Neutrophil dysfunction after biomaterial contact in an in vitro model of cardiopulmonary bypass

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Abstract

Objective: Cardiopulmonary bypass (CPB) induces neutrophil degranulation and superoxide anion production in vivo. We hypothesized that CPB-associated neutrophil dysregulation alters neutrophil adhesion to vascular endothelial cells and the extracellular matrix.

Methods: We, therefore, recirculated neutrophils in polyvinyl chloride (PVC) tubing using a roller pump model and thereafter measured adhesion to cultured microvascular endothelial cells and gelatin-coated surfaces. Recirculation-induced neutrophil priming or exhaustion was tested by boosting with phorbol myristate-acetate (PMA) or N-formyl-methionyl-leucyl-phenylalanine (FMLP) before quantification of adhesion.

Results and conclusion: After recirculation, neutrophils retained their adhesive capability to vascular endothelial cells, whereas adhesion to gelatin increased. This increase was not seen when the neutrophils were recirculated with a rotator instead of a roller pump, indicating that not only the pump mode but also foreign surface contact was of significance. The neutrophil PMA response after recirculation was not altered compared to resting neutrophils prestimulated with PMA. Recirculated neutrophils adhered less to cultured vascular endothelial cells after FMLP activation and more to gelatin compared to resting neutrophils prestimulated with FMLP. It is conceivable that dysregulation of neutrophil adhesive capability may play a part in the development of tissue damage after CPB.

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1. Introduction

Cardiopulmonary bypass (CPB) can provoke a severe inflammatory response inducing postoperative complications encompassing myocardial dysfunction, respiratory failure, coagulopathy, renal insufficiency, and neurocognitive dysfunction [1,2]. The etiology is multifactorial with contact between blood and the synthetic material of the extracorporeal circuit participating as well as endothelial and leukocyte activation related to endotoxemia, ischemia-reperfusion injury, and tissue damage, especially the pericardial wound [3–5]. Even though postoperative morbidity and mortality after CPB have been declining, they are still significant [6,7]. Among blood cells, neutrophils are believed to be the central effectors of the inflammatory response to CPB. They interact with the endothelium, transmigrate, and release toxic substances in the extracellular tissue.

It is well known that neutrophils exposed to CPB become activated. Whether they retain their ability to react normally to later stimuli or in reality are exhausted, however, is not known. A potential downregulation of neutrophil function could be a risk factor in the development of postoperative infections but at the same time could limit the consequences of the inflammatory reaction. In vivo studies of superoxide production, degranulation, and neutrophil apoptosis in patients undergoing CPB concluded with neutrophil hyperresponsiveness [8,9], but interactions with endothelium were not studied.

Biomaterial-induced neutrophil activation results in increased surface expression of adhesive receptors as the β2 integrin Mac-1 (CD11b/CD18) [10,11] and members of the immunoglobulin superfamily such as intercellular adhesion molecule-1 and 3 (ICAM-1 and 3) [12]. These changes, as well as L-selectin shedding, promote firm ligation between neutrophil receptors and ligands on the activated vascular endothelium or the biomaterial [13,14]. Adhesion is a crucial step in neutrophil activation and further induces degranulation of proteolytic enzymes mediating tissue damage as well as production of toxic reactive oxygen species [15,16]. There is a continuous release of neutrophils in vivo from the bone marrow during CPB and the first period thereafter.
They immediately become exposed to the biomaterials if still present as well as to the already released cytokines and activated endothelium, and their function may become deranged. When focusing on the biomaterial-related effects, neutrophils that become released during and after the period with extracorporeal circulation in vivo can obscure data and complicate interpretation. Thus, in vitro models may be helpful in understanding this phase of events.

To further clarify neutrophil function after biomaterial exposure we performed an in vitro study recirculating isolated neutrophils resuspended in heparin-anticoagulated plasma in polyvinyl chloride (PVC) tubing with a roller pump. Based on the hypothesis that neutrophil-adhesive properties would be deranged following recirculation, neutrophil adhesion was measured to cultured microvascular endothelial cells (HMEC-1) and gelatin-coated surfaces representative of collagen in the extracellular matrix. To further investigate whether recirculated neutrophils were exhausted by contact with the biomaterial or primed and could subsequently be boosted we also tested adhesion after subsequent treatment with phorbol myristate-acetate (PMA, positive control for intact intracellular signaling) or N-formyl-methionyl-leucyl-phenylalanine (FMLP, clinically relevant activator).

