Indices of Apoptosis Activation After Blood Cardioplegia and Cardiopulmonary Bypass

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Background—Cardioplegic arrest (CA) using cold blood cardioplegia (CBC) has been reported to reduce ischemia-reperfusion (IR)–induced myocardial injury via apoptosis. We studied key apoptotic mediators via the caspase-dependent and intrinsic pathways as well as poly(ADP-ribose)polymerase (PARP) activity in myocardial and peripheral tissues after CA and cardiopulmonary bypass (CBP).

Methods and Results—Right atrial (RA) and skeletal muscle (SM) was harvested from cardiac surgical patients with similar baseline characteristics (N = 6) before and after CPB and CBC. Total and modified caspase-3, Bcl-2, apoptosis-inducing factor (AIF), and PARP were quantified by immunoblotting. Terminal caspase-3 activity was assessed and immunohistochemistry was performed for PARP and AIF. TUNEL staining was used for identification of apoptotic cells. Microarray gene expression analysis was performed using Affymetrix U95 GeneChip. In RA tissue, CA with CBC significantly increased phosphorylation of Bcl-2 (Ser
tt20) (2.63 ± 0.4 and 1.77 ± 0.3-fold respectively; P < 0.05), and cleavage of the downstream caspase 3 (1.45 ± 0.1-fold; P < 0.05). There was no significant change in total protein levels. Also, there was an increase in mature AIF (57 kDa) levels (1.22 ± 0.01-fold; P < 0.05) and a trend toward nuclear translocation on histological staining. Caspase 3 activity was increased 1.5 ± 0.14-fold (P < 0.05). The number of apoptotic cells in atrial tissue increased after compared with before CPB/CA using TUNEL staining (1.55 ± 0.66 versus 0.325 ± 0.05%, respectively; P = 0.03). In contrast, SM samples did not show any of the changes observed in RA tissue after CPB.

Conclusion—Despite optimal current surgical myocardial protection, we found that CA with CBC induced both programmed cell death and survival signaling in myocardial tissue. (Circulation. 2006;114[suppl I]:I-257–I-263.)

Key Words: apoptosis ■ blood cardioplegia ■ extracorporeal circulation ■ ischemia reperfusion ■ myocardial protection ■ signal transduction

Myocardial dysfunction after cardioplegic arrest (CA) and cardiopulmonary bypass (CPB) has long been related to local processes that contribute to stress such as ischemia, ischemia-reperfusion injury (IRI), and the associated myocardial stunning. More recently, apoptosis has been implicated in many forms of cardiac pathology such as heart failure, cardiomyopathy and myocarditis.1,2 It has been shown that apoptosis can be triggered via either an intrinsic pathway caused by hypoxia and oxidative stress/reactive oxygen species or an extrinsic pathway triggered by elevated humoral factors released during CPB.3–5

Apoptosis has been considered as one mechanism of myocardial cell loss during IRI.2 However, the mechanism underlying IRI-induced apoptosis has not been fully elucidated. It has been shown that IRI-induced apoptosis is mainly regulated by Bcl-2 family of proteins and cysteine protease family of caspases.2 Bad is a pro-apoptotic Bcl-2 family protein, whereas phosphorylation of Bad inhibits its binding to and inactivation of anti-apoptotic Bcl-2.8 Thus, increases in phosphorylation of Bad and/or expression of Bcl-2 would prevent myocardial apoptosis. In contrast, cleavage and activation of caspase 3 and poly(ADP-ribose) polymerase (PARP) are recognized downstream effectors of apoptotic cell death.7 Also, apoptosis-inducing factor (AIF) is a ubiquitously expressed flavoprotein whose translocation from the mitochondrial inter-membrane space (molecular weight – 57 kDa) to the nucleus plays a critical role in caspase-independent apoptosis.8,9

Cold blood cardioplegia (CBC) has gained popularity in recent years and is currently the most commonly used form of...
cardiac arrest during cardiac surgery in North America. Animal studies have also demonstrated that CA during open-heart surgery was associated with induction of endothelial cell and cardiomyocyte apoptosis. Moreover, CBC has been reported to be superior to cold crystalloid cardioplegia in inhibiting myocardial apoptosis during ischemic arrest. Also, we have previously shown that intermittent blood cardioplegia is better than intermittent crystalloid cardioplegia in preserving myocardial function and endothelium-dependent microvascular response. Aebert et al demonstrated that serum from patients after CPB exerts a strong apoptosis inducing activity on human endothelial cells, which may be associated with the ensuing postoperative vascular dysfunction and permeability, likely through the extrinsic humoral apoptotic pathway.

