View metadata, citation and similar papers at core.ac.uk

brought to you by CO

provided by Elsevier - Publisher Conne

Journal of the American College of Cardiology
© 2001 by the American College of Cardiology
Published by Elsevier Science Inc.

Vol. 38, No. 4, 2001
ISSN 0735-1097/01/$20.00
PII S0735-1097(01)01469-3

# Moderate Hypothermia During Cardiopulmonary Bypass Reduces Myocardial Cell Damage and Myocardial Cell Death Related to Cardiac Surgery

Jaime F. Vazquez-Jimenez, MD,* Ma Qing, MD,† Benita Hermanns, MD,‡ Bernd Klosterhalfen, MD,‡ Michael Wöltje, PHD,§ Raj Chakupurakal, MD,† Kathrin Schumacher, MD,† Bruno J. Messmer, MD,* Götz von Bernuth, MD,† Marie-Christine Seghaye, MD†

*Aachen, Germany*

**OBJECTIVES** The goal of this study was to test the hypothesis that moderate hypothermia during cardiopulmonary bypass (CPB) provides myocardial protection by enhancing intra-myocardial anti-inflammatory cytokine balance.

**BACKGROUND** Moderate hypothermia during experimental CPB stimulates production of interleukin-10 (IL10) and blunts release of tumor necrosis factor-α (TNFα).

**METHODS** Twelve young pigs were assigned to a temperature (T°) regimen during CPB: moderate hypothermia (T°: 28°C; n = 6) and normothermia (T°: 37°C; n = 6). Intra-myocardial TNFα- and IL10-messenger RNA were detected by competitive reverse transcriptase polymerase chain reaction and quantification of cytokine synthesis by Western blot. Levels of cardiac troponin I (cTnI) in cardiac lymph and in arterial and coronary venous blood were examined during and after CPB. Myocardial cell damage was assessed by histologic and ultrastructural anomalies of tissue probes taken 6 h after CPB.

**RESULTS** Synthesis of IL10 was significantly higher, while that of TNFα was significantly lower, in pigs that were in moderate hypothermia during surgery than in the others. In contrast with normothermia, moderate hypothermia was also associated with significantly lower cumulative cardiac lymphatic flow during and after CPB, significantly lower lymphatic cTnI concentrations after CPB, significantly lower percentages of myocardial cell necrosis and a significantly lower score of ultrastructural anomalies of myocardial cells. While the percentage of apoptotic cells was not different between groups, the apoptosis/necrosis ratio tended to be higher in animals that were in moderate hypothermia during surgery. In all animals, TNFα synthesis correlated positively while IL10 production correlated negatively with necrosis and total cell death, respectively.

**CONCLUSIONS** Our results suggest that moderate hypothermia during CPB provides myocardial protection by enhancing intra-myocardial anti-inflammatory cytokine balance. (J Am Coll Cardiol 2001;38:1216–23) © 2001 by the American College of Cardiology

Myocardial damage is a relevant complication of cardiac surgery with cardiopulmonary bypass (CPB). Myocardial ischemia-reperfusion injury and perioperative cytokine balance are thought to play a central role in its pathophysiology (1,2). Cytokine balance during cardiac surgery can be modified by pharmacologic and physical interventions (2,3). In this respect, we recently showed that moderate hypothermia during CPB increases the synthesis of the anti-inflammatory cytokine interleukin-10 (IL10) while blunting the release of the pro-inflammatory tumor necrosis factor-α (TNFα) and provides organ protection (2). The influence of TNFα on myocardial cell damage has been well-recognized in septic shock, myocardial infarction, heart failure (4,5), and myocardial dysfunction after cardiac surgery (6). However, the role of intra-myocardial cytokine balance has not been addressed so far in the setting of cardiac surgery.

The systemic release of inflammatory mediators such as activated complement proteins and ischemia-reperfusion are inductors of TNFα (7,8), which binds specific receptors (4). By binding TNFα type 1 receptor (TNFR1), TNFα induces apoptosis, whereas by binding TNFα type 2 receptor (TNFR2), it activates nuclear factor κ B (NFκB), thus inducing the transcription of other pro-inflammatory cytokines that reinforce its cytotoxic effects and lead to cell necrosis (4). Interleukin-10, a natural monocyte-deactivating cytokine is a strong inhibitor of TNFα and has recently been shown to provide myocardial protection in an animal model of ischemia-reperfusion injury (9).

