Neutrophil-endothelial cells modulation in diabetic patients undergoing coronary artery bypass grafting

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Abstract

Objective: Diabetes mellitus is a well-known risk factor in patients undergoing coronary artery bypass grafting. Myocardial and pulmonary injury often occurs after cardiopulmonary bypass (CPB), mediated in part by neutrophil activation and adhesion to endothelial cells. The objectives of the present study are to compare the degree of neutrophil activation and neutrophil-endothelial cells adhesive interactions in diabetic patients after CPB. Methods: Nitro-blu tetrazolium scores, CD11b expression and neutrophil-endothelial cells adhesion were assessed in blood samples from 15 diabetic and 15 control patients who had undergone elective coronary bypass grafting. Blood samples were obtained at baseline, 30 min after beginning CPB, at the end of CPB and 60 min postoperatively. At the same sampling points as above, blood glucose levels were also checked in all patients. Results: Diabetes was associated with a significant basal increase in neutrophil CD11b expression and adhesion to endothelial cells as well as with an increased superoxide anion production. The increased adhesion of diabetic neutrophils persisted by the end of the CPB to 60 min postoperatively independently of the blood glucose levels. Antibodies directed against CD11b and CD18 significantly reduced the degree of neutrophil adhesion observed 60 min postoperatively. Conclusions: These results indicate that diabetes mellitus is associated with an increased neutrophil-endothelial cell adhesion probably mediated by the CD11b/CD18 molecule; this, in turn, might be responsible for the increased risk of postoperative complications observed in diabetic patients undergoing coronary artery bypass grafting. © 1998 Elsevier Science B.V. All rights reserved

Keywords: Neutrophils; Integrins; Diabetes; Endothelium; Bypass

Cardiopulmonary bypass (CPB) has been demonstrated to promote significant activation of polymorphonuclear leukocytes (PMNs) [1,2], that are now well recognized to play an important role in mediating both coronary and pulmonary endothelium injury associated with ischemia and reperfusion [3,4]. Neutrophil adherence likely contributes to such injury by both capillary plugging of the myocardial microvasculature and by the release of reactive oxygen species and proteolytic enzymes [5,6]. Specific ‘adhesion’ molecules on the neutrophil and endothelial cell surfaces directly mediate the adhesion of neutrophils to the endothelium [7]. Several studies indicate that the CD11b/CD18 complex of leukocyte adhesion receptors are involved in the adhesion of chemotactically stimulated normal human neutrophils to no-stimulated endothelial cells [8–10]. Granulocytes from diabetic animals show an enhanced superoxide radical production compared with non-diabetic animals [11], and diabetes has been demonstrated to be associated with exaggerated leukocyte-endothelial cell adhesion response to ischemia-reperfusion [12]. The aim of this study was to evaluate polymorphonuclear performance in 15 diabetic patients undergoing coronary artery bypass grafting (CABG) based on tests of the different cell functions: (1) adhesion molecule expression CD18/ CD11b, (2) adhesion test on endothelial cells, (3) superoxide anion production.

1. Material and methods

1.1. Study patients

Fifteen patients with non-insulin dependent diabetes
(NIDD) and 15 non-diabetic patients scheduled to have non-urgent aorto-coronary bypass grafting represented the subject of the study. Diabetes mellitus was considered present if a patient had been given this diagnosis and was receiving treatment (diet, tablet or insulin). The patients were classified as non-insulin-dependent by clinical history and according to the National Diabetes Data Group [13]. As a control group, 15 non-diabetic patients scheduled for elective coronary bypass and matched for age, weight and body surface to diabetic patients were selected. All patients in both groups had angina on effort on entry and were receiving some combination of β-adrenergic blocking agents, nitrate vasodilators and Ca2+ channel blocking agents. In the diabetes group, eight patients were under insulin treatment and seven were taking oral agents. The two groups were similar in mean age, male dominance, weight and body surface but diabetic patients had a higher proportion of multi-vessel stenoses (NYHA class (Table 1), but diabetic patients had a higher incidence of ischaemic heart disease (CHD) in the past than non-diabetics. The two groups were similar to diabetic patients had a higher proportion of multi-vessel stenoses (NYHA class (Table 1), but diabetic patients had a higher incidence of ischaemic heart disease (CHD) in the past than non-diabetics.