2. Materials and methods

2.1. Isolation of neutrophils

Neutrophils were isolated from fresh buffy coats from informed volunteer donors (Blood Bank, St. Olav’s Hospital, Trondheim, Norway) as previously described [17]. Isolated neutrophils were used as pilot assays employing whole blood gave too little neutrophil adhesion to differentiate between interventions in the model, especially during static conditions. For resuspension of neutrophils, pooled heparin-anticoagulated plasma from four donors was used, final heparin-concentration being 3 IE/ml (Leo Pharma, Ballerup, Denmark). The use of pooled plasma permitted employment of the same batch in all experiments, removing a potential source of variation. Neutrophil isolation and storage were performed at 4 °C to avoid in vitro activation.

2.2. Experimental model

Neutrophils were recirculated in a simplified model using a roller pump (MINI-Z with a two-roller system, MULTIFIX MC 1000 FEC, Schwäbisch Gmünd, Germany), as previously described [11] to simulate the influence of CPB before being tested in the adhesion assays described below. Isolated neutrophils at a final physiological concentration of \(2 \times 10^9/l\) were recirculated in polyvinyl chloride tubing with inner and outer diameter of 2 and 4 mm (Peristaltic pump tubing: Tygon R-3603, Saint Gobain, France) for 30 min. The flow rate was 85 ml/min resulting in a high shear rate of 1800/s and a flow velocity of 0.45 m/s, representative of CPB in vivo. As a control for the roller pump, neutrophils were also recirculated in PVC tubing at 0.132 m/s by a rotator (MX2, Dynal, Oslo, Norway) for 30 min, thereby exposing them to biomaterial without the physical manipulation caused by the pump. After recirculation, the tubing was emptied by gravity and the neutrophils were plated onto tissue culture plates for adhesion assays as described below. Control neutrophils kept with slight agitation on ice to avoid activation before the adhesion assays were also included. Fig. 1 outlines the various stages of the experiments in comparison with the clinical CPB setting.

2.3. Neutrophil adhesion assay

Neutrophil adhesion to cultured microvascular endothelial cells was compared with adhesion to gelatin simulating the extracellular matrix protein collagen. To this end, 96-well tissue culture plates (Becton Dickinson, Le Pont De Claux, France) were either pre-coated with gelatin, or microvascular endothelial cells (HMEC-1, a kind gift from Center for Disease Control and Prevention, Atlanta, GA, USA) were grown to confluency in the plates in Endothelial Basal Medium (Clonetics, Cambrex, New Jersey, USA) with Epidermal Growth Factor 10 ng/ml (BD Biosciences, San Jose, CA, USA), Hydrocortisone 1 μg/ml (Sigma Chemical Company, St. Louis MO, USA) and fetal bovine serum 10% (PAA Laboratories, Pasching, Austria). Each experiment was performed with neutrophils from recirculation plated onto two similar plates incubated in parallel at 37 °C for 30 min, one under static conditions and the other at 100 rpm rotation (Aerotron incubator, Infors HT, Bottingen, Switzerland), resulting in a representative shear stress of 1.25 dynes/cm² [18]. Static incubation was included as it allows testing of integrin function when selectins have been shed from the cell surface. All interventions were tested in triplicates. After incubation, the wells were washed three times with phosphate-buffered saline with 0.3% human serum albumin (Octapharma, Lachen, Switzerland). Adherent neutrophils in each well were solubilized with 100 μl hexadecyl-trimethyl-ammonium bromide 0.5% in sodium-phosphate buffer (pH 6) for 30 min. Myeloperoxidase released in the supernatants was quantified by tetramethylbenzidine oxidation as previously published [19,20] and the optical density (OD) was determined at 620 nm (Multiscan RC plate reader, Thermo Labsystems, Helsinki, Finland).

In the second part of the study neutrophils were again first recirculated as described with the roller pump or the rotator
for 30 min. The tubing was then emptied by gravity and the neutrophils were plated onto microvascular endothelial cells or gelatin-coated plates as described above. To test the responses of further stimulation the plated neutrophils were incubated for 30 min at 37 °C after addition of 40 ng/l PMA or 10⁻⁷ M FMLP, both from Sigma. As a control for the effect of recirculation in the tubing, resting neutrophils were incubated with PMA or FMLP for 15 min at room temperature before plating onto gelatin 3% or microvascular endothelial cells. Unstimulated control neutrophils were also included, and the adhesion assay was performed as described above.