This experimental study was intended to analyze the influence of core temperature (T°) during CPB on intra-myocardial cytokine balance and to test the hypothesis that intra-myocardial anti-inflammatory cytokine balance relates to myocardial protection.

## METHODS

The study was conducted according to the guidelines of the German Animal Protection Law ensuring humane care and was approved by the supervising state agency for animal experiments. Twelve female pigs (median weight: 40.5 kg,

From the *Departments of Thoracic and Cardiovascular Surgery, †Pediatric Cardiology, ‡Institute of Pathology and §Interdisciplinary Center for Clinical Research "BIOMAT," Aachen University of Technology, Aachen, Germany. Supported by a grant of the "Deutsche Forschungsgemeinschaft," DFG (SE 912/2-1).

Manuscript received February 1, 2001; revised manuscript received May 21, 2001, accepted June 11, 2001.

**Abbreviations and Acronyms**

BW = body weight
CPB = cardiopulmonary bypass
cTnI = cardiac troponin I
H&E = hematoxylin and eosin
IL10 = interleukin-10
mRNA = messenger RNA
NF$\kappa$B = nuclear factor kappa B
NO = nitric oxide
PCR = polymerase chain reaction
PVDF = polyvinylidene difluoride
T° = temperature
TNF$\alpha$ = tumor necrosis factor-$\alpha$
TNFR1 = tumor necrosis factor-$\alpha$ type 1 receptor
TNFR2 = tumor necrosis factor-$\alpha$ type 2 receptor
TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
US = ultrastructural score

range: 35 kg to 45 kg) were randomly assigned to a T° group during CPB (n = 6 each): normothermia (group 1, T° = 37°C) and moderate hypothermia (group 2, T° = 28°C).

General anesthesia was uniform and consisted of a combination of ketamine and pentobarbital. After endotracheal intubation, lungs of the animals were mechanically ventilated with an air/oxygen mixture (inspired oxygen fraction: 0.5). Core T° was continuously monitored using an esophageal T° probe (Probe 16561, Datex Engstrom, Germany). Catheters were placed preoperatively in the carotid artery and jugular vein and intraoperatively in the pulmonary artery and in the coronary sinus.

**Cannulation of the cardiac lymphatic system.** After sternotomy, the efferent cardiac lymph trunk, identified by epicardial injection of Evans blue (0.1 ml, 0.5% aqueous solution) into the right atrial and both ventricular walls, was cannulated with a 18G Cavafix-Certo-Basilica catheter (Braun, Melsungen, Germany), as previously described (10), and cardiac lymph was sampled.

**CPB and myocardial protection.** After cannulation of the cardiac lymphatic trunk, bovine lung heparin (400 IU/kg body weight [BW]) was given for anticoagulation, which was controlled by activated clotting time maintained at a value >450 s throughout the duration of CPB. Both caval veins and the ascending aorta were cannulated, and CPB was instituted.

Cardiopulmonary bypass equipment was uniform and consisted of a roller pump inducing a non-pulsatile flow, a disposable pediatric hollow fiber oxygenator, a hard-shell cardiotomy reservoir, an arterial blood filter and a bubble trap. The extracorporeal perfusion circuit was primed with a crystalloid solution. Cooling and rewarming were performed with a heat exchanger. Total duration of CPB was set at 120 min in all animals with a flow index of 2.4/min/$m^2$ to 2.7 l/min/$m^2$ body surface area. Mean systemic arterial pressure was maintained at about 60 mm Hg. After a perfusion time of 30 min, aorta was cross-clamped for a duration of 60 min and cardioplegia performed with a single dose of cold (4°C) crystalloid solution (Bretschneider solution, 30 ml/kg) injected into the aortic root. After removal of the aortic clamp, perfusion was continued for 30 min. Myocardial T° during CPB was monitored using a needle T° probe placed into the ventricular septum (T° Sensing Catheter, Medtronic Blood Management, Parker, Colorado). Anticoagulation was reversed by protamine. The circulating volume filling the CPB circuit was transfused by a venous passway. Mediastinal drains were placed and the chest closed.

**Postoperative care.** Pigs received standardized postoperative care according to our clinical practice. Postoperative monitoring included continuous registration of heart rate and rhythm, mean arterial blood pressure, left atrial and right atrial pressure, esophageal T° and urine output. Inotropic support consisting of dopamine and fluid administration with a crystalloid solution was adapted in order to optimize hemodynamics. Target value for mean arterial pressure was 60 mm Hg, for left atrial and right atrial pressure 5 mm Hg to 9 mm Hg, respectively, and for urine output 1.5 ml/kg BW. Routine blood examinations included blood gas analysis, arterial lactate concentrations and serum electrolytes. Six hours after CPB, pigs were euthanized with an overdose of pentobarbital. Lymphatic flow was calculated as the volume of lymph, which was drained by gravity over time and is expressed in ml/h.

