 then declined to 30 pg/ml in the 75th min (Fig. 1C). There was no statistically significant coronary veno-arterial difference for IL8 at any time point (Fig. 1D).

3.2. Expression of CD11b

On neutrophils, expression of CD11b in arterial samples rose to about 140% of the basal level in the 25th min of reperfusion, then declined again to the initial level (Fig. 2A). Monocytes showed a similar behaviour on a lower level of activation (Fig. 2C). CD11b expression was lower on neutrophils emerging from the coronary system during initial reperfusion (1st, 5th, 10th min) than the corresponding arterial values, and then again during later reperfusion (75th min, Fig. 2B). The early response of monocytes (1st, 5th, 10th min) was similar, but the v–a difference did not reach statistical significance (Fig. 2D).

3.3. Soluble ICAM-1

To check whether changes in CD11b detectability could arise from masking of this molecule by different levels of its soluble ligand sICAM-1 in the plasma, we also measured plasma levels of sICAM-1 during early reperfusion. The basal arterial concentration of sICAM-1 was 200 ng/ml, and did not change significantly during the first minutes of reperfusion (Fig. 3A). A coronary veno–arterial difference of sICAM-1 was detected in the 10th min of reperfusion (20 pg/ml), but not at earlier time points (Fig. 3B). The transcardiac rise in plasma levels at 10 min amounted to about 10%.

3.4. Platelet activation markers

The surface expression of both platelet activation

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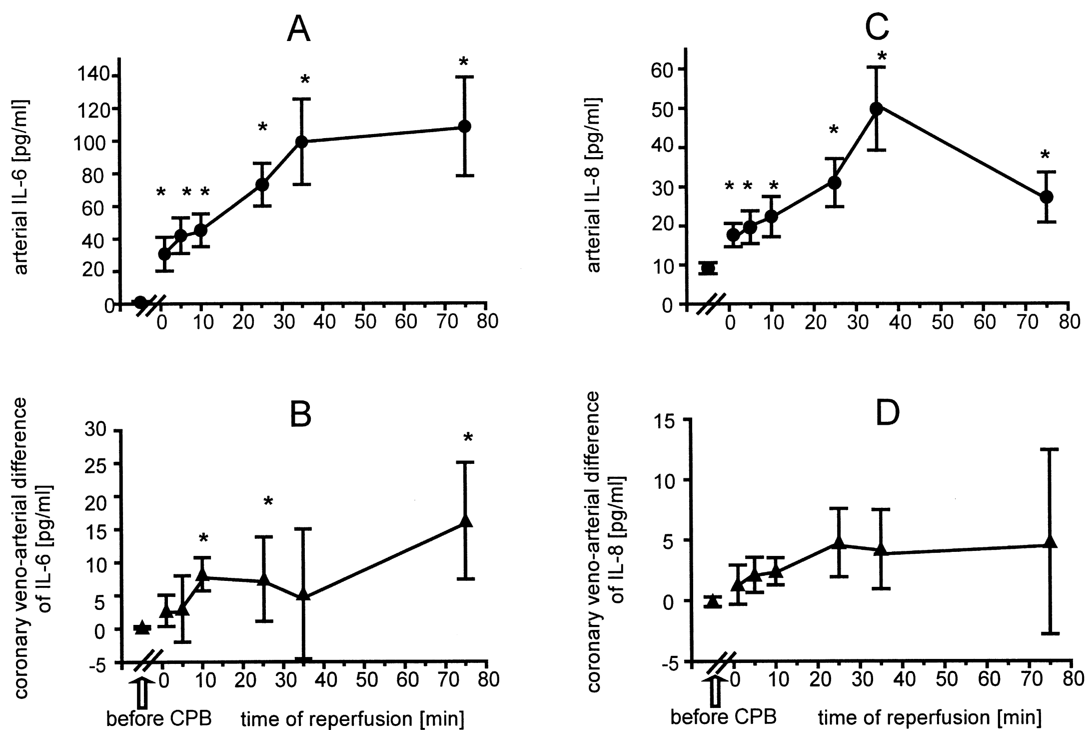

Fig. 1. Arterial plasma levels of IL6 (A) and IL8 (C) at various times before, during, and after CPB and coronary veno–arterial differences of IL6 (B) and IL8 (D). Values are means, bars represent S.E.M. $*p<0.05$ as compared to the initial value.

