Changes in neutrophil activity following cardiovascular surgery

Jarrold P Meadows^{1,2}

James A Tumlin³

Mark G Clemens⁴

Iain H McKillop^{1,4}

Susan Evans^{1,2}

¹Department of Surgery; ²FH "Sammy" Ross Jr Trauma Center, Carolinas Medical Center, Charlotte, NC, USA;

³Department of Medicine, Division of Nephrology, University of Tennessee College of Medicine at Chattanooga, Chattanooga, TN, USA; ⁴Department of Biology, University of North Carolina at Charlotte, Charlotte, NC, USA

Background: Neutrophils are implicated in initiating perioperative inflammation and postoperative outcome, particularly in patients with acute renal failure. The aim of the current study was to assess the role of neutrophil function in patients following cardiovascular surgery with mild preoperative renal dysfunction (PRD).

Materials and methods: Patients scheduled for cardiovascular operations were screened for renal function, and divided into those with glomerular filtration rate (GFR) < 60 mL/minute (PRD, n = 9), and GFR > 60 mL/minute (normal controls; [C] n = 7). Neutrophils were isolated from plasma preoperatively, and 2, 24, and 48 hours postoperatively. Neutrophil activity (respiratory burst; RB) and apoptosis were then determined.

Results: In the whole population, neutrophil apoptosis decreased from 67% preoperatively, to 42% postoperatively, with no significant difference between the groups (C = 64.8%–41.2%, PRD = 68.2%–42.6%). RB increased by 70% postoperatively in the whole population, with no significant difference between the groups (C = 68.1%, PRD = 72.9%, $P = 0.86$).

Conclusion: Collectively these data demonstrate that, in addition to decreasing neutrophil apoptosis, cardiovascular surgery leads to increased neutrophil RB, thus contributing to postoperative systemic oxidative stress. This can lead to clinically significant complications and tissue damage. Our data also suggest that differences in postoperative inflammation between patients with and without PRD, do not exist, at least in the setting of mild preoperative renal insufficiency.

Keywords: neutrophil, renal function, apoptosis, respiratory burst, glomerular filtration rate

Introduction

Renal dysfunction has a substantial detrimental impact on patient outcome following cardiovascular surgery.^{1–3} The mortality of patients who develop postoperative acute kidney injury (AKI) typically ranges from 25%–75%, depending on the definition of AKI,⁴ and the need for hemodialysis.⁵ Even patients with baseline renal dysfunction, who don't proceed to AKI, have more complications and higher mortality than those with normal preoperative renal function.^{6–9}

Preoperative renal dysfunction in patients undergoing cardiovascular operations exists in as many as 14% of the approximately 600,000 coronary artery bypass graft (CABG) patients treated annually.⁶ In the Cardiovascular Health Study, patients with chronic renal dysfunction had a greater incidence of associated cardiovascular disease.¹⁰ In this study the authors propose that the higher incidence of cardiovascular disease in these patients is attributable to the chronic inflammatory state associated with renal dysfunction, a factor that contributes to atherosclerosis.

Correspondence: Susan Evans
FH "Sammy" Ross Jr Trauma Center,
Department of Surgery, Carolinas
Medical Center, 1000 Blythe Blvd,
Charlotte, NC 28203, USA
Tel +1 704 355 3176
Fax +1 704 355 7202
Email susan.evans@carolinashealthcare.org

This chronic inflammatory process is demonstrated by elevated CRP, IL-6, and fibrinogen levels¹⁰ as well as neutrophil respiratory burst capacity,¹¹ an indicator of neutrophil activation. The neutrophil activation is related to the degree of renal dysfunction, with rates of respiratory burst capacity inversely correlated to glomerular filtration rate (GFR).¹¹ Interestingly, these same patients have a higher rate of neutrophil apoptosis,¹¹ suggesting that although the neutrophils are activated, they are fewer in number.