Few recent studies have shown that CA with cold crystalloid cardioplegia causes activation of the caspase cascade and myocardial apoptotic cell death in humans. However, the molecular indices of myocardial apoptosis induction have not been previously investigated using CBC in a clinical setting. The objective of this study was to assess the activation and expression of myocardial apoptosis mediators Bcl-2, Caspase 3, AIF, and PARP after CBC in humans. We also studied these mediators in peripheral skeletal muscle tissues exposed to CPB in the same patients to determine those effects specific for either CBC or CPB.

Methods

Tissue Harvesting

Samples of right atrial appendage and skeletal muscle were harvested from similar patients (n=6) undergoing coronary artery bypass graft surgery before and after exposure of the heart to blood cardioplegia and short-term reperfusion under conditions of CPB. Samples were harvested with cold sharp dissection and handled in a nontraumatic fashion. Double 3-0 polypropylene purse-string sutures (Ethicon) were placed in the atrial appendage. During placement of the venous cannula, the first sample of atrial appendage was harvested (pre-CBC/CPB). The superior suture was tightened to secure the venous cannula, the first sample of atrial appendage was harvested (pre-CBC/CPB). The inferior suture remained loose to allow this portion of the atrium to be perfused with blood, exposed to CPB and blood cardioplegia, and reperfused after removal of the aortic cross-clamp. The cardioplegia consisted of a 4:1 mixture of oxygenated blood and short-term reperfusion under conditions of CPB. Samples were incubated with the specific antibody diluted in Tris-Buffered Saline Tween (TBST) solution containing 5% bovine serum albumin or 2.5% milk overnight in cold room. After 4 washes with TBST, a second incubation was performed with anti-rabbit horseradish peroxidase-linked IgG (Cell Signaling Tech) at a dilution of 1:3000 in TBST containing 2.5% milk for 1 hour at room temperature. Immunodetection was validated with using the enhanced chemiluminescence Western blotting system (Pierce Biotechnologies, Rockford, Ill). Immunoblots were analyzed by digitalization and quantification of the radiographs using a flatbed scanner and NIH Image software (National Institutes of Health, Bethesda, Md).

Caspase 3 Activity Fluorometric Assay

Myocardial tissue (50 mg) was homogenized in lysis buffer (1% Triton X-100, 0.32 sucrose, NaCl, 1 mmol/L phenylmethylsulphonylfluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 10 mmol/L Tris/Hcl, pH 8.0) followed by centrifugation (16 000g, 4°C, 10 minutes). The supernatants (200 µg of protein) incubated with 2X reaction buffer (containing 10 mmol/L DTT) and 50 µmol/L DEVD-AFC (AF: 7-amino-4-trifluoromethyl coumarin) substrate at 37°C for 60 minutes (BioVision, Mountain View, Calif). Caspase-3 like activity was detected in a luminescence spectrometer (LS50B, Perkin-Elmer, Wellesley, Mass) by measuring the proteolytic cleavage of the fluorogenic sequence DEVD-AFC (7-amino-4-trifluoromethylcoumarin) substrate and AFC as standard at an excitation wavelength of 400 nm versus an emission wavelength of 505 nm. Fold-increase in caspase 3 activity was determined by comparing these results with level of the uncleaved controls.

Poly(ADP) Ribose, AIF, and Nitrotyrosine Histological Immunostaining

Right atrial slides were deparaffinized. For poly(ADP) ribose (PAR) and AIF immunostaining antigen was retrieved by incubation in boiling 0.1 mol/L sodium citrate (pH 6), then slides were rinsed in water. Slides were rinsed in phosphate-buffered saline (PBS), and then endogenous peroxidase activity was quenched with 1.5% (vol/vol) hydrogen peroxide in methanol for 15 minutes. Nonspecific binding sites were blocked using 2% (vol/vol) normal goat serum in PBS for 1.5 hours at 37°C. Preliminary experiments determined optimal antibody concentrations. Chicken antibody against PAR was used at 4 µg/mL dilution, rabbit anti-AIF antibody was used at 1.3 µg/mL dilution, and rabbit anti-nitrotyrosine antibody was used at 1:400 dilution. Slides were incubated overnight at 4°C, then washed in PBS, and as a secondary antibody, biotinylated goat anti-chicken IgG or biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame CA) was used for 30 minutes at 30°C. After PBS washes, slides were incubated with VECTASTAIN Elite ABC ( Peroxidase) standard kit (Vector Laboratory,) for 30 minutes at 30°C and developed using diaminobenzidine substrate. Slides were counterstained with nuclear fast red.

