Can preoperative modelling of individual neutrophil adhesion responses predict renal morbidity?§

David G. Healy a,c,*; Alfred E. Wood a,c; Amanda O’Neill b,c; James F. McCarthy a; John M. Fitzpatrick b,c; R. William Watson b,c

a Prof Eoin O’Malley National Centre for Cardiothoracic Surgery, Mater Misericordiae University Hospital, Eccles St, Dublin 7, Ireland
b Department of Surgery, Mater Misericordiae University Hospital, Ireland
© Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland

Abstract

Objective: Perioperative upregulation of the neutrophil adhesion molecule CD11b is associated with the development of renal impairment. We hypothesised that individual variation in neutrophil adhesion molecule responses to surgery influences renal outcomes and that this individual variability could be modelled prior to surgery and used to predict high risk patients. The developed model uses preoperative exposure of an individual patient’s neutrophils to a fixed inflammatory stimulus and assessment of the basal and stimulated adhesion molecule CD11b expression.

Methods: Neutrophils were isolated from human volunteers undergoing cardiac surgery with cardiopulmonary bypass support. Basal and stimulated CD11b expression was measured using flow cytometry in preoperative neutrophil samples and compared to postoperative clinical performance. Results: Patients with low levels of preoperative basal neutrophil CD11b expression had the greatest increase in CD11b following phorbol-12-myristate-13-acetate stimulation. This stimulated CD11b response correlated with changes in CD11b expression from preoperative to postoperative sampling. Preoperative basal CD11b expression showed an inverse relationship with postoperative renal function. However, preoperative CD11b stimulation was not related to postoperative renal function. In addition, preoperative basal CD11b expression correlated with adrenaline requirements and intra-aortic balloon pump usage. In contrast, stimulated CD11b expression was significantly related to length of hospital stay and changes in the A-a gradient. Conclusions: Preoperative CD11b expression assessment might enable preoperative identification of patients who will mount an exaggerated and damaging neutrophil response to surgery which contributes to renal injury. Identification of these patients would then allow selective application of immunomodulatory therapies.

Keywords: CPB; Inflammatory response; Inflammatory cells; Outcomes; Cardiac; Surgery; Complications

1. Introduction

Cardiac surgery is associated with operative immunological activation which contributes to adverse postoperative outcomes [1]. The neutrophil is of particular importance in mediating this damaging operative inflammatory response. The use of extracorporeal circulatory support causes neutrophil activation and the degree of neutrophil activation has been associated with increased hospital stay [2]. Cardiac surgery disrupts organ perfusion and neutrophils are major contributors to the tissue damage resultant from ischaemic reperfusion events [3]. Neutrophil-mediated tissue damage results from the release of oxygen free radicals and proteolytic enzymes produced on activation [4]. The amount of tissue damage is influenced by the number of neutrophils infiltrating the inflammatory tissue, which is determined by the rate of transendothelial migration. Neutrophil transendothelial migration is a three-stage process of selectin-mediated rolling and loose adhesion, followed by integrin-mediated firm adhesion and finally transendothelial migration. One of the important integrins in firm adhesion is the CD11b/CD18 complex.

Previous publications report that perioperative upregulation of neutrophil CD11b is associated with postoperative renal impairment [5]. Acute renal failure is an important contributor to prolonged intensive care stay and patients requiring renal replacement therapy following cardiac surgery have at 42% mortality [6,7]. We hypothesised that individual variation in neutrophil adhesion molecule response to surgery influences the development of renal impairment following cardiac surgery and that this individual variability could be modelled prior to surgery and used to predict renal injury. To date there have been no attempts to model the neutrophil adhesion molecule response to immunological stimulation in patients prior to undergoing cardiac surgery.

doi:10.1016/j.ejcts.2007.02.029

© 2007 European Association for Cardio-Thoracic Surgery. Published by Elsevier B.V. All rights reserved.
The model we have developed uses preoperative exposure of individual patient neutrophils to a fixed inflammatory stimulus and measurement of the basal and stimulated adhesion molecule CD11b response. This might enable preoperative identification of patients who will mount an exaggerated and damaging neutrophil response to surgery and who are likely to benefit most from selective application of immunomodulatory therapies.