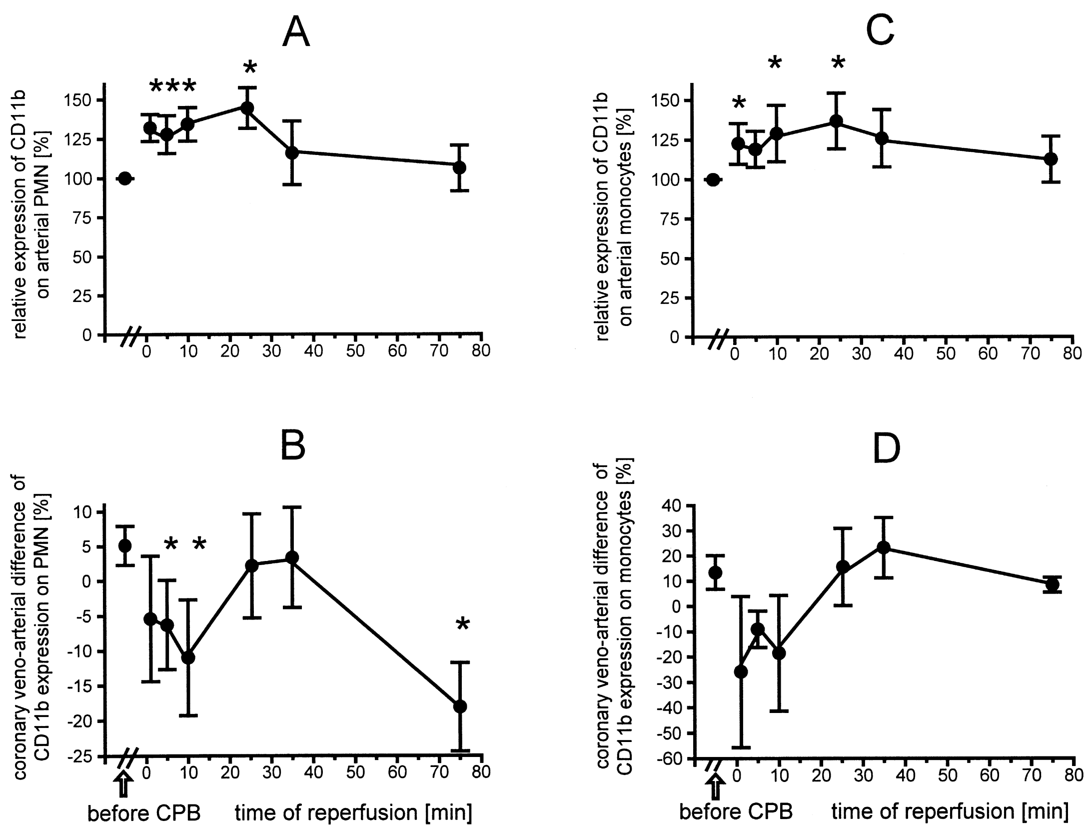

Fig. 2. Percentual changes of mean CD11b fluorescence on PMN (A) and monocytes (C) during and after CPB in arterial blood, and veno–arterial changes of these values for PMN (B) and monocytes (D). Values are means±S.E.M., $*p<0.05$ vs. the initial value.

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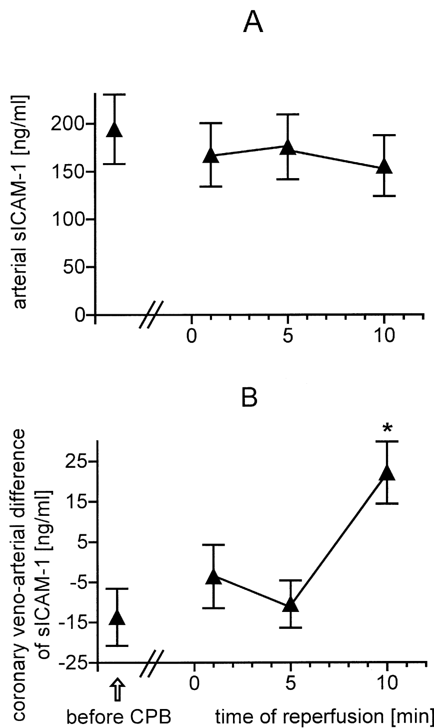

Fig. 3. Arterial plasma levels of soluble ICAM-1 (A) and coronary veno–arterial differences of sICAM-1 (B) before CPB as well as 1, 5, and 10 min after aortic declamping. Values are means±S.E.M., $*p<0.05$ vs. the initial value.

markers, CD62 (P-selectin) and CD41 (gpIIb/IIIa), increased rapidly on arterial platelets, with the maxima after protamine administration in the 35th min of reperfusion (Fig. 4A and C). Coronary passage led to neither a significant loss nor upregulation of the two markers during early reperfusion (Fig. 4B and D). However, a rise of CD62 expression of 25% above baseline was observed in the 75th min of reperfusion (Fig. 4B).