To our knowledge, the inflammatory response to surgical stress in patients with preoperative renal dysfunction remains unknown. An enhancement of the already activated inflammatory system, similar to patients who develop postoperative AKI, could explain the higher mortality and poorer overall outcome of this patient population. In patients who develop postoperative AKI, poorer outcomes are associated with enhanced inflammatory processes, extra-renal organ dysfunction, and even multi-system organ failure.¹² An enhanced inflammatory response following CABG has been characterized by decreased neutrophil apoptosis.¹³ Although a simultaneous increase in neutrophil respiratory burst activity has been proposed, it has not been demonstrated. In one patient population (Fung et al),¹⁴ respiratory burst was unchanged postoperatively until day 3, when it actually decreased. While the stimulus for increased neutrophil activity has yet to be clearly defined, experimental evidence suggests it may be related to interleukin-6 (IL-6) from renal tubular cells during stress.^{13,15}

Since heavily exaggerated inflammatory processes have been observed in both animal models and patient studies of CKD and AKI, it seems reasonable to hypothesize that this exaggerated inflammatory response may be the underlying culprit behind the complications following cardiovascular surgery experienced by patients with preoperative renal dysfunction. Additionally, patients with baseline renal dysfunction have a higher risk of developing postoperative AKI, and its consequent complications, than those with normal preoperative renal function.^{16,17} The aim of the current study was thus

to determine and compare postoperative respiratory burst and apoptotic rates in neutrophils isolated from patients with mild preoperative renal dysfunction following a cardiovascular operation with those patients with normal renal function.

Materials and methods

Patient population and institutional assurances

All studies were approved by the IRB of The Carolinas Medical Center and all patients participating in this study did so after appropriate informed consent was obtained. Patients undergoing elective thoraco-abdominal surgery requiring general anesthesia were screened. Those patients with a modification of diet in renal disease (MDRD) estimated GFR < 60 mLs/minute, and one (or more) of the inclusion criteria listed in Table 1, were included in the *preoperative renal dysfunction* group. Patients with an MDRD estimated GFR > 60 mLs/minute, and one (or more) of the inclusion criteria listed in Table 1, were included in the *normal preoperative renal function* group. Patients that met any of the exclusion criteria listed in Table 1 were not considered.

Materials

RPMI-1640 culture medium was purchased from Mediatech (Manassas, VA). Fetal bovine serum (FBS), the Amplex Red H₂O₂ detection kit, and the annexin V-fluoresceinisothiocyanate (FITC)/propidium iodide (PI) kit were purchased from Invitrogen (Carlsbad, CA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St Louis, MO). Anti-Fas antibody (human, activating; clone CH11) was purchased from Millipore (Temecula, CA). The Apo-ONE kit and Ac-DEVD-CHO were purchased from Promega (Madison, WI). All other chemicals used were purchased from Sigma-Aldrich.

Blood collection and neutrophil isolation

Heparinized venous blood (20 mL) was collected 1 hour prior to surgery and 2, 24, and 48 hours postoperatively. Blood was

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Age >60 years	Age <18 years
Insulin requiring type I or II diabetes mellitus	Neutropenia defined as WBC count <2500 cells/mL
Emphysema or COPD requiring chronic bronchodilator therapy	Patients receiving active chemotherapy
Dilated cardiomyopathy with ejection fraction <45%	Patients without IV access
Exposure to iodinated contrast agent within 7 days prior to admission	Hematocrit <20% or receiving regular transfusion of PRBC
Proteinuria (from any cause) >500 mg/24 hours	Live vaccine within 28 days of study enrollment
Peripheral vascular disease	Unable to give informed consent

Abbreviations: COPD, chronic obstructive pulmonary disease; PRBC, packed red blood cells; WBC, white blood cells.

layered on a discontinuous Histopaque-1077/1119 gradient and neutrophils isolated as per the manufacturer's instructions (Sigma-Aldrich). Briefly, following centrifugation ($500 \times g$, 30 minutes, 25°C), the upper two layers (plasma and lymphocytes) were aspirated and discarded. The polymorphonuclear leukocyte-containing layer was then collected and any contaminant erythrocytes removed using a hypotonic lysis buffer ($155 \text{ mM NH}_4\text{Cl}$, 10 mM KHCO_3 , $100 \mu\text{M EDTA}$). Neutrophil purity was determined by forward-side scatter analysis using a BD FACS Calibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ). Neutrophil viability was measured by trypan blue exclusion. Following isolation and purification, neutrophils were suspended in RPMI-1640 medium supplemented with 10% (v/v) FBS and antibiotics (apoptosis studies), or Krebs's Ringer phosphate buffer (KRP; 1 mM CaCl_2 , 1.5 mM MgSO_4 , 5.5 mM glucose , pH 7.4) for respiratory burst analysis.