2. Materials and methods

2.1. Patient selection and sample collection

The study was approved by the Mater Misericordiae University Hospital ethics committee. Twenty-four patients undergoing elective cardiac procedures were prospectively recruited and written informed consent obtained. Patients with evidence of infection were excluded. Venous blood samples were obtained on the day prior to surgery with subsequent sampling performed in an initial nine patients at the following time points: release of cross clamp (time = 0 h, +1 h, +6 h, +12 h and +24 h). The preoperative samples were processed immediately and these data were not available to the operative surgeon or the staff caring for the patient in the postoperative period. Data on differential white cell counts generated with an automated analyser were obtained on the sample taken the day prior to surgery. Fifteen patients underwent isolated coronary artery bypass grafting (CABG). One of the isolated CABG cases had undergone previous CABG surgery. In five patients coronary artery bypass grafting was performed with additional procedures: one aortic valve replacement, one mitral valve repair, one mitral valve replacement, one aortic root repair and one Dor procedure. In addition there were three isolated aortic valve replacements and one isolated mitral valve replacement. Left internal mammary grafts were used in 17 of the 20 CABG cases. In the four aortic valve replacements, a homograft was used in one case, a biological (Edward’s tissue valve) was used in one case and mechanical (St Jude) prosthesis used in two. In the mitral valve replacement cases a St Jude device was used in one and a Sorin device in the other. The mitral valve repair was performed with the use of an annuloplasty ring (MRS-Kohler-Chemie) and Cox Maze procedure was also performed. Patient profile data are shown in Table 1. There were two deaths prior to hospital discharge. One was a 74-year-old man with a EuroSCORE of 6 who underwent CABG \times 3. He developed a pulmonary embolism postoperatively requiring re-intubation and went on to develop mediastinitis and renal failure. He died 30 days after surgery. The second had a EuroSCORE of 11 and underwent an aortic valve replacement and CABG \times 1. His postoperative course was complicated by renal failure and a methacillin-resistant Staphylococcus aureus lower respiratory tract infection. He died at 53 days. In the first 24 h after surgery, intra-aortic balloon pumps were required in six patients. Twenty patients were extubated within 24 h of surgery and the mean extubation time was 24.6 (±37) h. Seven patients developed new onset atrial fibrillation prior to discharge.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>24</td>
</tr>
<tr>
<td>Male</td>
<td>20 (83%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.2 (±10.9)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>81.1 (±15.4)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>169.5 (±10.3)</td>
</tr>
<tr>
<td>BMI</td>
<td>28.1 (±4.3)</td>
</tr>
<tr>
<td>Current or ex smoker</td>
<td>19 (79.1%)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>18 (75%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7 (29.1%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>17 (71%)</td>
</tr>
<tr>
<td>EuroSCORE (applicable in 20)</td>
<td>5.5 (±3.5)</td>
</tr>
</tbody>
</table>

Data shown with means and standard deviations.

2.2. Perioperative management

Following standard anaesthesia techniques and invasive monitoring, the heart was exposed through median sternotomy. All procedures were carried out on the arrested heart with the aid of cardiopulmonary bypass. A roller pump was used with polypropylene tubing (arterial 3/8", venous 1/2", sump 1/4") and the circuit was primed with 1.2 l Hartman’s solution, 5000 iu heparin and 25 g of mannitol. Leukocyte filtration was not performed. Heparin was administered at a dose of 300 iu/kg and anticoagulation was monitored with an activated clotting time. Once a satisfactory level above 480 s was achieved cannulation and cardiopulmonary bypass were commenced. Pump flows of between 2 and 2.5 l/min/m² were maintained with a mean arterial pressure of 60–70 mmHg and moderate hypothermia (28 °C in 4 patients, 32 °C in 20). Blood cardioplegia was used in all cases. After decannulation, protamine sulphate was administered intravenously at 1 mg per 300 iu of heparin administered. Dual chamber pacing was used in all but one case.

