Endothelial apoptosis is induced by serum of patients after cardiopulmonary bypass

Hermann Aebert\(^a,\)\(^*\), Sylvia Kirchner\(^b\), Andreas Keyser\(^a\), Dietrich E. Birnbaum\(^a\), Ernst Holler\(^b\), Reinhard Andreessen\(^b\), Günther Eissner\(^b\)

\(^a\)Department of Thoracic and Cardiovascular Surgery, Regensburg University Hospital, Regensburg, Germany
\(^b\)Department of Internal Medicine I, Division of Hematology and Oncology, Regensburg University Hospital, Regensburg, Germany

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Abstract

Objective: Increased serum levels of a multitude of mediators like interleukins, tumor necrosis factor, elastase, adhesion molecules, and endotoxin have been described following cardiopulmonary bypass (CPB). The biological consequences of this complex response are unclear.

Methods: Serum samples of nine patients scheduled for elective coronary artery bypass grafting were obtained preoperatively and 1, 6, and 12 h after weaning from CPB. Additional serum samples were obtained perioperatively from four patients undergoing major lung resection and from four healthy volunteers. The apoptosis-inducing activity of serum samples on endothelial cells was examined using a tissue culture assay system. Endothelial cells were derived from human umbilical cords and incubated for 48 h with serum samples in various dilutions during their second passage. The culture plates were fixed with methanol/acetone and stained with the DNA dye diamidinophenylindole. Apoptotic and normal cells were identified and counted using phase contrast and fluorescence microscopy.

Results: The proportion of apoptotic endothelial cells was 5.6-fold higher in culture plates incubated with diluted (30%) serum samples obtained at 6 h after weaning from CPB when compared to plates incubated with preoperative samples (\(P < 0.0077\)). A smaller effect occurred already at 1 h in some patients, whereas at 12 h after weaning from CPB no increased endothelial apoptosis was observed. No proapoptotic activity was found in preoperative as well as in control samples from patients undergoing lung resection or from healthy volunteers.

Conclusions: Serum of patients after CPB exerts a strong apoptosis inducing activity on human endothelial cells. Apoptotic death of endothelial cells following CPB may be responsible for postoperative vascular and bypass dysfunction including phenomena like increased capillary permeability.

Keywords: Apoptosis; Endothelial cells; Cardiopulmonary bypass

1. Introduction

A plethora of triggers, effectors and mediators have been measured experimentally and in patients for biochemical characterization of the systemic inflammatory response resulting from cardiopulmonary bypass (CPB). However, the biological relevance of a single compound and even more so, of a complex concert of many different biologically active substances in the context of CPB, remains largely unclear [1].

Apoptosis or programmed cell death is a controlled biological process essential for cellular renewal and for clearance of damaged cells. A substantial role of programmed cell death in the pathophysiology of various diseases has been established [2]. With respect to the vasculature, apoptosis seems to be particularly important in the context of hypertension, atherosclerosis, inflammation, allograft rejection, and conditioning before bone marrow transplantation [3–5]. Septic shock is probably due to disseminated endothelial apoptosis resulting in increased vascular permeability, hypotension, and organ damage [6]. Additionally, endothelial cell apoptosis is an important trigger for disturbances of coagulation and in particular for local and disseminated thrombus formation by adhesion and activation of platelets [7].

The aim of this study was to examine the ability of serum of patients undergoing cardiac surgery with CPB to induce apoptosis in human endothelial cells.
2. Patients and methods

The study protocol was approved by the Institutional Committee on Medical Ethics. Nine patients scheduled for elective coronary bypass grafting (CABG) consented to removal of additional blood samples drawn from the central venous catheter at different times points: after induction of anesthesia (1); 1 h (2), 6 h (3) and 12 h (4) after weaning from cardiopulmonary bypass. Additional samples were acquired from four patients undergoing pulmonary lobectomy or pneumonectomy preoperatively and 1, 6, and 12 h postoperatively and finally from four healthy volunteers. Blood samples were collected in plastic tubes containing plastic pearls and allowed to clot at room temperature. Following centrifugation serum samples were aliquoted, frozen, and stored at −70°C until analysis.

