Endothelial apoptosis and circulating endothelial cells after bypass grafting with and without cardiopulmonary bypass

Franz-Xaver Schmid a,*, Nalini Vudattu b, Bernhard Floerchinger a, Michael Hilker a, Günther Eissner b, Markus Hoenicka a, Ernst Holler b, Dietrich E. Birnbaum a

a Department of Cardiothoracic and Vascular Surgery, University Hospital of Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany
b Department of Hematology and Oncology, University Hospital of Regensburg, Regensburg, Germany

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Abstract

Objective: We compared profiles of the numbers of circulating endothelial cells (CEC) and the apoptosis-inducing capacity of serum samples on human endothelial cells (hEC) in on-pump and off-pump coronary artery bypass grafting (CABG) patients. Methods: Blood samples from 30 patients undergoing CABG (randomly assigned to two groups: 15 patients off-pump and 15 on-pump (cardiopulmonary bypass, CPB)) were collected after induction of anesthesia (preoperatively), at weaning from CPB/end of bypass grafting (0 h), and 1, 6, 12, 24, and 48 h afterwards. CEC were isolated with immunomagnetic anti-CD146-coated Dynabeads, and counted in a Nageotte chamber. The apoptosis-inducing activity of serum samples on hEC was examined by a tissue culture assay system. Apoptotic and normal cells were identified using phase contrast/fluorescence microscopy after DNA dye staining. Results: CEC numbers and proportions of apoptotic hEC were significantly elevated during and after surgery in both groups (p < 0.01). Compared with the on-pump group, CEC and proportions of apoptotic hEC were significantly lower (p = 0.04 and p = 0.03, respectively) in patients having CABG performed off-pump. Starting at comparable baseline levels, the mean CEC-number was highest at 6 h postoperatively with 81.9 ml/C0 1 (range, 44—141) for on-pump patients and 63.3 ml/C0 1 (range, 48—105) for off-pump patients. hEC apoptosis peaked also at T4: 16.5/C6 2.8% versus 11.3/C6 2.2%. In both groups, CEC numbers and proportions of endothelial apoptosis were still elevated at 48 h after surgery. Conclusion: The number of circulating endothelial cells and apoptotic endothelial cell death are markers of endothelial activation and damage during CABG. This study provides evidence that CABG with the use of CPB in comparison to OPCAB surgery is associated with a significantly more pronounced endothelial response in the immediate postoperative period.

Keywords: Endothelial cell apoptosis; Circulating endothelial cells; Cardiopulmonary bypass; OPCAB surgery

1. Introduction

Cardiac surgery performed with the use of cardiopulmonary bypass (CPB) provokes a systemic inflammatory response syndrome (SIRS) [1]. Operative trauma, ischemia—reperfusion injury, and contact of blood with the artificial surface of the extracorporeal circuit are all possible causes. The complex pathophysiology may contribute to the hemodynamic alterations including myocardial dysfunction, respiratory failure, altered renal, liver and neurological function, postoperative bleeding, and ultimately multiple organ failure encountered sometimes in compromised patients at risk.

In an attempt to minimize the impact of SIRS on the outcome of cardiac surgery, several strategies, including modifications of CPB circuits, components and surfaces, have been investigated during the last few years, but a substantially improved clinical outcome could not be demonstrated. In this context, the performance of off-pump coronary artery bypass grafting (CABG) might offer advantages when compared with the need for CPB during routine CABG surgery. It is being increasingly used as an alternative to conventional CABG aiming to reduce cardiopulmonary bypass-induced inflammation.

Circulating endothelial cells (CEC) have recently been established as a specific and sensitive marker of endothelial activation and damage in a variety of vascular disorders [2, 3]. Endothelial activation and/or damage are considered a key process in the development of SIRS. Therefore, this study was designed to compare profiles of the numbers of CECs and the apoptosis-inducing capacity of serum samples on human endothelial cells in patients with coronary artery disease.
during CABG surgery with or without CPB in a prospective, randomized study.

2. Material and methods

Thirty patients, who were referred for elective and isolated coronary artery bypass surgery, were prospectively randomized to undergo CABG with \( n = 15 \) or without \( n = 15 \) CPB. There were 21 males and 9 females; median age was 67.4 years (range 59—75 years). The study protocol was approved by the Institutional Committee on Medical Ethics. All subjects gave written informed consent. Patients with ongoing infections, abnormal serum urea and creatinine, diabetes or on current treatment with steroids or nonsteroidal anti-inflammatory drugs, except acetylsalicylic acid, were excluded from the study. The EuroSCORE was calculated (Table 1). We also studied 10 age-matched healthy controls.