Table 1

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Control (N = 15)</th>
<th>Diabetes (N = 15)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>52.7 ± 2.3</td>
<td>50.3 ± 2.5</td>
<td>NS</td>
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<tr>
<td>Weight (kg)</td>
<td>61.8 ± 1.8</td>
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<td>Body surface (m²)</td>
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<td>Male-female ratio</td>
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<td>Accompanying drugs</td>
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<tr>
<td>β-Blockers</td>
<td>4</td>
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<tr>
<td>Nitrates</td>
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<tr>
<td>Ejection fraction</td>
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<td>0.50 ± 0.04</td>
<td>&lt;0.05</td>
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<tr>
<td>Total CPB time (min)</td>
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<td>100 ± 9.3</td>
<td>NS</td>
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<tr>
<td>Cross-clamp time (min)</td>
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<td>53.6 ± 7</td>
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<tr>
<td>No. of grafts</td>
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<td>2.8 ± 0.3</td>
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CPR, cardiopulmonary bypass; NYHA, New York Heart Association; LIMA, left internal mammary artery.

with pancuronium; analgesia was provided with fentanyl. The pump (Polystan, Vaerlose, Denmark) roller pump and Dideco hollow fibers oxygenator (Mirandola, Italy) was primed with 2000 ml of Ringer’s lactate. The heart was exposed through a median sternotomy, and sodium heparin at a dose of 300 units/kg was administered intravenously before cardiopulmonary bypass (CPB) to produce an activated clotting time greater than 400 s. The hematocrit level was maintained between 20% and 25%, pump flows were maintained between 2.0 and 2.5 l/min per m² during moderate hypothermia (28°C) to maintain a mean arterial pressure between 50 and 70 mmHg. Cardioplegia was achieved with ice-cold St. Thomas solution infused in the ascending aorta. The left ventricle was vented through the aortic root. After completion of the distal anastomosis, the aortic cross-clamp was removed and the aorto-venous anastomoses were performed while the patient was being rewarmed to 37°C. After decannulation, protamine sulfate (10 mg/ml, Lilly) was administered intravenously at a dosage of 1 mg for each 300 units heparin to neutralize the heparin. Throughout the procedure the patients received no exogenous glucose from either the anaesthesiologist or the priming fluid.

1.3. Study protocol

Blood samples were taken from radial artery catheter before induction of anesthesia, 30 min after the start of CPB, at the end of CPB, and 60 min postoperatively. The samples were collected in heparinized cooled syringes that were immediately capped and stored in ice until separation and analysis. Blood samples were assayed for oxygen and carbon dioxide tension, pH, oxygen saturation and glucose level. The study protocol was approved by the Ethics Committee of the Medical School of Catanzaro. Informed consent was obtained from each patient.

1.4. Hemodynamic measurements

From the moment patients arrived in the intensive care unit we monitored systolic, diastolic, and mean systemic and pulmonary arterial pressures, mean left atrial pressure, systemic vascular resistance, and cardiac output every 4 h until 24 h after arrival. We calculated left ventricular stroke work index and cardiac index using standard formulas. Hemodynamic instability was defined as mean arterial blood pressure less than 70 mmHg requiring dopamine support for stabilization.

1.5. Neutrophil isolation

Neutrophils were isolated using Ficoll–Hypaque density gradient centrifugation, dextran sedimentation and hypotonic lysis of erythrocytes which was conducted to remove red cells. Neutrophils were suspended in Hanks’ balanced salt solution, free of phenol red, Ca²⁺ and Mg²⁺ and contained 0.25% bovine serum albumin. The final cell prepara-
tion contained 98 ± 2% neutrophils. The neutrophils were then maintained on ice in Hanks’ balanced salt solution at 1–5 × 10⁶ cell/ml until usage. Isolated PMN were >99% pure as assessed by Wright’s stained cyt centrifuge preparation and >99% viable as assessed by exclusion of Trypan blue.