Anesthesia and patient management including CPB with moderate systemic hypothermia (30–32°C) and cardioplegia with Bretschneider solution were performed as described previously in detail [8,9]. No aprotinin was administered. Pertinent patient data are indicated in Table 1. All patients of the study had uneventful surgery.

2.1. Assay system for endothelial apoptosis

Endothelial cells were freshly prepared from human umbilical cord veins according to the method of Jaffe [10]. Purity of endothelial cells (greater than 95%) was assessed by indirect immunofluorescence staining directed against von Willebrand factor [11]. Serum endothelial cell growth medium free of lipopolysaccharide contamination (EGM; Promocell, Heidelberg, Germany) was used in the culture system described elsewhere [11]. During their second passage, 100 000 endothelial cells were seeded into 35 mm petri dishes (Nunc, Wiesbaden, Germany) and incubated for 48 h with serum samples in various dilutions in EGM. Several umbilical cords were used for harvesting of endothelial cells. However, analysis of all serum samples of each patient was carried out with endothelial cells derived from a single umbilical cord. Endothelial cells were fixed for 2 min with methanol/acetic acid and then washed in phosphate-buffered saline (PBS). Staining was carried out with 4,6-diamidino-2-phenylindole (DAPI; 0.5 µg/ml; Sigma) dissolved in 20% glycerin/PBS. Condensed nuclei shown by DAPI staining in the absence of Trypan Blue uptake are characteristic of apoptosis as opposed to necrosis [11].

For quantitative analysis, apoptotic and normal cells were identified using phase contrast and fluorescence microscopy. The number of apoptotic cells was counted in relation to all other identifiable cells from at least ten microscopic fields. The average number of cells amounted to 70 per field.

For better assessment of possible proapoptotic activity and of possible protective effects of human serum on endothelial cells in the assay system dilution experiments were performed, i.e. before incubation with endothelial cells samples were diluted down with culture medium to a serum content of 10, 30 and 50%, respectively.

Serum of healthy volunteers served as additional controls. Furthermore, during every assay with patient samples some petri dishes with endothelial cells were incubated with the culture medium only to serve as untreated intra-experimental control.

2.2. Statistical analysis

All continuous variables are presented as mean ± standard deviation (SD). Results are expressed as percentage of apoptotic cells ± SD. Mann–Whitney U analysis or Wilcoxon’s signed rank test using StatView (Abacus, Berkeley, CA), were performed as appropriate, to evaluate the differences between samples. Statistical significance was assumed at a P value less than 0.05.

3. Results

Increased endothelial apoptosis was observed with serum samples obtained at 1 and particularly at 6 h after weaning from CPB. Dilution experiments demonstrated strong proapoptotic activity if the samples were diluted to 30% and even down to 10% patient serum content (Fig. 1). Preoperative serum samples did show no difference when compared to serum samples of healthy volunteers or to pure culture medium (Fig. 1). Similarly, no increased apoptosis was found in endothelial cell cultures incubated with serum taken 12 h after termination of CPB. In addition, no increase in proapoptotic activity was observed in pre- or postoperatively collected serum samples of the patients undergoing lung resection (Table 2).

The cumulated results of all nine patients undergoing CPB with serum samples diluted to 30% are shown in Fig. 2. Six hours after weaning from cardiopulmonary bypass patient samples resulted in a 5.6-fold ± 1.7-fold (mean ± SD; P = 0.0077) higher proportion of apoptotic endothelial cells compared to preoperative samples. After 1 h there was a certain trend, however, due to a considerable SD originating from no proapoptotic activity in serum samples of some patients, this trend did not reach the required level of significance. No endothelial apoptosis was observed with samples taken 12 h after CPB.

