DOWN-REGULATION OF SURFACE MONOCYTE LIPOPOLYSACCHARIDE-RECEPTOR CD14 IN PATIENTS ON CARDIOPULMONARY BYPASS UNDERGOING AORTA-CORONARY BYPASS OPERATION

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Objectives: Major operative trauma like aorta-coronary bypass operation may lead to postoperative immunodisturbance, putting the patient at an increased risk for infection and sepsis. The monocyte/macrophage system and the endotoxin receptor CD14 are important in the early recognition and elimination of invading bacteria. The aim of this study was to analyze changes in membrane-associated CD14 and soluble CD14 during and after cardiac involving cardiopulmonary bypass. **Methods:** We studied numbers of leukocytes, monocytes, and monocyte subpopulations, expression of monocyte membrane-associated CD14 and plasma levels of soluble CD14 in 10 patients (63 ± 8 years of age), who underwent elective cardiopulmonary bypass. **Results:** Cardiopulmonary bypass induced marked postoperative monocytosis, which was maximal 20 hours after the operation (485 ± 242 cells/ μ l before, 1080 ± 264 cells/ μ l 20 hours after surgery). Expression of membrane-associated CD14 on classical CD14++ monocytes decreased significantly by 40%, reaching a nadir 20 hours after surgery ($p < 0.05$). At the time of maximal membrane-associated CD14 suppression, the levels of soluble CD14 measured by enzyme-linked immunosorbent assay were clearly increased (3.2 ± 1.0 μ g/ml before versus 5.6 ± 1.0 μ g/ml 20 hours after, $p < 0.001$). No significant change of the percentage of small (α) and large (β) forms of soluble CD14 was found. **Conclusions:** Cardiopulmonary bypass leads to reduced membrane-associated CD14 expression on peripheral blood monocytes and increased levels of soluble CD14 through shedding or secretion of membrane-associated CD14 from the cell surface. These findings indicate that bypass is associated with significant monocyte activation. (J Thorac Cardiovasc Surg 1998;115:1172-8)

Patients undergoing cardiopulmonary bypass (CPB) frequently manifest a generalized systemic inflammatory response syndrome. Clinically, these reactions are reflected in postoperative leukocytosis, fever, and extravascular fluid accumulation,

which may be associated with prolonged recovery and occasionally with serious organ dysfunction.¹⁻³ Current knowledge suggests that activation of circulating leukocytes, platelets, and vascular wall components, such as endothelial cells, during CPB is involved in this inflammatory response. However, neither the exact mechanism(s) of activation nor the sequelae of such inflammation are fully understood.

Complement activation and neutropenia occur during CPB, and functional signs of leukocyte activation after cardiac operation, including the release of proteolytic enzymes, free radical production, and changes in leukocyte adhesiveness, have been reported.⁴⁻⁶ Furthermore, measures of cell-mediated immunity, such as lymphocyte responsiveness to mitogens and antigen presentation, have been shown to be seriously impaired in patients after CPB.⁷

The monocyte/macrophage cell system is believed to play a central role in regulating acute inflamma-

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tory responses. During CPB, monocytes release proinflammatory mediators, such as interleukin (IL)-1 β , IL-6, and IL-8.^{3,8,9} Monocytes express specific cell surface antigens that are of principal importance in stimulation of these myeloid cells. CD14 is a 55-kD glycoprotein that exists in both membrane-associated and soluble forms. Membrane-associated CD14 (mCD14) has been identified as the main lipopolysaccharide (LPS) (endotoxin) receptor on leukocytes.¹⁰ mCD14 can be released by cells to yield a soluble protein (sCD14) that is able to attach to epithelial and endothelial cells.¹¹

Activation of monocytes through mCD14 triggers a complex inflammatory response, leading to cytokine release, production of reactive oxygen species, and production of prostaglandins. Elevated plasma concentrations of sCD14, together with a reduced expression of mCD14, have been reported in patients with pathologic conditions such as sepsis, infection, or multiple organ failure, and increased serum levels of sCD14 were associated with a higher mortality.^{12,13}

Because the systemic inflammatory response to CPB is mediated, in part, by monocytes and because increased plasma levels of sCD14 reflect monocyte activation we analyzed the effect of elective coronary artery bypass grafting with CPB on the monocyte CD14 system.

Patients and methods

Study design. To test the hypothesis that CPB activates the monocyte CD14 system, markers of monocyte CD14 activity were determined before, during, and after elective coronary revascularization operation performed with CPB. Primary measures of monocyte CD14 activity were the membrane expression of CD14 and plasma concentrations of sCD14. Because CPB may differentially effect the numbers of circulating leukocytes, numbers of leukocytes, monocytes, and monocyte subpopulations were also determined.