1.6. Neutrophil CD11b expression

Neutrophil CD11b expression was detected by indirect immunofluorescence and flow cytometry. Briefly, 1 ml of blood was drawn from the oxygenator into heparinized syringe that was kept at room temperature. 100 μl samples of blood were aliquoted in polypropylene tubes and stained anti-CD11b mAb CBL145 (Cymbus Biosciences, Hants., UK) as a primary reagent and fluorescein isothiocyanate-conjugated goat anti mouse immunoglobulin (Cymbus Biosciences, Hants., UK) as the secondary reagent. Controls included cells stained with the second reagent alone and cells stained with an irrelevant isotype-matched control mAb. No other primary mAbs were employed. Red blood cells were removed by hypotonic lysis. Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA). The granulocyte population was identified by their forward and orthogonal light scatter characteristics. Green and red amplifier gains were calibrated with beads before each experiment (Flow Cytometry Standards, Research Triangle Park, NC) to ensure that relative fluorescence values were comparable between experiments. The mean fluorescence for each specimen was calculated from the fluorescence distribution (5000 events) using the FACScan Research Software, version B (Becton–Dickinson). In the flow cytometry studies, the logarithmic mean fluorescence values obtained from the histograms were converted mathematically into a relative fluorescence value and expressed as percent increase over the observed baseline values.

1.7. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords by the method of Jaffe [15]. HUVECs were placed in 75 mm plastic culture flasks and grown in Medium 199 supplemented with 20% fetal bovine serum, 25 μg/ml of endothelial cell growth supplement and 90 μg/ml of heparin (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 95% air and 5% CO₂ atmosphere. All media were prepared with endotoxin-free water and filtered with Zetapore Filters (Cuno Life Science, Meriden, CT). Endotoxin-free plasticware and glassware were used in all experiments. For experimental studies, confluent HUVEC monolayers (passage 3–7) were trypsinized and replated on 48-well plated (Costar, Cambridge, MA) precoated with a sterile solution of 1.5% gelatin and grown to confluence for neutrophil adhesion studies. HUVECs were grown to confluence and used within 72 h. The identity of some endothelial cultures was checked by indirect staining with fluorescein isothiocyanate-labeled factor VIII antibody (polyclonal IgG, Sigma–Aldrich, Milan, Italy).

1.8. PMN adherence assay

PMN were fluorescently labeled with a hydrophobic fluorescent compound (3,3’-dioctadecyloxocarbocyanine perchlorate (DiO) (Fluka, Sigma–Aldrich, Milan, Italy) as described by Lo et al. [9]. Cells at 1–5 × 10⁶ cells/ml were incubated with 50 μg/ml DiI in HAP buffer for 10 min at 0°C, unbound dye was removed by three washes with HAP buffer, and labeled PMNs were resuspended in Medium 199 for the adhesion assay. DiO labeled PMNs (10 μl of 10⁴ cells/ml) were added to twice-washed EC monolayers. Adhesion was allowed to proceed for 15 min at 37°C, and unbound PMNs were removed by three washes with M199. Residual adherent PMNs on EC surfaces were counted manually, in a blinded fashion, on an inverted microscope equipped for fluorescence using the filter IF355–550 at a magnification of 100×. To examine the role of neutrophil integrins in endothelial adhesion, in another series of experiments, neutrophils from blood drawn 60 min postoperatively were preincubated for 15–20 min with blocking mAbs 44a (anti-CD11b, 10 μg/ml) and IB4 (anti-CD18, 10 μg/ml), both provided from Dr. P.F. Tassone (Dept. of Oncology, Medical School of Catanzaro, Italy). Neutrophil-endothelial adhesion was then assayed as above.

1.9. Nitro-blu tetrazolium

One specific form of neutrophil activation is the capacity to reduce nitro-blu tetrazolium (NBT) dye, which is associated with an enhanced generation of superoxide anion radicals. This is the pivotal phenomenon of the respiratory burst after neutrophil activation. The NBT test was performed on fresh blood as previously described [16]. In brief, 0.2 ml heparinized blood were incubated at 37°C for 10 min. Then, 0.2 ml of a 0.1% solution of NBT in phosphate-buffered saline was added. The mixture was incubated at 37°C for 15 min and then kept at room temperature (21–25°C) for another 15 min. Smears were prepared by allowing a drop of the NBT and blood mixture to rapidly run down a pre-cleaned slide. After air drying, the smears were stained by Wright’s stain. On each slide, 100 neutrophils were counted. The percentage of neutrophils containing formazan deposits of at least the size of a lobe of the nucleus was designated as positive NBT score.

1.10. Statistics

All values are expressed as mean (SE). Comparison between and within groups was made using two-way analysis of variance for repeated measures followed by Scheffe test for multiple comparison. Relationship between inde-
dependent variables was assessed by linear regression analysis. Statistical significance was set at $P < 0.05$.