Neutrophil culture and apoptotic analysis

Following isolation/purification, neutrophils ($5 \times 10^6/\text{mL}$) were cultured in RPMI-1640 medium supplemented with 10% FBS (v/v) and antibiotics in the absence or presence of anti-Fas (250 ng/mL) for 24 hours. At the end of this period, caspase 3/7 activity was determined using an Apo-ONE kit as per the manufacturer's instructions. Substrate specificity was confirmed using Ac-DEVD-CHO (50 nM). Neutrophil apoptosis was measured using an annexin V-FITC/PI kit as per the manufacturer's instructions. Briefly, neutrophils were washed twice with PBS and resuspended in binding buffer (10 mM HEPES/NaOH , 140 mM NaCl , 2.5 mM CaCl_2 , pH 7.4). Annexin V-FITC ($5 \mu\text{L}$ kit stock solution) and PI ($5 \mu\text{L}$ kit stock solution) were then added to $100 \mu\text{L}$ of cell-binding buffer suspension and incubated for 15 minutes at room temperature in the dark. Sample volume was then adjusted to $500 \mu\text{L}$ total volume and analysis performed by flow cytometry (20,000 events/sample, 485 nm excitation- 530 nm emission λ).

Respiratory burst/ampex red assay

Neutrophil respiratory burst was measured using the Amplex Red H_2O_2 kit under resting (untreated) or stimulated (200 nM PMA) conditions as per the manufacturer's instructions. Detection was performed using a Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Winooski, VT) (530 nm excitation- 590 nm emission λ).

Statistics

Student's *t*-test was used to compare group means. A $P < 0.05$ was considered significant.

Results

Patient demographics and surgical procedures performed

Sixteen patients met the inclusion criteria between March 2008 and May 2009, and consented to participate in the study. Of these patients, nine had preoperative renal dysfunction (PRD), and seven had normal renal function (Control; C). No significant difference in age, gender, or comorbidities existed between patients in the PRD and C groups (Table 2). Although the BUN was higher in the PRD group, the BUN/creatinine ratio was similar ($\text{PRD} = 17.7$ vs $\text{C} = 17.4$), supporting intrinsic renal dysfunction in the PRD group. Two of the patients in the PRD group had aortobifemoral bypass with aortic cross-clamping. All other patients, from both groups, underwent cardiac operations. One patient (PRD) had an off pump CABG, and the remaining patients had cardiopulmonary bypass (average pump time of 115 ± 40 minutes). There were no significant differences in the pump times, time to extubation, and ICU or hospital lengths of stay (LOS) (Table 2).

Neutrophil apoptosis

Neutrophil apoptosis/viability was measured using an annexin V-FITC/PI kit followed by FACS analysis (Figure 1a). Collective analysis of PRD and C patients demonstrated

Table 2 Patient demographics

Characteristic	Control (C) (n = 7)	PRD (n = 9)	P-value
Age – years (SD)	66 (± 8)	73 (± 6)	0.0816
Gender (% male)	71	69	0.83
Race – n (%)			0.23
Caucasian	6 (86)	7 (78)	
African American	0	2 (22)	
Native American	1 (14)	0	
BUN – mg/dL (SD)	15.7 (7.6)	26.5 (8.5)	0.01
Creatinine – mg/dL (SD)	0.9 (± 0.1)	1.5 (± 0.4)	0.0014
GFR – mL/minute (SD)	77 (± 10)	44 (± 9)	<0.0001
DM – n (%)	2 (29)	5 (56)	0.28
CAD – n (%)	7 (100)	6 (75)	0.15
PVD – n (%)	1 (14)	4 (44)	0.19
Pump – n (%)	8 (89)	7 (100)	
Pump time – minutes	119 (± 48)	110 (± 30)	0.15
LOI – hours	36 (± 80)	13 (± 13)	0.49
LOS ICU – days	4.4 (± 6.9)	3.8 (± 2.3)	0.81
LOS HOSP – days	8 (± 5)	13 (± 9)	0.23, 0.05*
D/C DISPO – n (%) home	6 (86)	4 (44)	

Note: *Wilcoxon rank sum test.