2.1. Anesthesiological and postoperative management

Anesthesia was induced with fentanyl (5 \( \mu \text{g/kg body weight} \)), etomidate and pancuronium (100 \( \mu \text{g/kg body weight} \)) and was maintained by continuous infusion of propofol (3—5 \( \mu \text{g/(kg h)} \)) with intermittent bolus doses of fentanyl (up to 20 \( \mu \text{g/kg} \)) and pancuronium (50 \( \mu \text{g/kg} \)). The patients were noninventilated using oxygen and air (FiO\textsubscript{2} 0.4), and enflurane was used as an inhalation agent both prior to and after CPB.

The postoperative management and care was identical in both groups of patients. According to our institutional protocol, all patients were ventilated postoperatively for at least 2 h and were weaned from the ventilator as soon as possible thereafter.

2.2. Cardiopulmonary bypass

CPB was established using a two-stage venous drainage and ascending aortic return. A roller pump (HL30\textsuperscript{R}, Jostra, Hirrlingen, Germany), a membrane oxygenator (Quadrox\textsuperscript{R}, Jostra, Hirrlingen, Germany), a cardiotomy suction device and a 40 \( \mu \text{m arterial line filter (Pall Biomedical, NY, USA) were integrated into the extracorporeal circuit. The system was primed with 1.5 l of Ringer's lactate. Heparin was given at 300 IU/kg body weight to maintain an activated clotting time (ACT) above 400 s during bypass. Pump flow rates averaged 2.4 l/(min m\textsuperscript{2}) body surface area with pressure maintenance at 50—60 mmHg. The patients were kept normothermic. Cardioplegic arrest was induced with 2 l of a single dose of Bretschneider's cold crystalloid solution, administered antegrade into the aortic root. All patients received an internal artery mammary graft to the left anterior descending coronary artery. The central aorto-venous anastomoses were performed during the reperfusion phase of CPB with the heart beating. After termination of bypass, heparin anticoagulation was antagonized by protamine sulphate at a 1:1 dosage. Tranexamic acid (2 mg/(kg h)) was administered throughout the surgical procedure. Operative characteristics are depicted in Table 1.

2.3. Off-pump technique

All operations were performed through a median sternotomy incision. Two to three traction sutures in the postero-lateral pericardium were placed. Regional myocardial stabilization was achieved with commercially available suction stabilizers. No preconditioning was performed. The target coronary vessels were snared with a pledget-armed tourniquet proximal to the anastomotic site. An intracoronary shunt was used during construction of the anastomoses. The left internal mammarian artery to LAD was the first anastomosis in all patients. During the operation all patients received tranexamic acid (2 mg/kg/h). In OPCAB patients heparin was given at a dosage of 150 IE/kg to achieve an ACT of \( \geq 250 \) s. The central aorto-venous anastomoses were established with partial occlusion of the ascending aorta. After completion of the final anastomosis heparin was antagonized with protamine sulphate at a 1:1 dosage to return the ACT to preoperative levels. A cell saving device was used to reduce the need for blood transfusion in all OPCAB-patients.

2.4. Isolation of circulating endothelial cells

Samples of peripheral blood were obtained from an indwelling arterial line at preselected time points: after induction of anesthesia but before CPB/start of bypass grafting, after weaning from cardiopulmonary bypass/end of bypass grafting, and 1, 6, 12, 24, and 48 h after termination of extracorporeal circulation. Additional blood samples were taken from 10 healthy volunteers and served as control samples.