Table 1

<table>
<thead>
<tr>
<th>Pertinent patient data (mean ± SD)</th>
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<tbody>
<tr>
<td>Sex (male/female)</td>
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<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Aortic cross clamping (min)</td>
</tr>
<tr>
<td>CPB (min)</td>
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<tr>
<td>Ejection fraction (%)</td>
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<td>Grafs (n)</td>
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4. Discussion

Serum samples of all patients taken 6 h after CPB resulted in increased endothelial cell apoptosis compared to samples obtained preoperatively. Some apoptosis was observed in part of the patients 1 h after weaning from CPB. In all patients the proapoptotic activity in serum had completely disappeared 12 h after CPB. Various control experiments including incubation of endothelial cells with different dilutions of serum samples, with culture medium only or with serum samples obtained from patients undergoing major lung resection and from health volunteers underscore that CPB and not a major surgical procedure or spontaneous onset of endothelial apoptosis in the assay system is responsible for the induction of endothelial cell apoptosis after CPB.

Table 2
Endothelial cell apoptosis with serum samples of patients undergoing major lung resection

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Patient number</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
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<tr>
<td>Preop</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>1 h postop</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>6 h postop</td>
<td>2.1 ± 1.4</td>
</tr>
<tr>
<td>12 h postop</td>
<td>0.9 ± 0.5</td>
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* The percentage of apoptotic endothelial cells counted in ten microscopic fields (cells ± SD) after incubation with 30% diluted serum samples for 48 h is indicated. Negative control: endothelial cells incubated with culture medium only for 48 h = 1.6 ± 0.6%; preop, preoperatively; postop, postoperatively.

A variety of soluble substances including tumor necrosis factor α (TNF), transforming growth factor β (TGF), interleukin 1, elastase, and endotoxin have been implicated in apoptosis of endothelial cells [3,6,12]. Increased serum levels of all these substances have been demonstrated following CPB [1,13–15]. However, for several mediators and particularly for TNF there are conflicting reports [1,15]. Aside from technical differences for measurement of serum levels, this may in part be due to the fact, that the preoperative status and conditions like reduced left ventricular function may influence basic serum levels and the intensity of cytokine response to CPB [16].

The time course of proapoptotic activity in the present study may suggest at first glance implication of mediators with slightly increased plasma levels around 1 h after CPB and peaking at around 6 h after weaning from bypass, followed by a rapid decrease and normalization at 12 h. Interleukin 1 or TNF could somewhat fit into this time scheme. However, data from our group indicate that only the transmembrane and not the soluble form of TNF is able to induce endothelial apoptosis [5]. In addition, we found that TGF and interleukin 1 do not result in apoptosis but in cell cycle arrest of endothelial cells which in turn reduces their ability to repair endothelial defects by proliferation [11]. We think that currently it is to early to speculate which maybe yet undefined factor may be responsible for endothelial apoptosis in the setting of CPB. With high probability the time course of proapoptotic activity observed in the present study is the result of a sum up effect of various factors including proinflammatory and anti-inflammatory mediators. CPB has a considerable impact on the cellular milieu of the endothelium in respect to modifications of temperature, osmolality and shear stress. All these factors may modulate endothelial cell apoptosis [17–19]. Therefore, it is very difficult to speculate on the extent of endothelial apoptosis in patients undergoing CPB. Furthermore, many cytokines and other mediators act primarily in a local paracrine way [1] and the proapoptotic

![Fig. 1. Example of dilution experiments with apoptotic potential of different serum concentrations of the same samples. Mean values with SD resulting from percentage of apoptotic cells in ten different microscopic fields are indicated and compared by the Mann–Whitney test. Control, untreated endothelial cells incubated with culture medium only; healthy volunt., healthy volunteer.](image1)

![Fig. 2. Bar graph summarizing induction of endothelial cell apoptosis by samples diluted down to 30% serum content in all patients undergoing CPB. Statistical analysis by Wilcoxon signed rank test.](image2)
activity of our serum samples may underestimate endothelial apoptosis at the place of production. This paracrine action includes induction of adhesion molecules in the neighboring endothelium by apoptotic endothelial cells [20]. In addition, many cytokines with increased plasma levels after CPB may activate leukocytes who then in turn are able to exert proapoptotic activity on endothelial cells [11]. Our experiments with cell free serum samples cannot reflect the influence of adherent and activated leukocytes.