Abbreviations: CAD, coronary artery disease; D/C DISPO, discharge disposition; DM, diabetes mellitus; GFR, glomerular filtration rate; LOI, length of intubation following operation; LOS, length of stay; PRD, preoperative renal dysfunction; PreCR, preoperative creatinine; Pump, cardiopulmonary bypass; PVD, peripheral vascular disease.

Figure 1 A) Representative fluorescent activated cell sorting (FACS) data from neutrophils isolated with Annexin V or propidium iodide (PI). In each instance the lower left quadrant represents viable neutrophils (Annexin V and PI negative), the lower right quadrant represents apoptotic neutrophils (Annexin V positive, PI negative), and the right upper quadrant represents necrotic neutrophils (Annexin V and PI positive). The number in each quadrant represents the percentage of total cells falling within this quadrant. **B)** Cumulative FACS analysis (mean $\pm$ SEM) of apoptotic (A) and viable (V) neutrophils isolated from the total patient population ($n = 16$), without, and with anti-Fas stimulation (250 ng/mL; Af and Vf).

Notes: * $P < 0.05$ apoptotic versus viable neutrophils; # $P < 0.05$ apoptotic versus viable neutrophils following fas-ligand.

a significant decrease in apoptotic neutrophils post-operation (post-op) as compared to pre-operation (pre-op), a maximal decrease occurring 24 hours postoperatively (66.7% $\pm$ 1.8% apoptotic [pre-op] versus 41.9% $\pm$ 2.9% 24 hours post-op, Figure 1b, $P < 0.001$). Concomitantly, viable cell number significantly increased postoperatively, a maximum increase being measured after 24 hours (62.5% $\pm$ 3.9% viable cells 24 hours post-op versus 17.7% $\pm$ 1.7% viable cells at baseline [pre-op], $P < 0.001$, Figure 1b). Analysis of neutrophils isolated 48 hours postoperatively demonstrated no further significant change in cell apoptosis/viability (Figure 1b).

The addition of proapoptotic antiFas (aF) to the assay did not significantly alter the number of apoptotic neutrophils (71.0% $\pm$ 2.6% [+ aF, pre-op] versus 66.7% $\pm$ 1.8% [- aF, pre-op], Figure 1b). The inclusion of aF also led

to apoptotic cell number remaining consistently elevated throughout the experimental time course (Figure 1b). As expected, the increased percentage of apoptotic cells in the presence of aF corresponded with a sustained decrease in viable cell number (Figure 1b). Analysis of neutrophil apoptosis/viability between the C and PRD groups did not demonstrate significant differences at the time points assayed, independent of aF addition (1, 24, and 48 hours post-op versus pre-op, Figures 2a and b).

To further analyze neutrophil apoptosis we next performed a caspase 3/7 enzyme activity assay. In neutrophils isolated from the study group as a whole (PRD + C) caspase 3/7 activity decreased by 35.4% $\pm$ 9.5% postoperatively (as compared to preoperative levels), and was sustained up to 48 hours postoperatively (46.7% $\pm$ 8.5% below pre-op;

Figure 2 A) Neutrophil apoptosis and viability Annexin V assay (mean $\pm$ SEM). * $P < 0.05$ apoptotic cells (A) versus viable cells (V). **B)** Neutrophil apoptosis and viability with anti-Fas (250 ng/mL).

Note: * $P < 0.05$ apoptotic cells (Af) versus viable cells (Vf).