Circulating endothelial cells were identified by immunomagnetic isolation. Paramagnetic M-450 Dynabeads (Dynal, Hamburg, Germany) were coated with anti-CD146 antibody (Biocytex, Marseille, France). CD-146 is expressed by mature endothelial cells, although various tumor cell lines also express the antigen. Blood was incubated with coated Dynabeads, and cells bound to anti-CD-146 coupled beads were separated from blood in a magnet, washed, mixed with acridine, and counted with fluorescence microscopy in a Nageotte chamber. To augment the specificity of the technique and to ensure that all isolated cells were indeed endothelial cells, Uleus europaeus lectin (UEA-1; Linaris, Wertheim, Germany) staining with counting was performed in parallel to acridine staining and counting.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline and clinical characteristics of patients</th>
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<tbody>
<tr>
<td></td>
<td>OPCAB</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.4 ± 9.8</td>
</tr>
<tr>
<td>Sex (m/f)</td>
<td>11/4</td>
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<tr>
<td>Additive EuroSCORE</td>
<td>3.9 ± 2.1</td>
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<td>Left ventricular function (EF%)</td>
<td>65 ± 9</td>
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<tr>
<td>Body mass index</td>
<td>24.7 ± 5.2</td>
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<tr>
<td>Op time (min)</td>
<td>167 ± 21</td>
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<tr>
<td>CPB time (min)</td>
<td>—</td>
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<tr>
<td>Aortic clamping time (min)</td>
<td>—</td>
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<tr>
<td>Average number of grafts</td>
<td>1.9 ± 0.9</td>
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<tr>
<td>LIMA use (%)</td>
<td>100</td>
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<tr>
<td>Time of intubation</td>
<td>8.2 ± 4.2</td>
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<tr>
<td>ICU stay &gt; 24 h</td>
<td>0</td>
</tr>
<tr>
<td>Blood loss (ml)</td>
<td>515 ± 63</td>
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</table>
2.5. Assay system for endothelial apoptosis

The apoptosis-inducing activity of serum samples on endothelial cells was examined using a tissue culture assay system and microscopic analysis of DNA fluorescence labeled cells. Endothelial cells derived from freshly prepared human umbilical cords were seeded and cultured in 35-mm Petri dishes (Nunc, Wiesbaden, Germany). These cultures were finally incubated for 48 h with serum samples diluted with culture medium to 50% serum content. Again serum of healthy volunteers served as control. Endothelial cells were fixed with methanol/acetone and washed in phosphate-buffered saline (PBS). The cells were stained with 4,5-diamidino-2-phenylindole (DAPI; 0.5 μg/ml; Sigma) and dissolved in 20% glycerine—PBS. Samples were mounted and subjected to fluorescence microscopic analysis. Nuclear condensation depicted by DAPI staining in the absence of trypan blue uptake is considered characteristic of apoptosis as opposed to necrosis [4]. Quantitative analysis comprised counting the number of apoptotic cells in relation to all identifiable cells from 10 microscopic fields. Seventy cells per field were identified in average. Results were determined as percentage of apoptotic cells.

2.6. Statistical methods

All data are presented as mean ± 1 SD. Results of the endothelial apoptosis in tissue culture systems are expressed as percentage of apoptotic cells ± 1 SD. Comparisons within one group were performed using the t-test for dependent samples or the Wilcoxon's matched pairs test, respectively. Intergroup differences at a single predetermined time point were assessed by using the t-test for independent samples in case of normal data distribution, and the Mann—Whitney U-test in case of non-normal data distribution. Comparisons within each group and between groups were further made using analysis of variance (ANOVA). All statistical analyses were performed using SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was assumed at a p-value less than 0.05.

3. Results

3.1. Baseline and clinical characteristics of patients

With respect to detailed preoperative and clinical data, patient characteristics were similar in both groups. No significant differences with respect to age, body mass index, sex, hemodynamics, the number of risk factors for coronary artery disease were observed between the groups. The average number of peripheral anastomoses was comparable between OPCAB and CABG group (OPCAB: 1.9 ± 0.9; CABG: 2.2 ± 0.8; n.s.). Perioperative patient findings and early outcome data are presented in Table 1. There were no significant differences in blood loss, blood product requirements, postoperative myocardial infarction or stroke, and new onset of atrial fibrillation. There was no reoperation for bleeding or early bypass occlusion. No wound infection occurred. There was no conversion from OPCAB to conventional CABG.