Blood samples were collected before anesthesia, after 15 and 60 minutes of CPB, and at 0, 6, 20, and 44 hours after completion of the operation. Blood obtained from five healthy donors (mean age 59 ± 7 years, three men and two women) was used to establish the normal range of monocyte CD14 activity.

Patients. Ten patients (eight men, two women, mean age 63 ± 8 years) with coronary heart disease undergoing elective coronary revascularization participated in the study. The study protocol was approved by the ethical board of the Faculty of Medicine at the Ludwig-Maximilians-University of Munich, and informed consent was obtained from each patient.

None of the patients had clinical evidence of acute infection or underlying immune disease, and none were

prescribed drugs known to interfere with the immune response before operation. Anesthesia was accomplished with a standard combination of a narcotic (isoflurane), muscle relaxant (pancuronium bromide), and analgesic (fentanyl). In all patients a polypropylene hollow fiber oxygenator (Maxima Plus PRF, surface area 2.3 m², ethylene oxide-sterilized, mean priming volume 2280 ml, Medtronic, Anaheim, Calif.) was used. The extracorporeal circuit was primed with standard electrolyte solution to which 50 mmol bicarbonate was added. CPB was instituted at a flow rate of 4.0 ± 0.4 L/min. Blood was anticoagulated by administration of 20,000 to 30,000 IU of heparin during CPB. The heparin was neutralized by protamine at the end of CPB. The total time on CPB was 101 ± 48 minutes, and the aorta was clamped for an average of 60 ± 31 minutes. Minimal body temperature reached was $28.4^\circ \pm 3.6^\circ$ C in 50 ± 31 minutes after initiation of CPB. A total of 2.6 ± 0.5 distal coronary anastomoses were performed.

All patients were extubated on the first postoperative day and transferred from the intensive care unit on the second postoperative day. Two patients had a rethoracotomy within the first postoperative hours because of an intrathoracic hemorrhage, but recovery was uneventful otherwise. Two patients were treated with antibiotics for uncomplicated urinary tract infections at the end of the first postoperative week.

Leukocyte count. The absolute number of total leukocytes was determined by use of a Coulter Counter T 840 (Coulter, Krefeld, Germany).

Immunofluorescence studies. Monocyte expression of CD14 and CD16 was studied using two-color immunofluorescence. Briefly, samples of ethylenediaminetetraacetic acid-anticoagulated blood were drawn and stored on ice for a maximum of 2 hours. Whole blood, 100 μ l, was reacted with monoclonal antibodies against CD14 (My4-PE, Coulter) and CD16 (3G8-Fitc, Coulter) or the respective isotype controls for 20 minutes on ice. Erythrocytes were lysed, and the leukocytes fixed using the Q-Prep Lysis Kit (Coulter). Samples were washed twice with cold phosphate-buffered saline and then analyzed in a FACS-Can flow cytometer (Becton Dickinson, San Jose, Calif.). Monocytes (5000 events) were acquired by gating on forward and side angle scatter. The percentage of nonviable cells determined by staining with propidium iodide (Sigma, Deisenhofen, Germany) was negligible ($< 2\%$). For analysis, the data were collected in log mode. The logarithmic data were transformed to a linear scale of specific mean fluorescence intensity, and results are expressed as relative change from preoperative values.

Within the human monocyte/macrophage system, most monocytes are CD16-negative and exhibit strong CD14 staining. These CD14++ cells (two ++ to denote the strong expression of CD14) account for about 90% of all monocytes and represent what is usually referred to as "monocytes." CD14+CD16+ positive cells form a subset of monocytes that is distinct from the classical monocyte population. These cells are characterized by a more mature phenotype and by the inability to produce IL-10.¹⁴ The number of CD14+CD16+ monocytes can be dramatically increased in patients with sepsis.¹⁵ Analysis of CD14+CD16+ positive cells was performed as previously

Fig. 1. Relative changes in the absolute numbers of leukocytes, total monocytes, and CD14++ monocytes. The absolute numbers of total monocytes and monocyte subpopulations were determined by FACS using directly fluorochrome-conjugated monoclonal antibodies against CD14 (My4-phycoerythrin) and CD16 (3G8-fluorescein). Preoperative values ($t = 0$) were taken as 100%. Subsequent data are given as relative difference to preoperative values ($\text{cells}/\mu\text{l}[t] \text{ divided by } \text{cells}/\mu\text{l}[t = 0]$), thus reflecting the factor of decrease or increase compared with $t = 0$. CPB, Cardiopulmonary bypass.