3.5. Leukocyte–platelet interaction

During the first 25 min of reperfusion, the occurrence of neutrophil–platelet coaggregates in arterial blood (measured as percentage CD41 positive neutrophils) was markedly elevated vs. the basal state before CPB and later time points (Fig. 5A). The same was true for monocyte–platelet coaggregates, however with a more protracted time course, and on a much higher level, with up to 60% of the arterial monocytes carrying platelets, as compared to maximally 15% for neutrophils (Fig. 5A and C). The coronary veno–arterial difference of leukocyte–platelet microaggregates was positive under basal conditions (Fig. 5B and D), i.e., new aggregates were being formed during coronary passage (31% increase vs. arterial values for PMN, 23% increase for monocytes). During the first minutes of reperfusion, as well as after 35 min (protamine infusion), the percentual coronary veno–arterial difference of both,

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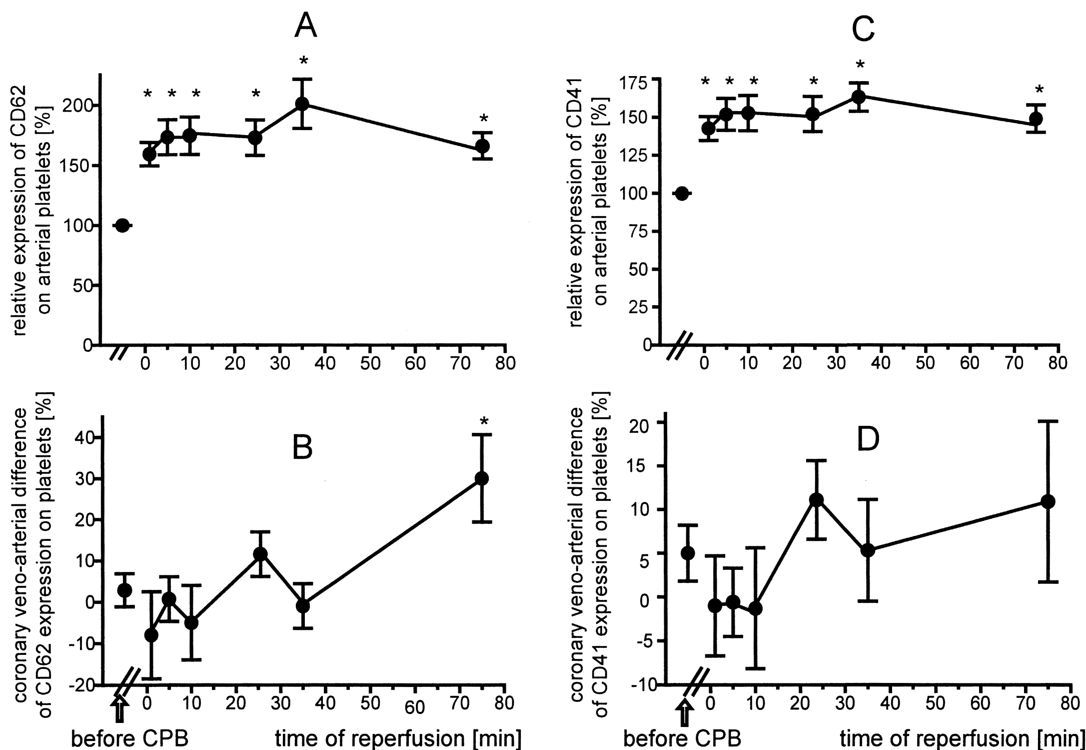

Fig. 4. Time course of the percentual mean fluorescence of CD62 (A) and CD41 (C) on platelets in arterial blood and percentual changes of fluorescence of CD62 (B) and CD41 (D) during coronary passage. Values are means±S.E.M., $*p<0.05$ vs. initial value.