2.3. Neutrophil isolation

Venous blood samples were obtained with 1 ml of 0.106 mmol/l sodium citrate solution per 10 ml of whole blood. Neutrophils were isolated by dextran (3%) sedimentation.
tion and centrifugation through a discontinuous ficoll gradient. Remaining red blood cells were lysed using 0.8% NH₄Cl. Isolated neutrophils at a concentration of 1 × 10⁶ were resuspended in Dulbecco’s modified eagle’s medium supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. Cells were incubated at 37 °C in a humidified CO₂ (5%) incubator in polypropylene tubes to prevent adherence. Neutrophil purity was assessed by size and granularity on flow cytometry and was consistently greater than 95%.

2.4. Neutrophil adhesion marker

Neutrophil cell surface marker measurement was performed on isolated neutrophils as described above. Surface expression of CD11b (Becton Dickinson) was assessed by flow cytometry as previously described [8]. Briefly, 500,000 neutrophils in 500 μl of medium were incubated with 10 μl of antibody at 4 °C for 20 min, washed and analysed by flow cytometry using a Coulter ELITE cytofluorometer and were compared to an unstained control. The preoperative ex-vivo stimulation consisted of treatment of 500,000 neutrophils in 500 μl of medium with phorbol-12-myristate-13-acetate (PMA) 3.2 nmol/l for 20 min at 37 °C. CD11b measurement was then performed as described above. This model was developed from our experience with the established use of PMA as a neutrophil stimulant for respiratory burst activity measurement [9,10]. PMA stimulation results in a significant rise in neutrophil CD11b expression on the cell surface (P < 0.001) and CD11b mRNA expression. The degree of individual response to PMA stimulation was calculated as a percentage of the basal CD11b expression.

2.5. Respiratory burst activity

The generation of reactive oxygen intermediates was assessed as previously described [10]. Briefly, neutrophils were incubated with dihydrorhodamine 123, 1 μmol/l for 10 min at 37 °C and then stimulated with phorbol-12-myristate-13-acetate 3.2 nmol/l for 20 min. Fluorescence intensity was assessed by flow cytometry and expressed as the log normal mean channel fluorescence.

2.6. Quantification of apoptosis

Spontaneous neutrophil apoptosis was quantified after 24 h culture in vitro as the percent of cells with hypodiploid DNA as previously described [10]. Cells were centrifuged at 200 × g for 10 min, then gently resuspended in 500 μl of hypotonic fluorochrome solution (50 μl/ml propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, 0.1% Triton X-100) and stored in the dark at 4 °C before analysis. A minimum of 5000 events were collected and analysed. All measurements were performed under the same flow cytometry settings.

2.7. Clinical outcome parameters

Protocol data were collected for the first 24 h. Peak creatinine measured using automated blood analysers in the first 24 h after surgery was compared with the preoperative measurement. During the first 24 h the usage of adrenaline and noradrenaline was recorded in addition to time to extubation. At 6, 12, 18 and 24 h arterial oxygen (A-a) gradient was calculated. The duration of hospital stay and significant clinical events were recorded.

2.8. Statistical analysis

Correlations among parametric data were performed with a Pearson correlation coefficient. Comparisons between paired samples from the same patient were made performed with a paired t-test. Comparison between two independent groups was made with an independent-sample t-test. Analysis of perioperative CD11b measurement was initially screened with a repeated measures ANOVA test followed by post-hoc analysis with a Dunnett’s test. Data are presented with means and standard deviations.