TUNEL Staining

Left ventricular tissues were fixed in formalin for 24 hours, embedded in paraffin, and a 4-µm section was obtained. The apoptotic cells were identified by dUTP nick-end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer’s protocol (Chemicon Inc, Temecula, Calif). Ten photographs (magnification 200X) of each tissue section were taken. The nuclei were viewed and manually counted by 2 observers, who were blinded to the experimental conditions. The number of TUNEL-positive cardiomyocytes, indicating apoptosis, was expressed in mean number per microscopic field.

Microarray Processing

Microarray gene expression data were used from 5 patients with similar baseline characteristics using the Affymetrix U95 GeneChip from right atrial and skeletal muscle mRNA using same technique as previously described. Array results were validated with Northern blotting, real-time polymerase chain reaction, and in situ hybridization. Median post-CBC/CPB to pre-CBC/CPB gene expression ratios
were computed for each gene by using the relative signal intensities of the probe cells. Fold change significance was set at a conservative P < 0.005.

**Statistical Analysis**

Values are shown as mean ± SEM. Statistical analyses were performed using the paired t test to compare fold change in pre-CBC/CPB to post-CBC/CPB samples. Statistical significance was accepted at P < 0.05. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

### Results

**Patient Characteristics**

Table 1 displays the clinical characteristics of the patients whose tissues were used for the microarray and validation studies. All subjects were white men aged between 52 and 83 years old, without significant comorbidity, who electively underwent 3 or 4 coronary artery bypass grafts with mildly hypothermic CPB and antegrade intermittent (every 20 minutes) hyperkalemic CBC. Mean left ventricular ejection fraction was 58 ± 8%.

**Increased Modified Bcl-2 and Bad Proteins in Atrial Tissue**

In atrial tissue, levels of p-Bcl-2 and p-Bad were found to be significantly increased after CA with CBC and a brief period of reperfusion (2.63 ± 0.4 and 1.77 ± 0.3-fold increase, respectively; P < 0.05; Figure 1) as demonstrated by Western blot analysis. In contrast, skeletal muscle tissue exposed to CPB did not have any significant changes in phosphorylated Bcl-2 and Bad. Total amount of Bcl-2 and Bad were found to be unchanged pre-CBC/CPB compared with post-CBC/CPB in both atrial and skeletal muscle.

**Increased Caspase 3 Cleavage and Activity in Atrial Tissue**

Western blot of Caspase 3 cleavage was significantly increased in atrial tissues after CBC/CPB compared with pre-CBC/CPB samples (1.45 ± 0.1-fold increase; P < 0.05; Figure 1). This increased level of caspase 3 cleavage was also accompanied by elevated caspase 3 activity revealed by fluorometric assay (1.5 ± 0.14-fold increase; P < 0.05; Figure 2) in atrial tissue. Total caspase 3 levels were not significantly changed after CBC/CPB in atrial tissue.

**TABLE 1. Baseline Patient Characteristics. Mean ± SEM**

<table>
<thead>
<tr>
<th></th>
<th>Myocardial (n=6) and Skeletal (n=6) Muscle Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>Men/women</td>
<td>6/0</td>
</tr>
<tr>
<td>HTN</td>
<td>6</td>
</tr>
<tr>
<td>Current tobacco</td>
<td>0</td>
</tr>
<tr>
<td>DM</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol &gt;200 mg/dL</td>
<td>0</td>
</tr>
<tr>
<td>Redo/combined procedures</td>
<td>0</td>
</tr>
<tr>
<td>N of CABG grafts [median (min, max)]</td>
<td>3 (3, 4)</td>
</tr>
<tr>
<td>LV ejection fraction (%)</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>Duration of CPB (min)</td>
<td>82 ± 19</td>
</tr>
<tr>
<td>X-clamp (min)</td>
<td>60 ± 14</td>
</tr>
<tr>
<td>Minimum CPB temperature (°C)</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Intraoperative blood transfusion</td>
<td>0</td>
</tr>
<tr>
<td>Postoperative LOS (days)</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>

CABG indicates coronary artery bypass graft; CPB, cardiopulmonary bypass; DM, diabetes mellitus; LOS, length of stay; LV, left ventricle.
Increased AIF Levels and Nuclear Translocation in Atrial Tissue

Levels of the mature form of AIF (57 kDa) were found to be significantly increased in atrial tissue after CBC/CPB by Western blot analysis (1.22 ± 0.01-fold increase; \( P < 0.05 \); Figure 1). This was accompanied by a trend for increased cytoplasmic AIF staining and translocation to the nucleus of cardiomyocytes on immunostaining (Figure 3). Skeletal muscle did not demonstrate any change in AIF levels pre- versus post-CBC/CPB.