**Collection of samples.** SAMPLING OF BLOOD. Blood (1.5 ml) taken in EDTA tubes was withdrawn preoperatively from the carotid artery, intraoperatively from the coronary sinus and the arterial line of the oxygenator and postoperatively from the coronary sinus and the carotid artery. Plasma was separated after centrifugation of the collected blood (3,000 rpm for 10 min) and stored at −80°C until assay (Table 1).

**Table 1.** Time Schedule of Sampling of Cardiac Lymph, Plasma and Myocardium

| | cTnI (L) | cTnI (CS) | cTnI (A) | RT-PCR | Histology | EM |
|---|---|---|---|---|---|---|
| Before CPB | X | | X | X | | |
| Before ACC | X | X | X | | | |
| End of CPB | X | X | X | | | |
| 1 h after CPB | X | X | X | | | |
| 6 h after CPB | X | X | X | X | X | X |

A = arterial blood; ACC = aortic cross clamping; CPB = cardiopulmonary bypass; CS = coronary sinus blood; cTnI = cardiac troponin I; EM = electron microscopy; L = cardiac lymph; RT-PCR = reverse transcriptase-polymerase chain reaction.

SAMPLING OF CARDIAC LYMPH. Lymph (1 ml) taken in EDTA tubes was separated after centrifugation (3,000 rpm for 3 min), and the supernatant was stored at −80°C until assay.

SAMPLING OF MYOCARDIAL TISSUE. Before institution of CPB and immediately post-mortem, tissue probes were taken from the anterior wall of the right ventricle, snap-frozen in liquid nitrogen and then stored at −80°C until reverse transcription and polymerase chain and Western blot, respectively. Immediately, post-mortem tissue specimens were taken from the anterior wall of the right ventricle for immuno- and standard-histology and from the left ventricular area supplied by the left anterior descending artery for electron microscopy.

**Cardiac troponin I (cTnI).** Cardiac troponin I was measured by a fluorometric enzyme immunoassay according to the manufacturers recommendations (Stratus, Dade International, Miami, Florida). This cTnI assay is an automated two-site immunoassay that utilizes two monoclonal antibodies specific for the human cardiac isotype of troponin I. The minimum detectable concentration is 1.9 $\mu$g/l.

**Standard histology.** Standard histology tissue specimens were flushed with 0.9% NaCl and fixed in 10% buffered formalin, embedded in paraffin and sectioned. The sections (4 $\mu$m) were stained with hematoxylin and eosin (H&E), elastica van Gieson and periodic acid Schiff.

**Histological score.** Histological alterations (edema, leukostasis, cell necrosis and focal bleeding) were scored from 0 to 3, which has been described previously (2), as follows: "0" no alterations; "1" slight alterations; "2" moderate alterations; and "3" severe alterations.

**Detection of necrosis.** Detection of necrosis was assessed in serial sections stained with H&E. Morphologic criteria of cell necrosis included cell swelling, disruption of cell membrane, karyolysis and hypereosinophilia (11). Necrotic cells were counted in five different fields using light microscopy at 400-fold magnification (an average of 500 cells was counted per sample), and the average number per field was calculated. Data were reported as the percentage of necrotic cells detected.

**Detection of apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling histochemistry [TUNEL]).** Detection of apoptosis (TUNEL-histochemistry) was performed on replicate of the paraffin sections using TUNEL assay (ApopTag Oncor, Gaithersburg, Maryland) according to the manufacturers recommendations. For quantification of apoptosis, samples with TUNEL-fluorescence staining were investigated by confocal laser scanning microscopy (Laser Scan Microscopy LSM 410 invert with Axiovert 135M, ZEISS, Germany). Apoptotic cells in 12 different fields were counted (an average of 1,000 cells were counted per sample). Data were reported as the percentage of apoptotic cells. Adjacent H&E sections were examined in order to assess typical morphologic findings of apoptosis (cell shrinkage, chromatin condensation and margination and apoptotic bodies) (11). Histological score, quantification of necrosis and apoptosis were assessed in blinded fashion by two investigators (M.Q. and B.K.).