described elsewhere¹⁶ (also in <http://www.med.uni-muenchen.de/immuno/ziegler>). The number of total monocytes/ μl blood was calculated as total leukocytes/ μl blood $\times$ % of all CD14 positive cells in the monocyte scatter gate. The number of CD14+CD16+ monocytes per μl was calculated as total monocytes per μl blood $\times$ % CD14+CD16+ monocytes among all CD14-positive monocytes.

sCD14 detection by Western blotting. Plasma was obtained by centrifugation of whole blood at 800g for 5 minutes at 4° C and stored at -80° C. For sCD14 analysis, plasma was thawed at 37° C and coagulated by addition of glass beads. Western blotting was performed as previously described.¹⁷ Briefly, plasma was diluted 1:50 with Laemmli's reducing sample buffer, boiled, and loaded onto precast 12.5% polyacrylamide gels (Phastgels, Pharmacia Biotech, St. Quentin, Yvelines, France). Electrophoresis was carried out in the presence of sodium dodecylsulfate in a PhastSystem (Pharmacia). Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom) in transfer buffer (48 nmol/L tris-hydroxymethyl-amino methane, 39 nmol/L glycine pH 9.0 with the addition of 20% methanol) for 16 minutes at 30 mA/gel using a semidry transfer cell (Bio-Rad). sCD14 glycoproteins were detected by incubation of the blots with the My4 monoclonal antibody (1 $\mu\text{g}/\text{ml}$) and visualized by enhanced chemiluminescence (Amersham). Luminescence was detected by short exposures on Hyperfilm MP films (Amersham). With short exposure times of the film, the Western blotting for sCD14 is at least

semiquantitative because in titration experiments a linear relationship between amounts of sCD14 and the densitometry signal was achieved.

sCD14 detection by enzyme-linked immunosorbent assay. Plasma concentrations of sCD14 were determined using a specific enzyme-linked immunosorbent assay (IBL, Hamburg, Germany).

Statistics. To determine the effect of CPB on monocyte activity, the time courses of monocyte CD14 expression and plasma CD14 concentrations were analyzed by use of an analysis of variance for repeated measures (SigmaStat, Jandel, San Rafael, Calif.). This method of analysis was chosen in recognition of the variability in monocyte populations that can occur between individuals. Where a significant effect of time was found, differences between individual time points were evaluated using a Bonferroni posthoc test. Data are presented as mean $\pm$ standard deviation. For clarity of presentation, the data presented in Figs. 1 and 2 are normalized to the preanesthesia value.

Results

CPB induces an initial decrease of monocyte count followed by postoperative monocytosis. Leukocyte and monocyte counts were within normal limits preoperatively (5.540 ± 2.093 leukocytes/ μl and 485 ± 242 monocytes/ μl). In contrast to earlier reports the total leukocyte count did not decrease significantly after start of CPB. Instead, total leuko-

Fig. 2. Expression of mCD14 on CD14++ monocytes. Expression of mCD14 on CD14++ monocytes was analyzed with the monoclonal antibody My4-PE and the respective isotypic control. Preoperative values ($t = 0$) were taken as 100%. Subsequent data are given as relative difference to preoperative values ($sMFI[t]$ divided by $sMFI[t = 0]$), thus reflecting the decrease compared with $t = 0$. Statistical differences are described in the text. *sMFI*, Specific mean fluorescence; *CPB*, cardiopulmonary bypass.

cyte count increased continuously up to factor 3 at 44 hours after operation ($p < 0.05$). Fifteen minutes after initiation of CPB, there was a mild decrease in monocyte count to 305 ± 237 cells/ μ l, followed 6 hours after the end of the operation by a monocytosis that reached a maximum at 20 hours (1080 ± 264 cells/ μ l, $p < 0.05$, Fig. 1). Because CPB might differentially influence monocyte subpopulations, we also studied the kinetics of classical CD14++ and CD14+CD16+ monocytes. The major population of CD14++ monocytes was decreased during CPB, with a nadir at 15 minutes (272 ± 214 cells/ μ l vs 457 ± 220 cells/ μ l during anesthesia). At 6 hours after operation the number of CD14+ monocytes in peripheral blood had already increased by a factor of 2.5, reaching a maximum at 20 hours with 911 ± 369 cells/ μ l ($p < 0.05$, Fig. 1). The CD14+CD16+ monocytes (48 ± 30 cells/ μ l initially) decreased, with a nadir at the end of operation (9 ± 6 cells/ μ l) and increased after operation by factor 1.5 with a maximum of 69 ± 33 cells/ μ l at 20 hours after operation (data not shown). Thus in the postoperative phase there is a relative decrease of CD14+CD16+ monocytes, which is maximal at 6 hours after operation ($9.3\% \pm 2.4\%$ vs $2.9\% \pm$