3. Results

3.1. Neutrophil adhesion molecule expression

Neutrophils isolated from the preoperative sample were stimulated with PMA. This resulted in significant increases in neutrophil CD11b expression after stimulation (Fig. 1, P < 0.001). However, a significant inverse correlation was seen between patients with lower preoperative CD11b expression and the response to PMA stimulation (Fig. 2, R = −0.425, P = 0.038), demonstrating that patients with low basal CD11b expression mount the greatest response to immunological activation with PMA.

Changes in neutrophil CD11b expression are seen in the perioperative period. A significant drop occurs in neutrophil CD11b expression in venous samples between the preoperative measurement and the expression at 24 h following cross clamp release (Fig. 3, n = 9, P = 0.02). Preoperative CD11b expression was not related to age (P = 0.393), or left ventricular function (P = 0.136), and no significant relationship is seen with stimulated CD11b and these variables. Basal (R = −0.74, P = 0.021) and PMA-stimulated (R = 0.696, P = 0.037) CD11b...
expression demonstrated significant relationships with the change in CD11b expression in the first 24 h following surgery. However, there are important differences in their relationships. An inverse relationship is seen between basal CD11b expression and postoperative CD11b expression, with patients with low preoperative CD11b expression demonstrating the smallest drop in CD11b expression over the 24-h period. In contrast a positive correlation is seen between PMA-stimulated CD11b expression and CD11b expression 24 h after surgery. This is, however, consistent with the initial observation that patients with low CD11b basal expression mount the greatest response to PMA stimulation.

3.2. Predictive value of preoperative CD11b on postoperative renal outcome

The mean preoperative creatinine was 91.4 μmol/l (±16.7). Six patients demonstrated a rise of >30% on their preoperative values and two patients required haemofiltration in the postoperative period. Both of these patients died. Preoperative basal CD11b expression demonstrated a significant inverse relationship with postoperative changes in creatinine in the first 24 h (Fig. 4, $R = -0.554$, $P = 0.005$), with patients with lower basal CD11b expression at greatest risk for renal injury. We then evaluated the utility of the neutrophil CD11b stimulation model for predicting postoperative renal injury. There was a significantly higher PMA-stimulated CD11b expression in the patients requiring renal replacement therapy (305 ± 89%) compared to other patients (253 ± 52%) ($P = 0.023$). However, looking at the pattern of creatinine changes across all patients, there was no relationship between PMA-stimulated CD11b expression and creatinine rise following cardiac surgery.

3.3. Predictive value of preoperative basal CD11b expression on other variables

Significant inverse correlations were seen between preoperative CD11b expression and postoperative adrenaline

---

Fig. 2. Neutrophils are isolated from patients prior to surgery. The basal and stimulated CD11b expression is measured using flow cytometry. Stimulated CD11b is measured in neutrophils exposed to 3.2 nmol/l PMA for 20 min at 37 °C. A significant inverse correlation was found between basal and stimulated CD11b expression ($P = 0.038$, $R = -0.425$), showing that patients with the lowest resting CD11b expression mounted the greatest response on immunological challenge with PMA. CD11b is expressed as the mean channel fluorescence. The increase in PMA is expressed as a fold increase of the patient’s basal CD11b expression.

Fig. 3. Neutrophil CD11b expression was measured in patients undergoing open heart surgery with cardiopulmonary bypass support. Samples were obtained on the day prior to surgery and at the following time points: release of aortic cross clamp (time = 0 h), +1 h, +6 h, +12 h and +24 h. A significant fall in CD11b expression is seen from the preoperative level to the 24 h time point (ANOVA $P < 0.01$; Dunnett’s test, $P = 0.02$).

Fig. 4. Neutrophils are isolated from patients prior to surgery. The basal CD11b expression is measured using flow cytometry. A significant inverse correlation was found between basal CD11b expression ($P = 0.005$, $R = -0.554$) and changes in creatinine in the first 24 h after surgery. CD11b is expressed as the mean channel fluorescence. Creatinine changes were calculated by deducting the preoperative level from the highest reading in the first 24 h and expressed in μmol/l.