**Electron microscopy.** Myocardial samples were fixed in 2.5% cacodylate-buffered glutardialdehyde for 30 min. After post-fixation in osmium, buffered in 0.1-M cacodylate, probes were dehydrated in ethanol and embedded in epon. Semithin sections (1.0 $\mu$m) were stained with ethylene blue-azure II. Ultra-thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Philips, EM400T). Ultrastructural changes with regard to cell membrane integrity, structure/number of mitochondria and integrity of myofibrils, compared with reference findings in normal pigs were scored as follows: "0" normal ultrastructure; "1" slight changes ≤50%; and "2" marked changes >50%.

**Reverse transcription polymerase chain reaction (PCR).** Total RNA was extracted using RNeasy Mini Kit (QIAGEN Inc., Germany). RNA (3 $\mu$g) was reverse transcribed to complementary DNA using random hexamers. Using primers for TNF$\alpha$, IL10 and $\beta$-actin, complementary DNA products were coamplified by PCR (35 cycles; 94°C for 45 s, 54°C for 45 s and 72°C for 45 s). Specific porcine primers used were as follows: TNF$\alpha$ (Genbank accession, no. X54859): sense primer ATCGGCCCCCAGAAGGAAGAG and anti-sense primer GATGGCAGAGAGGAGGTTGAC; IL10 (Genbank accession, no. L20001): sense primer GCATCCACTTCCCAACCA and anti-sense primer CTTCCTCATCTTCATCGTCAT, and $\beta$-actin (Genbank accession, no. U07786): sense primer GGACTTCGAGCAGGAGATGG and anti-sense primer GCACCGTGTTGGCGTAGAGG. The PCR products were subjected to electrophoresis in 1.8% agarose gel, stained with ethidium bromide and photographed. The predicted lengths of amplification products for TNF$\alpha$, IL10 and $\beta$-actin were 372, 446 and 233 base pairs (bp), respectively. Results are presented as the ratio of the band intensities of TNF$\alpha$- and IL10-messenger RNA (mRNA) over the corresponding $\beta$-actin-mRNA (Bio-Rad's Multi-Analyst, Bio-Rad,).

**Western blot.** Total protein homogenates (100 $\mu$g) were denatured and separated on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Millpore, Eschborn, Germany). The PVDF membranes were probed using primary antibodies against TNF$\alpha$ and IL10 (both polyclonal rabbit antihuman, Biermann, Germany) and $\beta$-actin (monoclonal mouse antihuman, Sigma, St. Louis, Missouri). Visualization of the protein bands was accomplished using an ECL chemiluminescence system according to the manufacturer's instructions (Amersham-Pharmacia, Freiburg, Germany). Signals for TNF$\alpha$, IL10 and $\beta$-actin produced bands of 17, 18 and 43 kDa, respectively. Protein signals for TNF$\alpha$ and IL10 were normalized for $\beta$-actin

signals that were developed on the same blot (NIH imaging 1.61b8. software, Bio-Rad,).

**Statistical analysis.** Results are expressed as the mean ± SEM. Differences between groups were analyzed by analysis of variance with the Scheffé procedure for repeated comparisons. Paired *t* tests were used for intergroup comparisons. Correlation analysis was done by Pearson's correlation test, and contingency tables with the Fisher exact test. A p value $<0.05$ was considered statistically significant. Data were analyzed with SPSS (SPSS Software GmbH, München, Germany).

## RESULTS

Esophageal and myocardial T° were similar in both groups before CPB. Esophageal T° before aortic clamping was significantly lower in pigs that were in moderate hypothermia during surgery (28.2 ± 0.3°C) (mean ± SEM) than in the other group (35.9 ± 0.7°C) ($p < 0.0001$). This difference persisted up to aortic declamping (28.9 ± 0.5°C vs. 37.4 ± 0.3°C, $p < 0.0001$) and to the end of CPB (35.6 ± 0.3°C vs. 36.9 ± 0.2°C, $p < 0.02$), respectively. In contrast, myocardial T° during CPB was not different between groups, with the exception of that measured before clamping of the aorta (28.1 ± 0.3°C vs. 34.6 ± 0.4°C, $p < 0.0001$). In both groups, myocardial T° reached its minimal value after cardioplegia (12.7 ± 0.6°C vs. 13.9 ± 1.7°C, p = NS).

**Hemodynamics.** Hemodynamic data—including heart rate, mean arterial pressure, left- and right-atrial pressures and urine output—were not different between groups (data not shown). Inotropic support after CPB was not necessary in animals that were in moderate hypothermia during surgery, by contrast with the others (dopamine: 0 ± 0 μg/kg BW/min vs. 5.3 ± 1.5 μg/kg BW/min) ($p < 0.05$).