2.4. Statistics

Nonparametric statistics were used due to non-normal distribution of variables and few observations. Results are reported as median ± range as a 95% confidence interval can only be calculated with six or more observations. The p-values < 0.05 were considered statistically significant. For comparisons of parallel interventions within a series of experiments, Friedman’s nonparametric analysis of variance was employed [21]. SPSS-PC software version 13 was used for statistics (SPSS-PC software, Chicago, Illinois, USA).

3. Results

3.1. Neutrophil adhesion after recirculation

Recirculation of neutrophils with the roller pump increased their adhesion to gelatin-coated plates (p < 0.05) compared to neutrophils that had been resting at 4 °C or recirculated by the rotator (Fig. 2). Adhesion to endothelial cells was comparable for all three interventions. These results were similar under static and nonstatic conditions.

3.2. Neutrophil adhesion after recirculation and activation with PMA

Neutrophils recirculated with the roller pump or rotator and further stimulated with PMA adhered to gelatin-coated wells or microvascular endothelial cells to a similar extent as control neutrophils pre-stimulated with PMA (Fig. 3A). Adhesion of unstimulated control neutrophils was comparable both to gelatin and endothelial cells under static and nonstatic conditions, whereas the activated cells adhered to a much greater extent to gelatin than to endothelial cells. There were no differences between static and nonstatic conditions.

3.3. Neutrophil adhesion after recirculation activation with FMLP

Neutrophils recirculated with the roller pump and further stimulated with FMLP adhered to a larger extent to gelatin-coated wells than did control neutrophils prestimulated with
FMLP or unstimulated control cells, both under static and nonstatic conditions \((p_{\text{static}} < 0.05, p_{\text{nonstatic}} < 0.01)\) (Fig. 3B). Neutrophil adhesion to gelatin under nonstatic conditions increased after recirculation with the rotator and subsequent FMLP stimulation. In contrast, neutrophils recirculated with the rotator and then stimulated with FMLP adhered to a smaller extent to microvascular endothelial cells under both conditions \((p < 0.05)\). Adhesion of neutrophils under static conditions also decreased after recirculation with the roller pump and further FMLP stimulation \((p < 0.05)\).

The only increase in adhesion to microvascular endothelial cells compared with unstimulated control cells was observed for control neutrophils pre-incubated with FMLP \((p < 0.05)\).

4. Discussion

The present study demonstrated that neutrophils retained their ability to adhere to microvascular endothelial cells and increased their adhesiveness to gelatin both under static and nonstatic conditions after being recirculated with a roller pump. The increase in neutrophil adhesion to gelatin was not found after biomaterial contact without the physical trauma of the roller pump. Neutrophil responsiveness to PMA was unaffected by previous recirculation in simulated CPB or biomaterial contact. On the other hand, FMLP further increased neutrophil adhesion to gelatin after recirculation in the roller pump both under static and nonstatic flow conditions, whereas adhesion to microvascular endothelial cells decreased after recirculation with the roller pump or biomaterial contact.

The interactions between biomaterial used in the extracorporeal circuit and neutrophils have been the object of thorough investigations both in vivo and in vitro to clarify the underlying mechanisms involved in induction of the inflammatory reaction \([4,8–10,22]\). As neutrophil adhesion to intact or damaged microvascular surfaces is a major event leading to neutrophil-related organ damage and postoperative complications, we focused on this function. To investigate whether biomaterial contact during CPB induces neutrophil dysfunction, models including relevant factors are useful as they enable focusing on selected aspects of an otherwise extremely complex situation. We employed a simplified in vitro model including a roller pump and representative biomaterial to better simulate the flow dynamics and physical trauma of CPB than with a rotator only. Heparin-anticoagulated plasma was selected as heparin is the most commonly used anticoagulant in CPB even though it has complex pro- and anti-inflammatory effects, affecting both the complement system and neutrophils.

The study has certain limitations as only one level of shear stress and temperature was used and a short incubation time. Omission of an oxygenator is another important difference from clinical CPB. Since the adhesion assays necessitated use of isolated blood cells, the data must be interpreted with caution, acknowledging that the lack of red blood cells can reduce neutrophil margination in the tubing and that omitting platelets decreases neutrophil activation \([11]\). In combination, the above mentioned limitations expectedly tend to reduce the inflammatory responses observed. The fact that the recirculated neutrophils showed an exaggerated response to FMLP stimulation is in keeping with previous data demonstrating hyperresponsiveness with respect to superoxide production and degranulation in patients undergoing clinical CPB \([8]\). Adhesion to biomaterial was not measured in that study.