48 hours post-op, Figure 3a). We next analyzed the effect of aF addition to the medium in the C and PRD groups individually. In the absence of aF the percentage change in caspase-3/7 activity trended toward greater decreases 24 hours postoperatively. However, these data were not significant

and no significant differences were measured between the PRD and C groups (Figure 3b). The addition of aF to the medium led to smaller, though not significant differences in caspase-3/7 activity as compared to measurements in the absence of aF (Figures 3b and c). As with assays performed

Figure 3 **A)** Neutrophil apoptosis as determined by change in Caspase 3/7 activity relative to preoperative neutrophils. (mean $\pm$ SEM). $n = 16$. **B)** Change in Caspase 3/7 activity in neutrophils isolated from Control ($n = 7$) or preoperative renal dysfunction (PRD, $n = 9$) patients, without anti-Fas ligand. **C)** Change in Caspase 3/7 activity in neutrophils isolated from Control ($n = 7$) or preoperative renal dysfunction (PRD, $n = 9$) patients, with anti-Fas ligand (250 ng/mL).

in the absence of aF, no significant differences in caspase-3/7 activity were observed between the C and PRD groups (Figure 3c). Reaction/assay specificity was confirmed by the inclusion of Ac-DEVD-CHO (a pan-caspase inhibitor) (data not shown).

Respiratory burst

Neutrophil respiratory burst was determined using an amplex red assay. Using neutrophils isolated from the study group as a whole (PRD and C) we demonstrated a $70.7\% \pm 18.6\%$ increase in respiratory burst 1 hour postoperatively as compared to preoperative levels, an effect that occurred independently of PMA stimulation (Figure 4a). Within 24 hours,

respiratory burst returned to baseline (Figure 4a). In addition to change in total amplex red formation, no significant difference was detected in the rate of amplex red formation independent of PMA stimulation (data not shown). As previously demonstrated, no significant difference in amplex red formation/respiratory burst was identified between the C and PRD groups at the time points analyzed (Figures 4b).

Discussion

In this report we demonstrate that cardiovascular surgery results in activation of inflammation. This is demonstrated by increased neutrophil respiratory burst and decreased neutrophil apoptosis. Although the decrease in apoptosis has

Figure 4 A) Change in respiratory burst from preoperative value in total patient population. Neutrophils were either unstimulated or stimulated with phorbol 12-myristate 13-acetate (PMA). (mean $\pm$ SEM) $n = 16$, * $P < 0.05$ versus post-op. **B)** Change in respiratory burst in neutrophils isolated from Control (C, $n = 7$) or preoperative renal dysfunction (PRD, $n = 9$) patients. Neutrophils were either unstimulated or stimulated with PMA.

Note: * $P < 0.05$ versus post-op.

been previously reported following cardiovascular surgery, this is the first time that an increase in neutrophil respiratory burst has been demonstrated. Collectively these changes may contribute to systemic oxidative stress following cardiovascular operations that, in turn, challenges postoperative recovery. Interestingly, the inflammatory response of the PRD

group did not differ from that of the normal renal function group. This finding differs from our original hypothesis that a postoperative decrease in neutrophil apoptosis and increase in neutrophil respiratory burst would be more exaggerated in patients with preoperative renal dysfunction. The explanation for this deviation may be due to a number of factors.

The inflammatory response in patients with renal dysfunction has been mixed.¹⁸ Chronic renal dysfunction has consistently been described as an inflammatory state.¹⁹ However, the stimulation of some inflammatory mediators may not translate to decreased neutrophil apoptosis and increased neutrophil respiratory burst. Patients with acute and chronic renal dysfunction may have different inflammatory responses to stressors. Even those patients with long-standing renal dysfunction seem to have variable rates of baseline apoptosis depending on the degree of, and response to, uremia.¹⁸ In fact, chronic renal dysfunction has also been described as an altered inflammatory state with the potential for increased neutrophil apoptosis, decreased superoxide production, and phagocytosis.²⁰ Although our patients had mild degrees of renal dysfunction, with essentially no uremia, they may have had some early inflammatory changes which preceded the azotemia, and uremia, causing a chronic kidney disease (CKD) type of behavior.