**Lymph flow rate.** Pigs that were in moderate hypothermia during surgery had a significantly lower lymphatic flow rate during and after CPB than pigs in normothermia during surgery. In fact, in the former, the lymphatic flow rate did not increase at all during or after CPB (Fig. 1). Cumulative lymphatic flow rate during and after CPB averaged 8.9 ± 3.0 ml versus 26.4 ± 8.7 ml in pigs that were in moderate hypothermia during surgery and those in normothermia, respectively ($p < 0.05$).

**cTnI levels.** In all animals, plasma cTnI levels were undetectable before CPB and rose significantly during and after CPB in both arterial and coronary venous blood, without any difference between groups (Table 2). In all animals, cTnI concentrations in cardiac lymph averaged 400 ± 145 μg/l before the institution of CPB. The cTnI was not significantly released in cardiac lymph during CPB, but only later, reaching its peak level 6 h after CPB. At that time, cTnI concentrations in cardiac lymph were significantly lower in pigs that were in moderate hypothermia during surgery than in those in normothermia (Table 2). There was no correlation between cTnI levels in the cardiac lymph and in the coronary venous blood or arterial blood, respectively.

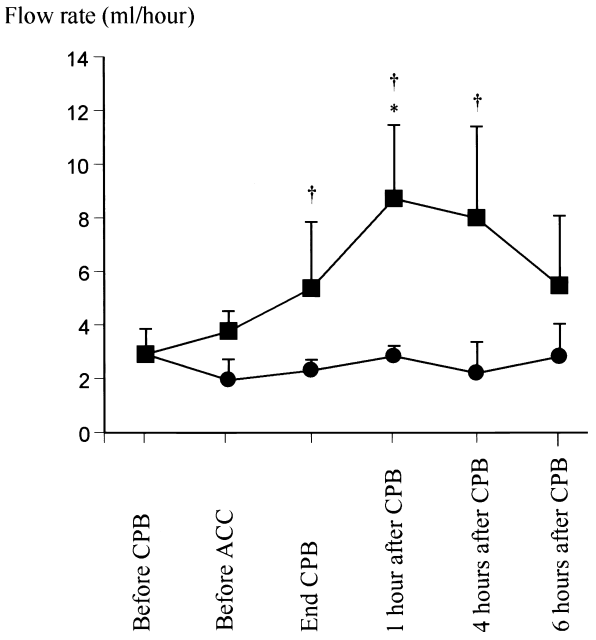


**Figure 1.** Flow rate of cardiac lymph before, during and after CPB in pigs that were in moderate hypothermia during surgery (temperature [T°]: 28°C) **(solid circle)** or in normothermia during surgery (T°: 37°C) **(solid square)**. *$p < 0.05$ between groups; †$p < 0.05$ versus lymph flow measured before CPB in all animals. ACC = aortic cross clamping; CPB = cardiopulmonary bypass.

**Histologic score and percentage of cell necrosis.** Compared with animals that were in normothermia during surgery, pigs in moderate hypothermia showed significantly lower histologic scores (1.0 ± 0.0 vs. 2.8 ± 0.2, $p < 0.001$) and a significantly lower percentage of cell necrosis (0.2 ± 0.1% vs. 3.2 ± 0.3%, $p < 0.0001$). Percentage of necrotic myocardial cells and lymphatic or blood concentrations of cTnI were not correlated with each other.

**Apoptosis.** The TUNEL-positive myocardial cells were located in the subendomyocardium of the ventricular tissue. The percentage of apoptotic cells was not significantly different between both groups and averaged 1.0 ± 0.4% in the hypothermia group and 0.6 ± 0.4% in normothermia group, respectively. The apoptosis/necrosis ratio tended to be higher (4.1 ± 2.9 vs. 0.3 ± 0.2, p = 0.05), and total cell death by necrosis and apoptosis was significantly lower, in animals that were in moderate hypothermia during surgery than in the others (1.3 ± 0.6 vs. 3.8 ± 0.3, $p < 0.01$).

The percentage of apoptotic myocardial cells and lymphatic or blood concentrations of cTnI were not correlated with each other.