2. Results

There were no deaths and all patients are alive and well. The median number of grafts per patient performed in the diabetic group and the control group was 3.4 and 3, respectively ($P = NS$). No significant difference was observed in the diabetic groups when compared with matched controls with regard to the mean total pump run and mean cross-clamp time (Table 1). Four patients in the diabetic group and two in the control group required administration of dopamine for mild low cardiac output state ($P = NS$). No significant difference was found between the two groups in terms of postoperative blood loss through the thoracic drains and the duration of ventilatory assistance during the permanence in intensive care unit.

2.1. Glucose variations during CPB

All patients in the control group were normoglycemic before induction of anesthesia, whereas, at the corresponding time, the diabetic patients were slightly hyperglycemic (151 mg/dl, range 115–183 mg/dl). At the initiation of operation there was a slight increase in blood glucose levels in all two groups, but the values did not deviate significantly from the baseline values. During the course of CPB and throughout 60 min postoperatively, both groups exhibited a significant increase in blood glucose level compared with baseline values. Despite insulin infusion, blood glucose levels were significantly higher in the diabetic patients at any sampling point during and after CPB (Fig. 1).

2.2. NBT

The values of NBT scores are shown in Fig. 2. The baseline superoxide anion production by PMNs from diabetic patients was significantly higher than that of controls (6 ± 0.36% and 3.8 ± 0.25% respectively; $P < 0.05$). Through the CPB course, there was a progressive increase of superoxide anion production, which peaked in both groups at the end of CPB (control 13.6 ± 0.72% $P < 0.01$ vs. baseline; diabetic 14.4 ± 0.77%, $P < 0.01$ vs. baseline). In both groups, values of NBT scores although tending downward, were still significantly higher compared with baseline values 60 min postoperatively. When analyzed by ANOVA, no significant differences were found between groups at any sampling point during and after CPB.

2.3. Neutrophil CD11b expression

Fig. 3 shows neutrophil CD11b expression in control and diabetic patients. Under basal condition, CD11b expression was significantly greater in neutrophils from diabetic patients compared with controls value (mean channel fluorescence: control = 79, diabetes = 94, $P < 0.05$). A significant increase over baseline value in CD11b expression was observed in both groups throughout the whole experimental period, with peak values at 60 min postoperatively. However, when analyzed by ANOVA, no significant difference in neutrophil CD11b expression was observed between the two groups at during and after CPB.
2.4. Neutrophil-endothelium adhesion

Values of five replicates from each circuit were averaged, and the coefficient of variations between replicates were small (<10%). Under basal conditions, neutrophils from diabetic patients showed a greater adherence to vascular endothelium than those from control patients (6.8 ± 0.36% vs. 4.3 ± 0.3%, \( P < 0.05 \)). Compared with the baseline values, a significant increase of neutrophil-endothelial adherence was observed in both groups throughout the experimental period, with peak values at 60 min postoperatively (control \( P < 0.01 \) vs. baseline; diabetic \( P < 0.01 \) vs. baseline). When the values between the two groups were compared, a significant difference in PMNs adherence was observed at the end of CPB and 60 min postoperatively (Fig. 4). When analyzed by linear regression, no significant correlation was found between PMN adherence to ECs and either blood glucose levels or CD11b/CD18 expression.

2.5. Blocking mabs to CD11b and CD18

Fig. 5 summarizes the effects of mAbs directed against CD11b and CD18 adhesion molecules on PMNs adherence 60 min after the end of CPB. MAbs against CD11b reduced leukocyte adherence by 60.7% and 64.2%, respectively, in the control and diabetic group, whereas MAbs against CD18 reduced leukocyte adherence by 80.1% and 83%, respectively, in the two groups. No significant difference in adherence was observed between the two groups after neutrophil treatment with mAbs.

3. Discussion

Diabetes mellitus is a well-known risk factor in the patients undergoing coronary artery bypass grafting [17,18] and with increasing frequency diabetic patients are referred for CABG [19]. Although the results of many studies suggest that CABG is a safe operation for the diabetic patient [19–21], from the fact that alteration of the neutrophil morphology and functions have been described in patients with diabetes [11,22,23], the presence of a hyperglycemic state might be expected to increase the risk of post-operative complications. Granulocytes isolated from diabetic animals and humans are less deformable [23] and generate larger quantities of toxic oxygen radicals [22] than granulocytes isolated from non-diabetics. Moreover, a larger percentage of the circulating neutrophils are activated in human diabetics compared with control populations [24]. In an animal model of ischemia-reperfusion, Panes et al. [12] demonstrated that diabetes mellitus was associated to an exaggerated inflammatory response manifested as a greater accumulation of adherent and emigrated leukocytes and a larger increase in albumin extravasation.