2.1% , $p < 0.001$) and still persists at 20 hours ($6.7\% \pm 3.3\%$) (data not shown).

mCD14 expression decreases on CD14++ monocytes after CPB. Before operation, mCD14 expression by CD14++ monocytes was within the normal range compared with five healthy donors. After the start of CPB, a temporary increase in mCD14 expression was noted (Fig. 2). mCD14 expression then decreased, reaching a nadir 40% below initial values at 20 hours postoperatively ($p < 0.05$). The CD14+CD16+ monocytes are not included in the above analysis. Cell surface expression of mCD14 did not significantly change in the CD14+CD16+ monocytes (data not shown).

Plasma sCD14 is increased at the time of low monocyte mCD14 expression. At the time of maximal mCD14 suppression, the levels of sCD14 were clearly increased by factor of 1.7 (before CPB 3.2 ± 1.0 vs 5.6 ± 1.0 μ g/ml 20 hours after operation, $p < 0.001$, Fig. 3, A). Levels of sCD14 in plasma (total sCD14) derive from a small membrane form of CD14 (sCD14 α) and from intracellular pools (sCD14 β).¹⁷ In our study no significant change was found in the relative contribution of the α -form and the β -form to total sCD14 levels (α -sCD14: at 0

Fig. 3. Levels of sCD14 before and 20 hours after aorta-coronary bypass operation. **A**, Total levels of sCD14 were determined by Western blotting at 0 hours and 20 hours after operation with the My4 monoclonal antibody and were visualized by enhanced chemiluminescence. Values are given as mean $\pm$ standard deviation. Statistical differences are described in the text. **B**, Representative Western blot of the α -form and β -form of sCD14 in patient No. 1 at 0 hours and 20 hours after operation. Here, the α -form (lower band) accounts for 59% of total sCD14 before and 64% 20 hours after operation.

hours $55\% \pm 2\%$ of total vs $51\% \pm 3\%$ of total 20 hours after). In Fig. 3, B, representative data obtained from one patient are shown. In this patient the relative contribution of sCD14 to total sCD14 was essentially constant (59% at 0 hours and 64% 20 hours after operation). Thus secretion of intracellular material and shedding of mCD14 from the monocyte surface appear to contribute equally to the increase of total sCD14.

Discussion

Despite considerable progress in the use of CPB, the so-called postperfusion syndrome still plays a role in the morbidity and mortality of patients undergoing cardiac operations, particularly after prolonged extracorporeal circulation. The underlying mechanism(s) of this inflammatory response is still not fully understood. Functional and phenotypic signs of leukocyte activation during extracorporeal circulation for cardiac operation have been reported previously.^{1,2} In this regard leukocyte-mediated vascular endothelial damage is believed to play a major role, with most of the studies focused on the generation of activated complement components (C5a) or the release of leukocyte-derived cytokines (IL-1, IL-6, IL-8, tumor necrosis factor- α).

Besides generation of proinflammatory soluble cell products, monocytes express a specific 55-kD glycoprotein on their surface, the CD14 antigen that exists in both the membrane-associated and soluble form. The CD14 antigen system is of principal importance in stimulation of monocytes by endo-

toxin and endotoxin-like substances. In this study the effect of cardiac bypass operation on the monocyte CD14 system was investigated.

Herein a profound alteration of the monocyte CD14 system was found in patients undergoing elective coronary revascularization. After cardiac operation with the use of CPB, a significant monocytosis was observed, reaching a maximum at 20 hours after operation. In parallel to this increase of monocyte cell number, a decrease of mCD14 antigen expression and a significant increase in levels of sCD14 antigen occurred in all patients studied.

Bacterial LPS is a potent activator of leukocytes, with CD14 on the cell membrane as the main LPS receptor. LPS may associate with soluble serum proteins (LPS binding protein) before the resulting complex is bound by mCD14, leading to characteristic cell responses such as synthesis and release of inflammatory mediators. In addition, CD14 can be released by leukocytes, and the soluble form of CD14 has been shown to enable responses to LPS by cells that do not express CD14 as an LPS receptor,¹⁸ as well as by mCD14-bearing cells.^{19,20} These activities indicate that the membrane form behaves as an LPS receptor, and its soluble pendant may serve as a coligand for LPS-mediated cell activation.