**Electron microscopy.** Compared with normal porcine myocardium, all animals investigated showed a normal contractile apparatus with regular myofibril texture, intact Z and intercalated discs with only sparse signs of myolysis (ultrastructural score [US]: 0.75 ± 0.71 vs. 1.29 ± 0.76 in the hypothermia and normothermia groups, respectively; p = NS). A similar slightly increased number of mitochondria was observed in all (US: 0.5 ± 0.76 vs. 1.0 ± 0.58 in the hypothermia and normothermia groups, respectively;

**Table 2.** Course of Plasma and Lymph cTnI Levels During and After CPB in Pigs in Moderate Hypothermia During Surgery or in Normothermia

| | Coronary Venous Blood | | | Arterial Blood | | | Cardiac Lymph | | |
|---|---|---|---|---|---|---|---|---|---|
| **Time Points** | **28°C** | **37°C** | **p Value** | **28°C** | **37°C** | **p Value** | **28°C** | **37°C** | **p Value** |
| Before ACC | 0.48 ± 0.1 | 0.37 ± 0.05 | NS | 0.18 ± 0.05 | 0.34 ± 0.08 | NS | 169 ± 62 | 200 ± 77 | NS |
| End of CPB | 3.75 ± 0.8* | 5.37 ± 1.4* | NS | 2.8 ± 0.8* | 2.82 ± 0.5* | NS | 324 ± 109 | 239 ± 90 | NS |
| 1 h after CPB | 7.93 ± 1.7* | 8.97 ± 2.4* | NS | 6.1 ± 0.9* | 7.64 ± 2.0* | NS | 1,047 ± 488* | 1,428 ± 272* | NS |
| 6 h after CPB | 18.9 ± 3.7* | 13.9 ± 3.9* | NS | 16.8 ± 2.3* | 20.3 ± 4.2* | NS | 648 ± 438* | 2,186 ± 358 | <0.05 |

Cardiac troponin I (cTnI) levels are expressed in μg/l, results as mean ± SEM. Coronary venous and arterial cTnI levels significantly increase over time (*$p < 0.05$ vs. levels measured before ACC) without any difference between pigs in moderate hypothermia during surgery (28°C) and in normothermia (37°C); cTnI concentrations in cardiac lymph, which were by far higher than in plasma already before CPB and ACC, did not increase during, but after, CPB (*$p < 0.05$ vs. levels measured before CPB and ACC).
ACC = aortic cross clamping; CPB = cardiopulmonary bypass.

p = NS). Animals that were in moderate hypothermia during surgery showed significantly fewer structural mitochondrial anomalies such as swelling, cristae rarefaction and ruptures (US: 0.13 ± 0.35) than animals in normothermia during surgery (US: 1.14 ± 0.69, $p < 0.005$). Discrete focal membrane lesions were observed in the hypothermia group (US: 0.13 ± 0.35), while recurrent membrane ruptures were present in the normothermia group (US: 0.86 ± 0.90; $p < 0.05$) (Fig. 2). Ischemic lesions such as absent myofibrillar banding due to thickening of the Z-disc were not observed.

**Gene expression and synthesis of TNFα and IL10 in the myocardium.** Tumor necrosis factor-α- and IL10-mRNA were not detected in the myocardium before the institution of CPB but 6 h after CPB. At that time, pigs that were in moderate hypothermia during surgery showed a significantly lower expression of TNFα-mRNA than those in normothermia ($p < 0.0001$) and tended to show higher expression of IL10-mRNA ($p < 0.1$) (Fig. 3). In fact, only 2/6 pigs in moderate hypothermia during surgery, but 6/6 in normothermia, showed myocardial TNFα-mRNA expression ($p < 0.05$). Conversely, IL10-mRNA was detected in 6/6 pigs and in 2/6 pigs that were in moderate hypothermia and normothermia, respectively ($p < 0.05$).

Considering all animals, TNFα-mRNA expression positively correlated with myocardial cell necrosis and total myocardial cell death (Pearson correlation coefficient: 0.87, $p < 0.001$ and 0.95, $p < 0.0001$), respectively, while IL10-mRNA expression negatively correlated with the percentage of myocardial cell necrosis (Pearson correlation coefficient: −0.63, $p < 0.05$).

Western blot analysis showed the absence of both TNFα and IL10 in the myocardium before the institution of CPB. However, 6 h after CPB, pigs in the moderate hypothermia group showed significantly higher intra-myocardial production of IL10 and significantly lower production of TNFα than those in the normothermia group ($p < 0.05$, respectively) (Fig. 4). Tumor necrosis factor-α production correlated positively with myocardial cell necrosis (Pearson correlation coefficient: 0.70, $p = 0.05$), while production of IL10 correlated negatively with total cell death (Pearson correlation coefficient: −0.80, $p < 0.02$).