Irrespective of what the molecular mechanisms may be, we clearly demonstrated a strong proapoptotic activity in patient serum samples taken during the early postoperative period after CPB. Endothelial apoptosis occurring 6 h after experimental administration of a noxious stimulus has been shown to be the pathophysiologival basis for increased capillary permeability and circulatory derangements including hypotension during a systemic inflammatory response [6]. The time course of proapoptotic activity after CPB in our study corresponds to the clinical phenomena of decreased myocardial function and decreased vascular resistance after CPB reaching their nadir around 5 h postoperatively [21]. In this context findings indicating negative correlations between systemic vascular resistance and serum levels of several proinflammatory cytokines during this time of the postoperative period [13,16] are of particular interest. Furthermore, endothelial apoptosis may have profound impact on the coagulation system. Apoptotic endothelial cells become proadhesive even for non-activated platelets [7]. This is probably even more true for platelets activated during CPB. Formation of microthrombi and microemboli in the course of CPB could be the result of aggregating platelets at the place of apoptotic endothelial damage in addition to activation on artificial surfaces. Plasma of patients with various forms of thrombotic angio-pathies induces endothelial apoptosis [22]. Moreover, if repair of defects in the endothelial lining of blood vessels is decreased due to elevated levels of TGF and interleukin 1 following CPB, the procoagulative stimuli increase even further. Like in other forms of apoptosis several damaging factors may result in an additive effect. As a consequence, bypass grafts seem to be most prone for local thrombus formation since vein grafts in particular may undergo considerable mechanical, osmotic and thermal stress followed by unaccustomed hypertension. All these kinds of stress have been implicated in endothelial cell apoptosis [17,18,23]. Increased endothelial apoptosis in vein grafts will result in a high risk of thrombotic occlusion in the early postoperative period which corresponds to clinical observations. On the other hand, apoptotic endothelial cells and gaps in the endothelial lining of blood vessels may contribute to consumption of platelets and coagulation factors following CPB.

Our assay system is well-established in research on endothelial cell apoptosis [5,11]. In the present pilot study this assay system seems promising for further elucidation of the biological consequences of CPB. Endothelial cell apop-


tosis is able to explain many adverse effects of CPB. Further insight into the causative mechanisms and possibly anti-apoptotic treatment may result in reduction of the whole body inflammatory response to CPB and ultimately in better clinical results.

Acknowledgements

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Appendix A. Conference discussion

Mr J.R.L. Hamilton (Newcastle-upon-Tyne, UK): You anticipated my question by your final comment. Where do you think this research should go now? What will be your next step?

Dr Aebert: First, we have to look at patients not undergoing cardiopulmonary bypass. Second, we will try to block certain pro-apoptotic substances, because reduction of endothelial apoptosis following cardiopulmonary bypass is the ultimate goal of all this research.

Mr David O’Regan (London, UK): Apoptosis requires three modalities to define: Histological appearance, TUNEL, and FACS scanning. You’ve used a single modality here to determine apoptosis in your endothelial cells. Could you comment, did you look at any other modality for determining apoptosis? Can you exclude necrosis using your method? Did you measure lactate levels in the cell culture medium as well?

Dr Aebert: We do these experiments in co-operation with our hematologists. They have compared DAPI stain and FACS in different settings of endothelial cell apoptosis and obtained equivalent results. We have considerable experience with apoptosis of cardiac myocytes after cardioplegic arrest and reperfusion. The TUNEL technique was not very reliable and this has also been published many times in the literature.

Mr O’Regan: The only other comment is, is that compared to your controls, the serum from the patients who have been on bypass, that has got heparin, cardioplegia and protamine in as well. So just to point out that the serum that you’ve obtained from patients who have been on bypass has got other additives in as well, that being heparin, cardioplegia and protamine in as well.

Dr Aebert: We are well aware of this. Osmolarity, temperature, and shear stress all may modulate endothelial cell apoptosis. Shear stress actually protects endothelial cells from apoptosis. The low shear stress during cardiopulmonary bypass may have some pro-apoptotic activity. But we just checked the serum. In our experiments we could not examine the influence of other factors like low shear stress or the rolls of activated leucocytes being in contact with endothelial cells. Since these and all the other factors you have mentioned are probably pro-apoptic, the total pro-apoptotic activity in patients following cardiopulmonary bypass may even be larger than that of serum as shown in these experiments.