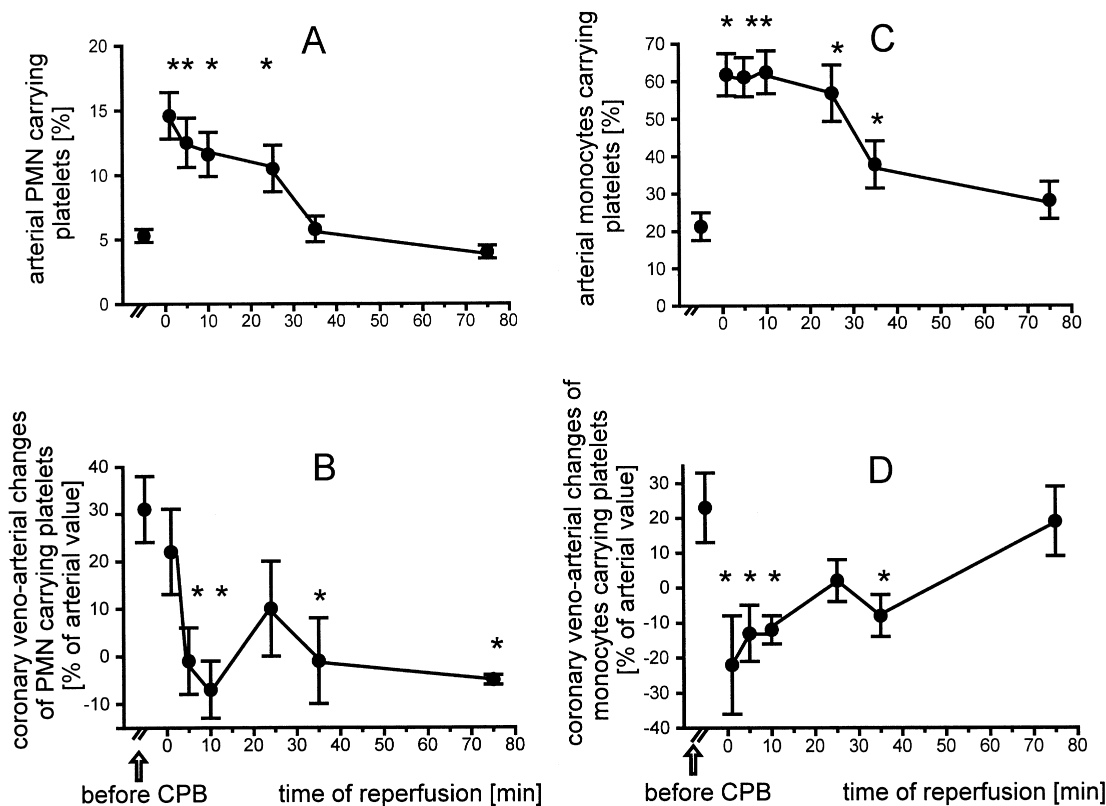

Fig. 5. Upper panel: Percentage of PMN carrying platelets (CD41 positive PMN) (A) and monocytes carrying platelets (C) in the arterial blood before and at various time points during and after CPB. Lower panel: changes of PMN carrying platelets (B) and monocytes carrying platelets (D) during coronary passage as percent of the respective arterial value. Values are means±S.E.M., $*p<0.05$ vs. the initial value.

neutrophil– and monocyte–platelet aggregates, decreased significantly below these basal values (Fig. 5B and D). In fact, less aggregates were emerging from the coronary system than were entering it (negative v–a-difference).

3.6. Cell counts

The time courses of the veno–arterial differences for leukocytes and platelets are given in Table 2. In the 1st min of reperfusion, as well as immediately after reperfusion of the grafts (about 25th min), there was a tendencial increase of both leukocyte and platelet counts during coronary passage. At all other time points, leukocytes tended to be retained in the coronary system. However, statistically significant retention (Table 2) was attained only after 35 min (platelets) and 75 min (leukocytes).

4. Discussion

The present study demonstrates that, after CPB, the human heart is a site of postischemic inflammation and a source of proinflammatory stimuli. Findings leading to this conclusion are (I) increase of IL6 concentrations after coronary passage early in reperfusion, (II) trapping of CD11b positive leukocytes in the coronary system, and (III) trapping of leukocyte/platelet aggregates in the coronary bed during initial reperfusion. These effects occur in addition to the systemic inflammatory changes evoked by the extracorporeal circulation.

4.1. Cytokines

A systemic increase of the cytokines IL6 and IL8 due to extracorporeal circulation has already been reported by a

Table 2
Veno–arterial differences of leukocyte- and platelet counts[a]

Time of reperfusion (min)	1	5	10	25	35	75
Leukocytes	577±200	−109±220	−144±146	228±241	−343±230	−688±387*
Platelets	6550±3759	−3579±3351	6053±4501	1789±5547	−11 000±3424*	−1333±5290

[a] Cells/µl, means±S.E.M., $*p<0.05$ vs. zero difference). Negative numbers indicate coronary cell retention, positive numbers reflect washout of cells from the coronary system.