Undetectable Change in PAR or Nitrotyrosine Staining

On examining the immunohistochemical staining performed on atrial samples, we found no significant change in PAR staining at the time of harvest. Nuclear PAR staining was seen in myocytes, endothelial cells, and white blood cells within vessels before and after CBC/CPB (Figure 4a).

Similarly, peroxynitrite formation as measured by nitrotyrosine staining was not significantly changed in response to CBC/CPB in cardiomyocytes and vessel walls (Figure 4b).

TUNEL Staining

Figure 5 shows the TUNEL-positive myocyte nuclei photographed at a magnification of 100×. Percent TUNEL-positive cells was significantly increased post-CBC/CPB compared with pre-CBC/CPB (1.55 ± 0.66 versus 0.325 ± 0.05; respectively, \( P < 0.03 \)).

Upregulation of Early Stress Genes in Myocardium

In atrial tissues, we found no significant change in the gene expression of the apoptosis mediators studied at the time of specimen harvest after CBC/CPB. However, we did find a significant gene upregulation of c-Fos and c-Jun, known early mediators of cellular stress and apoptosis signaling\(^1\) (4.2 ± 0.6 and 1.9 ± 0.2-fold increase, respectively; \( P < 0.005 \)).

In contrast, skeletal muscle gene expression was not significantly changed for the apoptosis intermediates studied or early mediators of cellular stress. Microarray gene expression for these selected genes in atrial and skeletal muscle is summarized in Table 2.

Discussion

CA during cardiac surgery is a tool for mitigating the iatrogenic IRI. Recent studies indicate that apoptosis is induced in animal and human cardiomyocytes after CA, suggesting that apoptosis may play a key role in myocardial dysfunction after open-heart surgery.\(^5\)\(^10\) These studies have
TABLE 2. Gene Expression Changes in Myocardial and Skeletal Muscle Tissues When Comparing Pre- to Post-CBC/CPB Samples

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene</th>
<th>Right Atrium</th>
<th>Skeletal Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>836</td>
<td>Caspase 3</td>
<td>0.13</td>
<td>0.31</td>
</tr>
<tr>
<td>596</td>
<td>Bcl 2</td>
<td>0.11</td>
<td>0.52</td>
</tr>
<tr>
<td>572</td>
<td>Bad</td>
<td>0.62</td>
<td>0.23</td>
</tr>
<tr>
<td>581</td>
<td>Bax</td>
<td>0.60</td>
<td>0.18</td>
</tr>
<tr>
<td>9131</td>
<td>AIF</td>
<td>0.85</td>
<td>0.28</td>
</tr>
<tr>
<td>842</td>
<td>Caspase 9</td>
<td>0.30</td>
<td>0.48</td>
</tr>
<tr>
<td>355</td>
<td>Fas</td>
<td>0.33</td>
<td>0.05</td>
</tr>
<tr>
<td>356</td>
<td>FasL</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>2353</td>
<td>*c-Fos</td>
<td>&lt;0.0001</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.2±0.6 fold ↑)</td>
<td></td>
</tr>
<tr>
<td>3725</td>
<td>*c-Jun</td>
<td>0.002</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.9±0.2 fold ↑)</td>
<td></td>
</tr>
</tbody>
</table>

Early mediators of cell stress c-Fos and c-Jun were found to be significantly increased only in atrial tissues (P<0.005). Upregulation in other apoptosis-related genes was not detected after the short reperfusion period at the time of post-CBC/CPB tissue harvest.

*Significance for atrial tissue only.

demonstrated that crystalloid cardioplegia infusion in pigs and humans during CPB impairs the morphological integrity of the coronary endothelium and induces cardiomyocyte apoptosis. Mounting evidence points to improved clinical outcomes in cardiac surgical patients after blood-based compared with crystalloid-based cardioplegia. This study is the first to examine the role of apoptosis mediator activation after CBC, the most commonly used form of CA.

Our study demonstrates that despite ongoing advances in myocardial protection and cardioplegia formulations, myocardial tissue undergoes significant activation of caspase-dependent and caspase-independent apoptosis mediators. We showed that CBC, although the most commonly used form of CA, has not significantly improved the profile of apoptosis mediator activation. Moreover, our results show that nonischemic peripheral tissues exposed to CPB, such as skeletal muscle, are essentially protected from these changes.