Fig. 5. Neutrophils are isolated from patients prior to surgery. The basal CD11b expression is measured using flow cytometry. A significant inverse correlation was found between basal CD11b expression ($P = 0.043$, $R = -0.415$) and the amount of adrenaline required in the first 24 h after surgery. CD11b is expressed as the mean channel fluorescence. Adrenaline usage is measured in ml/h/kg of a 3 mg in 50 ml solution.
patients requiring intra-aortic balloon pump placement had significantly lower preoperative CD11b basal expression (48.0 ± 10.4 vs 60.5 ± 9.1; P = 0.01). However, basal preoperative CD11b expression demonstrated no relationship with survival (P = 0.151) and length of hospital stay (P = 0.26). Postoperative noradrenaline requirement (P = 0.546), development of atrial fibrillation (P = 0.637), time to extubation or any postoperative respiratory measurement in the first 24 h was also unrelated to preoperative basal CD11b expression.

3.4. Predictive value of preoperative PMA-stimulated CD11b expression

In relation to length of hospital stay, the PMA-stimulated CD11b response was significantly positively correlated with prolonged hospital stay (R = 0.622, P = 0.001). PMA-stimulated CD11b expression showed a significant inverse relationship with the changes in A-a gradient over the first 6 (R = -0.408, P = 0.047), 12 (R = -0.46, P = 0.023) and 24 h (R = -0.473, P = 0.026) compared to the preoperative A-a gradient. There was no demonstrable relationship between preoperative PMA stimulation, noradrenaline (P = 0.966) or adrenaline (P = 0.640) usage in the first 24 h after surgery, intra-aortic balloon pump placement (P = 0.64) or postoperative development of atrial fibrillation (P = 0.936). The time to extubation was also not associated with PMA-stimulated CD11b expression.

3.5. Predictive value of other neutrophil measurements

No significant relationship was demonstrable among any of the postoperative variables and neutrophil counts, reactive oxygen intermediate production and spontaneous apoptosis rates.

4. Discussion

Neutrophil activation in response to microbial invasion is a desirable response; however, when seen in the sterile inflammatory response to cardiac surgery, this activation contributes to adverse clinical outcomes [11]. Neutrophil activation is associated with an increase in the expression of the CD11b/CD18 adhesion molecule complex [12]. The expression of CD11b in arterial sampling is upregulated with CPB [13] and Rinder et al. have shown that the upregulation of CD11b over the cardiac surgery operative time course is associated with renal injury [5]. A number of approaches have been tested to ameliorate the adverse effects of neutrophil activation in cardiac surgery but have not been successful enough to be widely adopted in clinical practise, although animal data suggest potential benefits [14]. This may reflect individual variation in neutrophil responses. Patients with over-exuberant neutrophil responses could potentially benefit from immunomodulatory therapy, whereas those with less robust responses might suffer higher infection complications and loss of the physiological contribution of inflammation to wound healing. If these groups could be separated prior to surgery, it could allow the application of tailored immune therapies. Prior to this study, modelling the response of a neutrophil to surgery had not been used to predict clinical performance in cardiac surgery.

Our studies have demonstrated a large individual variation in preoperative neutrophil CD11b expression. We hypothesised that higher neutrophil CD11b expression would predispose to transendothelial migration and neutrophil infiltration resulting in tissue damage at an inflammatory site. Instead, we have seen that patients with low initial CD11b expression are the patients with more severe postoperative adverse outcomes. However, we have also observed that patients with low initial CD11b expression were the individuals who were able to mount the greatest increase in CD11b expression on stimulation. This may provide evidence that it is the ability to adapt to an immunological challenge which impacts most on post-cardiac surgery tissue injury. Alternatively, we may be measuring indirectly other co-factors expressed with de novo transport of CD11b to the neutrophil cell surface that influences their ability to cause tissue damage.