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number of authors [1,5,15]. In our present study, we have found similar systemic increases of both IL6 and IL8 in the blood of patients undergoing cardiopulmonary bypass (Fig. 1A and C). In addition to this systemic response, the cardiac production and release of cytokines might be of special interest, because cytokines act negatively inotropic [10]. They could, thus, directly contribute to myocardial stunning. Moreover, the cytokines produced by the heart itself could further locally upregulate coronary endothelial adhesion molecules [16], leading to retention and activation of passing leukocytes, and generate a chemotactic gradient for inflammatory cells into the cardiac parenchyma. Indeed, rapid cardiac release of the cytokines IL6 and IL8 has been shown in humans after myocardial infarction and subsequent recanalization, and has been interpreted as a sign of postischemic cardiac inflammation [9]. In experimental models of cardiac ischemia and reperfusion, the induction of IL6- and IL8-mRNA has been demonstrated [8,17], and during pediatric CPB, the induction of IL8 in human heart muscle has been shown [18].

In our study we have, for the first time, measured coronary veno–arterial differences of IL6 and IL8 levels during acute post-crossclamp reperfusion. Surprisingly, IL6 concentrations rose during coronary passage already after 10 and 25 min of reperfusion (Fig. 1B), a timepoint much too early to allow de novo synthesis of a protein. The absence of this effect in the case of IL8 (Fig. 1D) suggests that it was no mere washout phenomenon after ischemia, but the specific increase of a proinflammatory agent. Thus, it may be concluded that preformed IL6 has been released from cardiac tissue, with cardiac endothelial cells or macrophages being the most likely sources. In contrast to this early rise of IL6, the secondary increase after 75 min might well be due to gene induction by reperfusion [8].

4.2. Activation of leukocytes

Leukocytes, especially neutrophils (PMN), are widely accepted as one of the main causes for reperfusion injury [11]. After adhesion to endothelium, they are chemotactically activated, transmigrate through the endothelial barrier and undergo a so-called 'burst reaction' [19], liberating proteolytic enzymes and reactive oxygen species. These processes cause microvascular leakage, vasoconstriction and, ultimately, tissue damage [20]. Extracorporeal circulation leads to rapid upregulation of CD11b (1), an early activation marker on leukocytes and an adhesion molecule which is important for the attachment of leukocytes to the endothelium [21]. We, too, observed the systemic upregulation of CD11b on both PMN and monocytes during CPB (Fig. 2A and C). However, this increase was transient, probably due to the retention of the most activated leukocytes in the tubing and the oxygenator of the CPB.

The transcardiac difference for CD11b on PMN and (tendencially) on monocytes was negative during the first minutes of reperfusion. This finding conflicts with the upregulation of CD11b (i.e. activation of PMN) observed during coronary passage after myocardial infarction and PTCA [9]. Lower mean expression of CD11b could mean (A) deactivation of leukocytes, (B) masking of CD11b by binding of a soluble physiologic ligand, or (C) retention of the most activated leukocytes in the reperfused coronary system. Hypothesis (A) is very improbable, because no mechanism has been described for a rapid downregulation of CD11b (proteolytic shedding, as in the case of L-selectin, does not seem to occur). To test the second hypothesis (B), we measured transcardiac concentrations of soluble ICAM-1, a natural ligand for CD11b [22]. However, after 5 min of reperfusion, when there already was a notable decrease of CD11b, no enhanced veno–arterial differences of sICAM-1 were to be found (Fig. 3B). Interestingly, there was a significant rise of sICAM-1 after 10 min of reperfusion. This may well be taken as a sign of endothelial activation. To date, however, there is no consensus on the pathophysiological significance of the presence of soluble adhesion molecules in plasma. With respect to explanation (C), an actual sign of leukocyte retention is the tendencially negative veno–arterial difference of leukocyte counts at those time points, at which CD11b decreased most (Table 2). One has, however, to keep in mind that leukocyte adhesion does not necessarily depend on upregulation of surface CD11b, but can also be induced by an increase of CD11b affinity [23]. Thus, a strict correlation of CD11b expression and leukocyte retention may not be expected.