During CPB, neutrophils are exposed to intact, damaged, and denuded vascular endothelium as well as the biomaterial. After endothelial transmigration, neutrophils migrate in the extracellular spaces along various matrix components. Exposure to extracellular matrix proteins was imitated by gelatin-coated wells as collagen is a heterogeneous mixture of polypeptides that can be denatured to gelatin. Neutrophils were clearly more adhesive to gelatin after recirculation by the roller pump than after exposure to biomaterial with circulation by the rotator. We have earlier shown that the unphysiological manipulation neutrophils were subjected to by the roller pump in the model induced degranulation and increased expression of the adhesion molecule. Simultaneously, \(\zeta\)-selectin was removed from the neutrophil membrane \([11]\). Mac-1, the CD11b/CD18 heterodimer, is an important cell-surface receptor for extracellular matrix proteins like collagen. \(\zeta\)-Selectin plays a central role in neutrophil rolling on endothelium before firm adhesion during normal blood flow. This function may be less important in neutrophil adhesion to gelatin as no decrease in adhesion was found under nonstatic conditions.

Recirculated neutrophils were also found to have retained their capability to increase adhesion to gelatin after PMA stimulation. PMA activates through a member of the protein C kinase family and thereby bypasses membrane receptors, supporting that the intracellular signaling pathways remained intact. On the other hand, FMLP interacts with a G-protein-linked membrane receptor, and is a good model for activation in the clinical setting. Neutrophils recirculated with the roller pump showed increased adhesion after FMLP stimulation, consistent with priming caused by changes in surface receptor number or avidity. The same tendency was observed after contact with biomaterial using the rotator, although to a lesser degree, perhaps because of great individual variation in the response. Again, the presence or absence of flow during adhesion assays seemed to be of little importance.

Altogether, differences in neutrophil adhesion to the cultured microvascular endothelial cells were much more subtle. Neutrophils recirculated by the roller pump behaved like the unstimulated neutrophils in their ability to adhere to the endothelial cells regardless of incubation under static or nonstatic conditions. Furthermore, neutrophils recirculated by the roller pump and stimulated with PMA or FMLP neither increased nor decreased their adhesiveness to endothelial cells as opposed to the nonrecirculated neutrophils stimulated with PMA or FMLP. This indicates a functional hyporesponsiveness that was not seen in the interaction with gelatin, perhaps because of the involvement of different sets of adhesion molecules. One could hypothesize that the necessary endothelial counter receptors were missing. However, this was unlikely, as a previous study has shown that there is substantial complement activation in the model \([11]\). Both complement and PMA are well known endothelial cell activators.
5. Clinical implications and conclusions

The present findings indicate that neutrophils exposed to simulated CPB or biomaterial lose some of their ability to adhere to microvascular endothelial cells and that their response to later activation in the vasculature may be reduced. On the other hand, the study indicated that neutrophils exposed to CPB that further get in contact with the extracellular matrix, e.g. because of endothelial damage, adhere to a larger extent and respond with increased intensity to subsequent stimuli. Under such circumstances, neutrophils that have undergone CPB may contribute to aggravating organ damage. The short-lived nature of neutrophils may limit the observed neutrophil dysfunction to the period during and shortly after cardiopulmonary bypass. This is a critical period where the patients are at risk for triggering complications because of extensive damage of myocardial and other tissues, possible bacterial passage from the ischemic gastrointestinal tract to the circulation, a hormonal stress situation, and the systemic inflammatory reaction. We may speculate that the observed neutrophil dysfunction may especially be detrimental in patients undergoing CPB twice in a short time because of the need of a reoperation. If no complications develop during the first critical period, the intense neutrophil release from the bone marrow gradually normalizes the situation at the same time as the inflammatory mediators disappear and the vascular endothelium regains its normal function.

In conclusion, one may speculate that a certain down-regulation of neutrophil-adhesive function is favorable in the early period after CPB and may contribute to explaining why most patients do not develop serious organ dysfunction. In patients where other factors contribute to more extensive endothelial damage, the balance may tip toward increased neutrophil-related damage. Thus, CPB-related neutrophil dysfunction is complex and novel methods to reduce it are warranted.

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References