Patients with acute kidney injury (AKI), on the other hand, demonstrate an increased inflammatory response to stressors.²¹ This may be because the etiology of AKI is often accompanied by a whole body inflammatory state, which is often the result of an ischemia/reperfusion injury common in cardiovascular operations. We had initially assumed that patients with mild preoperative renal dysfunction would respond to the stress of an operation with ischemia/reperfusion much like those with AKI, due to the fact that their mild renal dysfunction would put them at higher risk of developing AKI. This assumption appears to be incorrect.

An alternative explanation is that our patients' pathophysiology functioned as a mix of acute and chronic renal dysfunction. The contribution of the neutrophil suppression of chronic renal dysfunction and neutrophil stimulation of acute kidney injury may have resulted in a zero net difference from patients without renal dysfunction. This may help explain the extreme variability in each patient's inflammatory markers. In our population, most of the patients had minimal change in BUN and creatinine throughout the study period, suggesting that they were appropriately categorized. One of the patients in the PRD group had a slight decrease in creatinine (1.1 mg/dL pre-op to 0.7 mg/dL at 48 hours) and a starting BUN of 34 mg/dL, which suggests that her azotemia may have been due to volume depletion, rather than intrinsic renal dysfunction. This acute kidney injury was difficult to determine with only one available preoperative creatinine value.

Additionally, the presence of non-renal diseases may contribute to poorer outcomes of many patients with associated

renal disease. In our population, these non-renal diseases were present equally in our control and PRD groups. Therefore, this isolated the impact of renal dysfunction on outcome. It is possible that previous suggestions of poorer postoperative outcome in patients with preoperative renal dysfunction were limited by paired diseases, which even regression analysis is unable to eliminate. Similarly, it is possible that overall improved postoperative critical care results in less stress and better outcomes. A number of initiatives have changed the postoperative management of CABG patients. These changes (ie, early extubation, perioperative beta blockade, statin use)²²⁻²⁵ may disproportionately blunt the inflammatory response, as well as improve the outcomes, of the most diseased patients, consequently eliminating differences which once were pronounced.

Alternatively our findings may differ from our original hypothesis due to a lack of statistical power, or a beta error. Some of the variables had trends toward significance (for example, postoperative caspase activity), which may become significant with larger numbers of patients in each group. The point at which larger numbers would make the finding clinically relevant is not clear. Acquiring patients who met inclusion criteria, proved somewhat more difficult than was initially estimated. The difference may also be related to the mild level of renal dysfunction, which we selected as inclusion criteria. It is possible that choosing a PRD group with a greater degree of renal dysfunction (ie, GFR < 50, 40, or 30) while maintaining a GFR > 60 for the control group, may have resulted in significant differences in our findings. This would have required a different query, as we wanted to answer the question as to how mild renal dysfunction affected inflammatory outcome. We did attempt to answer the question of which threshold of GFR may result in poorer outcome by doing post hoc analysis. However, there was not an identifiable GFR threshold, which demonstrated differences in the variables of inflammatory outcome.

In addressing the patient population, it is also important to highlight that not all of the patients in the PRD group underwent cardiopulmonary bypass. Cardiopulmonary bypass has been demonstrated to cause a decrease in neutrophil apoptosis, which is slightly greater than the decrease in patients undergoing off-pump CABG.¹⁴ Therefore, the lower incidence of cardiopulmonary bypass in the PRD group may have masked a trend toward more enhanced inflammation in that group. Using an overall view of our population, it does not appear that patients who underwent cardiopulmonary bypass had differences in the inflammatory parameters. However, the contribution of cardiopulmonary

bypass to respiratory burst was not evaluated. Additionally, two of the patients in the PRD group who did not undergo cardiopulmonary bypass underwent aortic cross-clamping, a procedure that has previously been demonstrated to enhance the inflammatory process.

In conclusion, we have demonstrated that, in addition to decreasing neutrophil apoptosis, cardiovascular surgery leads to an increase in neutrophil respiratory burst capacity, thus contributing to postoperative systemic oxidative stress. This can lead to clinically significant complications and tissue damage. Our data also suggest that differences in postoperative inflammation between patients with and without preoperative renal dysfunction do not exist, at least in mild preoperative renal insufficiency.

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Disclosure

No conflicts of interest were declared in relation to this paper.

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