## DISCUSSION

In this study, we demonstrate for the first time gene expression and synthesis of the pro-inflammatory cytokine TNFα and of the anti-inflammatory cytokine IL10 in the myocardium of animals submitted to cardiac surgery with CPB. Synthesis of TNFα was significantly lower, and that

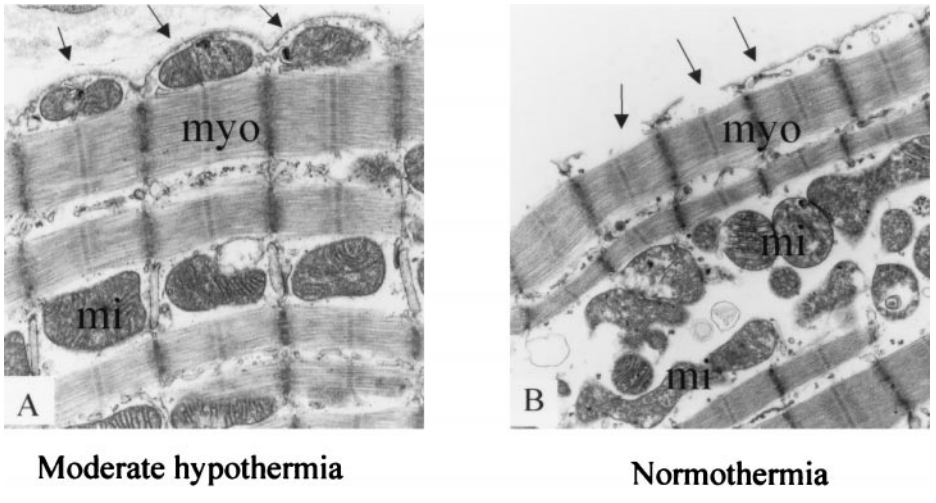


**Figure 2.** Example of ultrastructural examination showing discrete focal lesions of cell membrane **(arrows)** and mitochondria (mi) in animals that were in moderate hypothermia during surgery **(A)** compared with membrane rupture **(arrows)**, mitochondrial swelling with cristae rarefaction and rupture (mi) in animals in normothermia during surgery **(B)**. Both groups show normal contractile apparatus. myo = myofibril.