Apoptosis activation has been described to be increased in diseased myocardial states. For this reason, patients in this study were matched for similar baseline characteristics, thus hoping that we decrease the impact of confounding. In this set of patients, we also report an increase in apoptosis mediator activation via post-translational protein modification rather than transcriptional regulation of apoptosis-related genes in the early stages after CA with CBC. After 10 to 20 minutes of reperfusion, all mediators studied except for PARP were found to be modified through phosphorylation or cleavage. Caspase 3 cleavage and phosphorylation of Bcl-2 and Bad were significantly increased in myocardium when comparing samples harvested pre- to post-CBC/CPB. This significant finding is supported by an increase in myocardial caspase 3 activity using a separate assay. PARP cleavage and activity are considered very late processes within the apoptosis cascade and would thus also not be expected to increase this early after the apoptosis inducing insult. Meantime, this short reperfusion period was not enough time to effect a change in mRNA expression of these intermediates. By the same token, total protein levels of caspase 3, Bcl-2, and Bad were unchanged early in this clinical setting.

We also report a small but significant overall increase in myocardial AIF levels after CBC/CPB. This was accompanied by a trend toward nuclear translocation on histological examination in atrial tissues after CBC/CPB. AIF, a caspase-independent mediator of apoptosis, is normally localized to the mitochondrial intermembrane space and released in response to apoptotic stimuli. It is synthesized as a precursor protein of 67 kDa and converted to a mature 57 kDa protein on mitochondrial import and removal of the amino-terminal mitochondrial localization signal. Susin et al reported that treatment of isolated nuclei with recombinant AIF leads to early apoptotic events such as chromatin condensation and large-scale DNA fragmentation. Until now, the role of AIF in myocardial tissue apoptosis after CBC/CPB has not been examined. Our study demonstrates that this represents another possible pathway for apoptosis induction in a clinical setting.

Interestingly, we found that myocardial ischemia-reperfusion activates both pro-apoptotic (caspase 3, AIF) and anti-apoptotic (p-Bad) mediators after CBC/CPB in the clinical setting. It is still widely controversial whether the phosphorylation of Bcl-2 is a pro-apoptotic or anti-apoptotic event. The Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability and the release of cytochrome c. The anti-apoptotic proteins of Bcl-2 reside in the outer mitochondrial wall and inhibit cytochrome c release. Figure 6 provides a summary of possible apoptosis activation pathways after CBC/CPB observed.

The pro-apoptotic Bcl-2 proteins, such as Bad and Bax, reside in the cytosol but translocate to mitochondria and form a pro-apoptotic complex with Bcl-xl or Bcl-2. This translocation is inhibited by survival factors that induce phosphorylation of Bad, leading to its cytosolic sequestration. Thus, phosphorylation of Bad may promote cell survival. Kuwahara et al reported that cardioprotection by insulin is possibly mediated, in part, via Bad inert phosphorylation. In addition, overexpression of Bcl-2 attenuated apoptosis and protected against myocardial IRI. The present data are the first to show that CBC and CPB induce phosphorylation of Bad and Bcl-2 in human tissue. Furthermore, it indicates that there exists a balance between pro-apoptotic and anti-apoptotic mediators after IRI in patients after CBC/CPB. However, this balance is likely to be in favor of pro-apoptotic signaling as the cleavage and activity of downstream effectors such as caspase 3 is a relatively terminal event in the cascade. This conclusion is further supported by the evident increase in TUNEL positive apoptotic cardiomyocytes we observed after CBC/CA.

It is a point of controversy whether ischemia alone or ischemia-reperfusion activates the apoptotic machinery in cardiomyocytes. In this study we demonstrate, in a clinical setting, that myocardial ischemia for ≈1 hour fol-
lowed by a brief period of reperfusion during using CBC/CPB does cause activation of upstream and downstream effectors of apoptosis via pathways dependent and independent of caspases. It is difficult to speculate the extent to which the brief period of reperfusion played in this activation process as this would require deviation approved clinical practice. Another limitation of this study is that myocardial samples were obtained from the right atrium rather than the more clinically relevant ventricular tissue. To obtain sufficient quantity of tissue from patients to perform the molecular work, however, would have raised ethical concerns by the Institutional Review Board. Moreover, we have previously reported, in a large animal model, the similarity between atrial and ventricular tissues harvested using this approach.25 These results provide clinical evidence that supports the role of apoptosis in myocardial injury after CA using CBC. Improvements in cardioplegic solutions would therefore likely benefit from incorporation of anti-apoptotic strategies for myocardial protection and improved clinical outcomes.

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Disclosures
None.

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Figure 6. Summary of possible apoptosis activation pathways after CBC/CPB observed.


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