An alternative explanation for the association of adverse postoperative consequences with low preoperative CD11b expression may rest in the sampling method chosen in this study. All blood samples chosen here were obtained from venous blood. This was chosen as the most practical blood collection method for the development of a preoperative test. The expression of the adhesion molecule CD11b/CD18 in arterial sampling is increased with CPB [13]. On venous sampling CPB is associated with a significant initial rise in neutrophil CD11b expression, but this later drops to below the preoperative level [15]. This fall in venous neutrophil CD11b in CPB patients is sometimes explained incorrectly as a dilution effect. However, assessing neutrophil CD11b expression in renal artery and veins simultaneously on renal reperfusion demonstrates a fall in CD11b expression from arterial to venous blood which is proportional to neutrophil recruitment into the kidney and is explained by the recruitment and infiltration of the organ by neutrophils strongly expressing CD11b [16].

In this study of open heart cases utilising cardiopulmonary bypass support, we were able to demonstrate a relationship between the perioperative change in neutrophil CD11b expression and a novel preoperative stimulation method using PMA. We demonstrated that markers of neutrophil adhesion potential were significantly associated with clinical correlates. In contrast no such correlations were seen with neutrophil numbers in circulating blood, reactive oxygen intermediate production or neutrophil lifespan. This might suggest that these aspects of neutrophil function are not as relevant to clinical outcomes. However, neutrophil function is altered following transendothelial migration and measurement of these aspects of neutrophil function in circulating blood might not reflect their activity after transendothelial migration. Neutrophil CD11b/CD18 adhesion receptors, however, may have additional properties. Not only does CD11b play a role in neutrophil adhesion but it has been shown that CD11b is also fundamental to adhesion-dependent delivery of destructive reactive oxygen intermediates to the myocyte’s intracellular compartment [17]. Preventing neutrophil myocyte interactions with antibodies to CD11b and CD18 inhibits this damage [18]. Previous work by our group has also shown a significant relationship between
preoperative neutrophil CD11b expression and early rejection cardiac transplantation [19].

This study focused primarily on renal injury, but a number of other interesting relationships were noted. In relation to cardiac function neutrophils are recognised to play an important role in myocardial ischaemia reperfusion injury and low cardiac output syndromes. In this study we demonstrated an association between preoperative CD11b expression and adrenaline requirements, but not with noradrenaline usage. A significant difference was also noted in preoperative neutrophil CD11b expression in patients requiring intra-aortic balloon pump placement following surgery. Renal function following cardiac surgery is an important factor in clinical outcomes and we have observed a significant relationship between preoperative neutrophil CD11b expression and changes in creatinine following surgery. Previous studies have noted a relationship between the increase in neutrophil CD11b expression following surgery and the risk of acute renal injury as measured by creatinine following CPB [5]. The major factor contributing to impaired function is renal hypoperfusion, and in light of our observations on postoperative cardiac performance the relationship with CD11b may be a reflection of cardiac output. Associations between stimulated neutrophil CD11b expression and pulmonary A-a gradients are also seen, which may reflect adverse changes in lung function as utilised in previous publications [20]. Perioperative inflammatory damage is a recognised factor in lung function postoperatively [21]. Preoperative measurement of stimulated neutrophil CD11b expression may therefore offer a potential marker of postoperative lung function. However, the A-a gradient is limited as an index of pulmonary function as it is influenced by the FiO2, cardiac output and metabolic state.

Our investigation has demonstrated significant relationships between assessment of a preoperative neutrophil sample and subsequent postoperative renal injury. This potentially clinically applicable simulation model highlights the contribution of individual variation in immunological responses and the importance of those responses in cardiac surgical practice. It could be used as a tool for selecting patients likely to benefit most from immuno-modulatory strategies in cardiac surgery and avoiding infection risks in patients who will have a minimal response to surgical stress. Among the many strategies that could be adopted to address this perioperative response, targeting the neutrophil and transendothelial migration may offer the greatest therapeutic potential.

References