In the present study we evaluated the abilities of PMNs from 15 non-insulin-dependent diabetes mellitus subjects undergoing coronary artery bypass to produce the \( \text{O}_2^- \) anion, to express superficial adhesion molecules and adherence of cell to the vascular endothelium. The results of the present study, accordingly with those of other reports, confirm the occurrence of PMN activation during CPB, as evidenced by an enhanced production of superoxide, and an increased PMN adhesion to endothelial cells. Moreover, our data suggest that neutrophils in diabetic patients are in a sort of pre-activated state, consisting in both increased basal \( \text{O}_2^- \) production and adherence to vascular endothelium. Under basal conditions, superoxide production by PMNs, as expressed by NBT score, was in fact significantly greater in the diabetic groups compared with controls. However, during the time course of the study, an increased superoxide production was observed in all groups, and the peak of \( \text{O}_2^- \) production at 60 min postoperatively by PMNs of diabetic patients was comparable with that of PMNs from control subject, with a percent increase over baseline values greater in control group compared with diabetics. Extracorporeal circulation caused a significant increase in the number of adherent leukocytes in both diabetic and non-diabetic groups, and leukocyte adhesion after reperfusion were significantly higher in diabetic than control patients. Interestingly, the neutrophil adhesion to endothelium was enhanced under basal condition in diabetic patients compared with
controls, and this increased adhesion, except 30 min after CPB beginning, persisted throughout the whole sampling period independently of the glycemic levels. Another interesting finding of this study is that diabetics are associated with an increased baseline level CD11b/CD18 leukocyte expression. Although we could not demonstrated any significant correlation between CD11b/CD18 upregulation and basal PMN adherence, nevertheless, a substantial role for this integrin as a determinant of CPB induced leukocyte adherence can be inferred from our experiments with blocking mAbs. When PMNs of patients from both groups were incubated with blocking antibodies against Cd11b/CD18, the number of neutrophils adhering to endothelial cells diminished significantly, and no difference was observed between the two groups at any sampling point.

The mechanisms by which hyperglycemia could activate CD11b/CD18 induced binding has not been evaluated in this study. However, recent studies suggest that the increased expression of neutrophil integrins per se could be not sufficient to explain the higher neutrophil-endothelial adhesion observed in the diabetic patient. A number of potential mechanisms have been described that may explain the increase in leukocyte accumulation in diabetic vessels. These effects are an indirect effect of glucose on proteins or cells such as platelets. However, the direct effect of elevated glucose on endothelial neutrophil interactions has not been determined. There are several possible explanations for the effects of hyperglycemia on neutrophil-endothelial interactions, including changes in arachidonic acid metabolism [25], non-enzymatic glycation of proteins [26], increases in diacylglycerol and protein kinase C activity [27,28], and reduction of endothelial cell nitric oxide activity [29]. Finally, glucose can cellular dysfunction by increasing free radical formation [30].

In conclusion, our study demonstrated that PMNs from diabetic patients produce a large amount of O2 reducing more efficiently NBT, and present an upregulation of CD11b expression. Correspondingly, there is an intrinsic adhesive dysfunction of diabetic neutrophils that enhanced adherence to endothelium and which might predispose diabetic patients undergoing cardiovascular bypass to an increased risk of post-operative complications.

3.1. Limitations of the study

Our conclusions are limited in that, in the present study, changes of neutrophil adherence were studied in an experimental model of cultured HUVEC. At this regard, at least two considerations are mandatory. Firstly, although the adherence assay that we used is very reliable with regard to that specific vascular bed, nevertheless, it should be emphasized that it is not possible to affirm whether similar changes also occur with systemic endothelium. Therefore, further examination of endothelium from other clinically important vascular beds might be worthwhile.

Secondly, once microvascular cells from any specific organ are placed into culture and passaged, the differences between cells of one organ and another may become blurred, as cell phenotypes change in response to the in vitro conditions during passage. Moreover, concerns that isolation procedures, particularly the effect of the temperature, may alter neutrophil function have been reported in literature [31,32].

Finally, it must be stressed that isolated neutrophils used in this study have lost their physiological environment.

References

[16] Chello M, Mastroroberto P, Celvi V, Romano F, Marchese AR, Colonna A. Reduction by indobufen of neutrophil activation in per...