3.2. Endothelial cell apoptosis

Increased proapoptotic capacity of serum samples obtained from CPB patients early after weaning from CPB was demonstrated. There was a measurable but not statistically significant difference in baseline values between groups. When compared to preoperative and off-pump control samples, the proportion of apoptotic endothelial cells was significantly increased in EC cultures with serum samples derived from CPB patients from 1 to 12 h postoperatively, particularly at 6 h after termination of CPB. Six hours after CPB patient serum samples resulted in a proportion of 16.5 ± 2.8% of apoptotic endothelial cells in comparison to a proportion of 8.9 ± 2.1% (p < 0.01) preoperatively, and of 11.3 ± 2.2% (p = 0.03) in samples from OPCAB patients at the same sampling time point. The results of both groups are shown in Fig. 1. In both groups, proportions of apoptotic endothelial cells were still elevated at 48 h after surgery, but due to considerable distribution of the results, this difference did not reach a level of significance (Fig. 2).
3.3. Circulating endothelial cells

Healthy controls had low numbers of circulating endothelial cells (12 ± 6 cells/ml; median, 9 cells/ml). CEC numbers were significantly elevated before, during, and after surgery in both groups. Cell numbers were significantly elevated already at baseline, that is, before bypass surgery and/or start of CPB in both groups (OPCAB: 30 ± 13 cells/ml; CABG: 31.1 ± 15 cells/ml, p < 0.01 when compared with healthy controls). There was a further significant increase in cell numbers after CPB (maximum increase at 6 h after CPB: 81.9 ± 23 cells/ml, range, 44—141 cells/ml, p < 0.001). The increase of CEC numbers was significantly lower in patients having coronary surgery performed off-pump (maximum increase at 6 h after surgery: 63.3 ± 17 cells/ml, range 48—105, p = 0.04). Total numbers of CECs obtained 1, 6, 12, and 24 h after surgery were significantly lower in the OPCAB group when compared to the CPB group (55.2 ± 11.9 vs 74.9 ± 19.8; 63.3 ± 17 vs 81.9 ± 23; 60.7 ± 15.9 vs 78.9 ± 17.9; 41.9 ± 9.9 vs 65.8 ± 14.5 cells/ml; p < 0.05).

Cell numbers decreased during the first and second postoperative day. We were able to identify still significantly increased levels of CEC at the end of the observation period in comparison with healthy controls (p < 0.005). CEC levels 48 h after termination of CPB or coronary surgery (OPCAB: 43.2 ± 20 cells/ml, CABG: 47.5 ± 24 cells/ml) were not significantly different to levels before starting extracorporeal circulation due to a considerable scattering in some patients.

4. Discussion

In this prospective, randomized study of OPCAB versus on-pump CABG, we found two similar patterns: the CPB group had a significantly more pronounced endothelial activation demonstrated by the numbers of circulating endothelial cells, and serum derived from patients after CPB exerted a stronger apoptosis inducing activity on cultured human endothelial cells. Levels of CECs were increased throughout the observational period of 48 h before and especially after both surgical procedures and peaked at high levels 6—12 h after termination of the procedure.

Our findings are in accordance with other studies showing massive endothelial activation during and after early after cardiac surgery performed with the use of CPB in comparison to OPCAB surgery [5—7]. The activated state of the endothelium immediately after on-pump, as evidenced by increased CEC numbers and the proportion of apoptotic endothelial cells, may reflect the overall systemic inflammatory response and could theoretically contribute to the organ dysfunction associated with CPB. Immediately after OPCAB, there is much less endothelial activation, and this may have been a contributing factor in the better reported clinical outcome [8,9]. Moreover, a less proinflammatory response may explain the reduced consumption of clotting factors with subsequently reduced postoperative blood loss and transfusion requirements, lower expression of cytokines, a reduction in renal injury, and less myocardial injury that have been reported after OPCAB surgery [10,11]. However, this will need to be confirmed in larger studies.

Levels of CECs were increased from the first postoperative hours after both surgical procedures and peaked at high levels 6—12 h after termination of bypass grafting. Enumeration of circulating endothelial cells in the peripheral blood is a novel method for assessing endothelial damage. We here demonstrate the occurrence of circulating endothelial cells in peripheral blood in CABG patients exposed to extracorporeal circulation in comparison to OPCAB surgery. Circulating endothelial cells have recently been evaluated as a marker of endothelial injury in a variety of pathological conditions including vascular inflammatory diseases, acute myocardial infarction, and critical limb ischemia [2,12,13]. We assume that detection of elevated numbers of circulating endothelial cells represent the most direct marker of endothelial activation or injury, and perhaps may enable quantification of the inflammatory response in conjunction with CPB. Since these cells are very rarely found in the blood of healthy people, as evidenced by our control group of healthy people, the increased number may reflect the degree of endothelial perturbation and even represent a prognostic indicator in patients developing an overwhelming inflammatory response after a period of CPB. Surprisingly, patients already had slightly elevated cell numbers before bypass surgery. It is tempting to speculate that this finding may reflect induction of anesthesia or surgical trauma by thoracotomy. Alternatively, elevated cell numbers may also reflect the underlying disease. In this context, elevated numbers of circulating endothelial cells have been reported in patients with symptomatic coronary artery disease [13].