The decrease of membrane CD14 on blood monocytes and the concomitant increase of sCD14 in plasma reported herein suggest that the sCD14 is derived from monocytes by shedding of the cell surface molecule. In addition, it is possible that tissue macrophages contribute to the increased

sCD14 by the same mechanism. At this point it is, however, not known whether mCD14 of tissue macrophages also becomes down-regulated after CPB.

Although anti-CD14 monoclonal antibodies preferentially stain monocytes and macrophages, reactivity with other cells, such as granulocytes, B cells, and mammary cells, is also observed.²¹ Besides activation of monocytes, CPB may lead to significant alterations in granulocytes. Indeed, increased plasma concentrations of granulocyte proteinases and oxygen radicals have been reported.²² Therefore granulocytes and monocytes should be considered as the source of sCD14 found after CPB.

CD14 in plasma is found as a mixture of two soluble proteins, the smaller molecular weight sCD14 α , deriving mainly from membrane-bound protein material by shedding, and the larger molecular weight sCD14 β , released from intracellular pools.¹⁷ In this study we found no significant difference in the ratio of the α and the β forms of sCD14 between samples taken before and after operation. These results indicate that secretion of intracellular material and shedding of mCD14 contribute equally to the increase of total sCD14 found in plasma after coronary bypass operation.

In vitro LPS stimulation of monocytes induces an increase of mCD14 expression within 30 minutes followed by a slow decrease after 1 to 3 hours.²³ Furthermore, this decrease of mCD14 coincides with an enhanced sCD14 release by the stimulated cells.²⁴ In our study a transient increase of mCD14 expression immediately after the start of CPB was followed by a continuous decrease of mCD14. Similar to the in vitro results reported, these changes in membrane-bound antigen activity paralleled the increasing levels of sCD14, reaching a maximum at 20 hours after the end of operation. Our results indicate that CPB poses a strong stimulus to previously unaffected circulating monocytes.

Several mechanisms of cell activation during CPB have been proposed. Direct interaction of immunocompetent blood cells with the foreign surfaces of the extracorporeal circuit has been favored as the primary mechanism.¹ However, with its biologic function as the main LPS receptor on leukocytes, the CD14 system could mediate activation of cells by endotoxin itself. Indeed, in patients undergoing CPB, increased intestinal permeability and significantly elevated levels of plasma endotoxin activity measured by the *Limulus* amoebocyte lysate assay have been reported.²⁵ Furthermore, as found with other extracorporeal

circuits, such as hemodialysis and hemofiltration, monocyte activation could be due to contamination of blood, tubing lines, the membrane oxygenator, or the priming solution with endotoxin or endotoxin-like material.²⁶ Interestingly, a reduced expression of mCD14, together with elevated serum levels of sCD14, was also described in patients undergoing intermittent hemodialysis.²⁷ In addition, the operative trauma itself, ischemia-reperfusion injury caused by crossclamping of the aorta, and reinfusion of shed blood could contribute to the cell-activating process.

On the basis of our results, we speculate that endothelial cell responses after CPB occur through different pathways. One pathway, probably the principal pathway, is activation of monocytes through mCD14 triggering a complex inflammatory response, including the production and release of cytokines, reactive oxygen species, and prostanoids, all of which are known to be able to induce vascular endothelial cell damage directly. In addition, activation of the CD14 system could induce increased shedding and release of sCD14. The soluble form of CD14 then could interact with endothelial cells, promoting cell activation indirectly in concert with inflammatory cytokines.²¹ In our study we did not include the measurement of cytokine production. However, in patients with sepsis, LPS-mediated signaling and cytokine transcription were reported unchanged despite a significant decrease of CD14 expression on blood monocytes.²⁸ Finally, endothelial cell activation and damage may also be induced by direct leukocyte endothelial interaction mediated by adhesion molecules.²⁹

In conclusion, our results demonstrate that cardiac operation with the use of CPB results in significantly decreased mCD14 and elevated sCD14 levels, indicating a profound derangement of the CD14 receptor system. The clinical importance of these findings is not yet clear. In septicemic patients both decreased as well as unchanged CD14 expression by blood monocytes has also been reported.^{16, 30} These different results might be explained by different subsets of patients or different times of cell surface antigen analysis. Furthermore, elevated plasma levels of sCD14 were associated with a higher mortality in patients with sepsis or multiple organ failure. However, whether these changes contribute to a quantitative and functional impairment of the immune response in patients undergoing cardiac operation cannot be concluded from this study.

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