The partial conflict of these results with previous data [9] suggests that different clinical forms of myocardial ischemia and reperfusion (e.g. acute myocardial infarction and subsequent rescue PTCA as compared to cardioplegic global ischemia during cardiosurgery) might well result in quite different extents and relations of leukocyte activation and retention. The pathophysiological consequences have still to be evaluated.

4.3. Activation of platelets

During reperfusion of previously ischemic myocardium, activated platelets with their thrombotic potential are a risk factor for restenosis [24]. Furthermore, anti-platelet drugs have turned out to be promising tools in the prevention of reocclusion [25]. During CPB, marked activation of platelets has been described [2]. In our study, we measured the surface expression of two activation markers on platelets: the adhesion molecule CD62 or P-selectin, which enables platelets to adhere to leukocytes [26], and CD41, a subunit of the integrin GPIIb/IIIa [27]. Both adhesion molecules have been described to be rapidly upregulated on platelets upon stimulation [26,27]. We have found both markers to be upregulated systemically throughout reperfusion, with a peak after protamine application (Fig. 4A and C). Furthermore, we investigated whether global myocar-

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dial reperfusion after cardioplegia leads to a specific activation of platelets during coronary passage. Surprisingly, mean veno–arterial differences of both P-selectin and gpIIb/IIIa tended to be negative during early reperfusion. This would suggest retention of activated platelets in the coronary system rather than washout of activated thrombocytes. In the 5th min of reperfusion, the negative veno–arterial difference of platelet counts (Table 2, not significant) strengthens this hypothesis.

The clear retention of platelets in the coronary system after administration of protamine (Table 2) correlates with a transient decrease of veno–arterial CD41 and CD62 levels (Fig. 4). In a later phase of reperfusion (75 min, about 40 min after the onset of blood passage through the new grafts), P-selectin was markedly upregulated by coronary passage, indicating platelet activation, and, perhaps, beginning of acute reocclusion (Fig. 4B).

4.4. Leukocyte–platelet interaction

Over the last years awareness has grown that blood cell populations do not act independently, and that mutual functional modulation is common. For example, platelets readily adhere to leukocytes, priming them for subsequent chemotactic stimulation [26,28]. The formation of such leukocyte–platelet coaggregates has been shown after coronary angioplasty, and may even be a predictor for the development of restenosis [14]. Also in the situation of CPB, platelet association with leukocytes has been detected [3,4]. In our study, systemic (arterial) formation of PMN–platelet as well as monocyte–platelet microaggregates peaked in the 1st min of reperfusion, then declined continuously (Fig. 5A and C). This suggests the formation of these aggregates in the extracorporeal circulation and their subsequent sequestration, e.g. in the reperfused coronary and pulmonary vascular beds. As described in the literature [3], the tendency to bind platelets was more pronounced for monocytes than for PMN (Fig. 5A and C). However, both types of coaggregates were numerically reduced by coronary passage in the first minutes of reperfusion, and then again after protamine administration (Fig. 5B and D). This could be interpreted as dissolution of coaggregates in the microcirculation, but is more probably due to retention in the coronary circulation, there being no known reason why protamine should dissociate the aggregates. The retention of aggregates could, in part, contribute to the observed lower leukocyte counts (Table 2).

That systemically activated PMN or leukocyte–platelet microaggregates might also be retained in other organs, especially the lung, cannot be completely excluded by the present data. However, the biphasic time course of retention suggests that this action is not caused by unspecific trapping, but regulated by specific adhesion molecules. Furthermore, it can be assumed that the retention of activated leukocytes in the reperfused heart, as compared to other organs, has by far the most important consequences for the clinical outcome of cardiothoracic surgery.

4.5. Pathophysiological implications

In the present study we found signs of very rapid postischemic cardiac inflammation. In the first minutes of reperfusion, a net trans-coronary increase of IL6 and sequestration of activated neutrophils and of leukocyte–platelet coaggregates occurred in the coronary system. A second wave of these inflammatory responses took place at later time points, namely with the application of protamine and after onset of reperfusion of the fresh grafts. This time course suggests that reperfusion injury is a consequence of a positive feedback, initiated by early reperfusion. This early phase could induce expression of pro-inflammatory genes, and, thus, set the stage for later events. If coronary retention of activated leukocytes and platelets during early reperfusion really is the trigger for the subsequent pathophysiologic effects, patients might greatly profit from anti-inflammatory and anti-platelet interventions during, and early after cardiosurgery.

The crucial point of future studies has to be, how far the experimental findings on inflammatory parameters correlate with the individual clinical outcome after coronary bypass surgery.

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