The origin and the fate of detached endothelial cells in the circulation remains a matter of speculation. This issue and the time frame of these events have not been investigated so far. We have documented that in routine CABG patients who do not suffer from peri- or postoperative complications, irrespective of the surgical strategy (on-pump or off-pump), CEC decreased to levels comparable to levels before bypass grafting, indicating that the endothelial injury initiating factor has disappeared but complete endothelial restoration has not yet been accomplished at that time. The demonstration of significant increases in CEC and in apoptosis-inducing capacity at the same time in on-pump and off-pump surgery suggests that this may be to a certain extent a response secondary to any surgical procedure as the surgical trauma in both groups was comparable.

Apoptosis is a form of genetically induced programmed cell death. The biochemical hallmark of apoptosis is the fragmentation of genomic DNA, an irreversible event that commits the cell to die. We used in situ labeling of fragmented DNA (TUNEL stain) to analyze numbers of cells with DNA damage. TUNEL technique is a well-established and reproducible tool in research on endothelial cell apoptosis [4,14]. Apoptosis is regulated by a complex interplay between cell surface signals and the expression of specific intracellular gene products. Thus, different pathways may be responsible for inducing apoptosis in endothelial cells. It has been reported that cytokine receptors of the tumor necrosis factor family regulate a number of stages of inflammation [14—16] especially endothelial cell activation [1,17,18]. In experimental studies, the reperfusion injury mechanism has been increasingly recognized responsible to induce cell death by means of TNF-α-mediated apoptosis [19]. In a previous study, our group
was able to demonstrate that serum taken from patients who had surgery with the use of CPB may induce endothelial apoptosis [20]. CPB induces a whole body inflammation with generation of oxygen-free radicals, cytokine release, and altered nitric oxide release presumably due to the contact of blood with foreign surfaces [21,22]. Possibly CPB per se may promote apoptosis, as increased serum levels of the caspase blood with foreign surfaces [21,22]. Possibly CPB per se may alter nitric oxide release presumably due to the contact of generation of oxygen-free radicals, cytokine release, and apoptosis [20]. CPB induces a whole body inflammation with endothelial activation of the coronary vascular system and an inflammatory endothelial response of the whole body. As a matter of fact, predominantly the reperfused myocardium is a possible source of apoptosis-inducing mediators, where the execution phase of programmed cell death is triggered by a specific nuclear factor kappa B-initiated inflammatory response [24]. Endothelial apoptosis induction by serum taken selectively from coronary sinus blood and experimental evaluation of intermittent blood cardioplegia for myocardial and endothelial protection should provide further insights into the concept of endothelial apoptosis induction in the setting of cardiac surgery. Obviously, apoptosis plays a role in the tissue injury induced by CPB but its extent and its mechanism remain to be elucidated.

The profiles of a number of circulating endothelial cells and the time course of proapoptotic capacity of serum samples on human endothelial cells in on-pump and off-pump CABG patients in our investigations correspond well with the well-described occurrence of acute myocardial dysfunction in conjunction with increased capillary permeability and subsequent early recovery occurring predominantly at 6—12 h postoperatively.

Limitations of our study are the use of a cardiotomy suction device in the CPB group and of a cell saver system in the OPCAB group which both may by itself have had impact on the results. We were concerned about comparability but we believe that this represents ‘the real world’ in CABG surgery.

In conclusion, the number of circulating endothelial cells and the proapoptotic activity of serum from patients undergoing coronary artery surgery are early and reliable markers of the endothelial activation and/or damage. CECs appear to indicate vascular endothelial activation because they are specific, stable, and circulating components of injured vessel wall. This prospective, randomized study provides evidence that CPB, in comparison to off-pump surgery, is associated with a significant more pronounced endothelial response in the immediate postoperative period. Further research is needed to determine the origin of these cells, corroborate the clinical significance of our findings, and delineate the influence of factors associated with cardiopulmonary bypass and coronary artery surgery as well. Understanding the endothelial cell response to injury is central to appreciating the role that dysfunction plays in the preoperative, operative, and postoperative course of almost all cardiovascular surgical procedures.

References