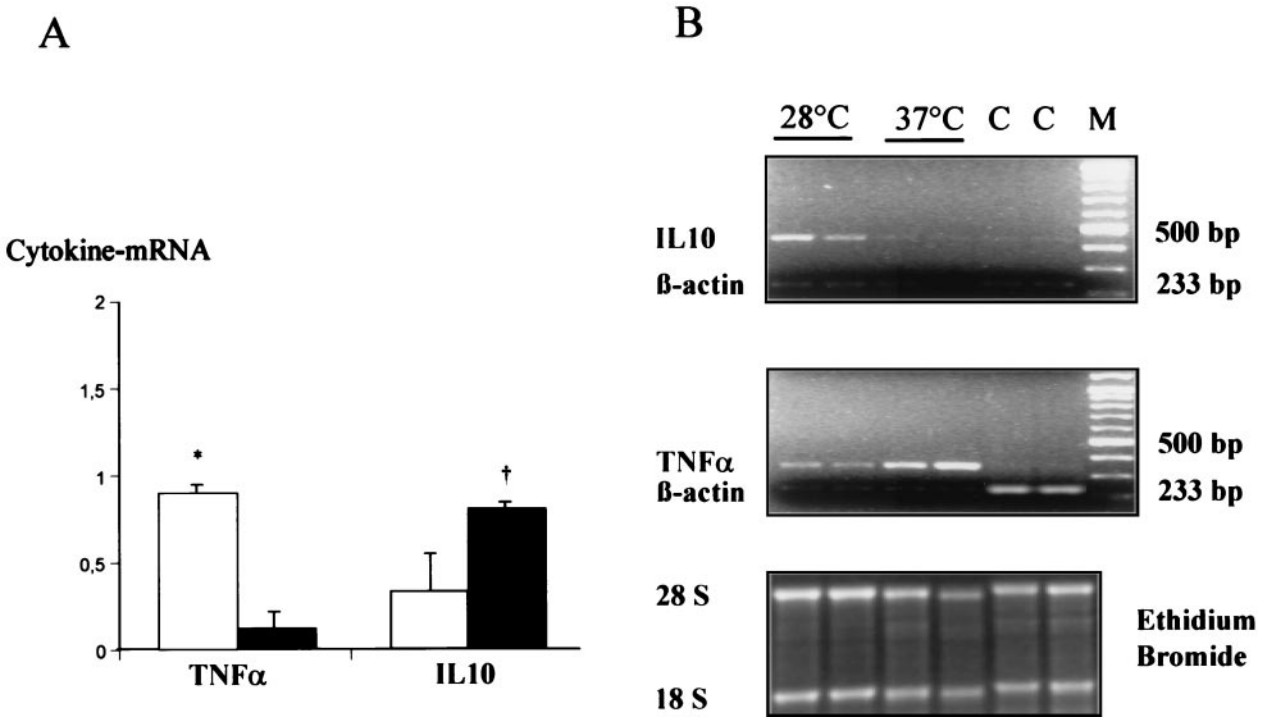


**Figure 3. (A)** Gene expression of tumor necrosis factor-$\alpha$ (TNF$\alpha$) and interleukin-10 (IL10) detected in the myocardium taken 6 h after cardiopulmonary bypass (CPB) by reverse transcript polymerase chain reaction in pigs that were in moderate hypothermia during surgery (temperature [T°]: 28°C) **(solid bar)** and in normothermia during surgery (T°: 37°C) **(open bar)**. Results are expressed as the ratio of cytokines messenger RNA (mRNA)/$\beta$-actin mRNA and are shown as mean ± SEM. *$p < 0.0001$ and †$p < 0.1$ between groups, respectively. **(B)** Exemplary gel representative for six independent experiments in each group (moderate hypothermia: T°, 28°C and normothermia: T°, 37°C) showing the effect of core T° during CPB on TNF$\alpha$ and IL10 gene expression. **Lower panel** shows corresponding 18S and 28S ribosomal RNA bands as loading control. C = control sample before CPB; M = molecular-weight marker.

of IL10 significantly higher, in pigs that were in moderate hypothermia during surgery than in those in normothermia.

**Effects of TNF$\alpha$ on myocardial cell damage and cell death.** Potential stimuli for intra-myocardial TNF$\alpha$ synthesis during cardiac surgery are inflammatory mediators such as activated complement proteins (7) released into the systemic circulation, the presence of which precedes increased circulating levels of pro-inflammatory cytokines (12) and ischemia followed by reperfusion (8). Tumor necrosis factor-$\alpha$ exerts immediate negative inotropic effects on cardiomyocytes that are mediated by sphyngosine disruption of calcium currents, independent of nitric oxide (NO) and delayed NO-dependent negative inotropic effects due to myofilament desensitization to calcium (13). Besides this, TNF$\alpha$ has strong cytotoxic effects on myocardial cells, leading to gap formation in the cell membrane, cell membrane rupture, interstitial edema and, finally, cell necrosis (14). Furthermore, by binding its receptor TNFR1, the cytosolic component of which relates to the death domain, TNF$\alpha$ induces apoptosis (4). Finally, TNF$\alpha$ promotes apoptosis by activating the pro-apoptosis protein Fas, by sphyngosine formation and by inducting NO production (4,14).

Our results, showing myocardial cell damage, mitochondrial anomalies and the presence of necrotic and apoptotic cells in animals undergoing cardiac surgery, suggest that TNF$\alpha$ is, at least in part, responsible for the lesions observed. This is supported by the correlation we observed between intra-myocardial synthesis of TNF$\alpha$ and myocardial cell necrosis. The functional importance of these findings is documented by the loss of cTnI into the myocardial interstitial space, and thus into the cardiac lymph, as shown here for the first time in an in vivo model of cardiac surgery. Lymphatic cTnI concentrations were shown to be far higher than arterial or coronary venous concentrations, pointing out the superiority of investigating cardiac lymph for the estimation of myocardial cell damage related to cardiac surgery. As expected (15), cTnI loss into the interstitial space was not correlated with myocardial cell death. Indeed, cTnI lymphatic and blood concentrations are likely to reflect global myocardial cell damage, including transient disruption of cell membrane as well as myocardial cell death by necrosis and apoptosis.

**Cardiac surgery and intra-myocardial cytokine balance.** Our study provides evidence for the first time that the anti-inflammatory cytokine IL10 is synthesized in the myocardium during and after cardiac surgery. The cellular origin of IL10 has not been investigated in our series. In a recent study, however, IL10-mRNA was shown to be expressed by CD5-positive lymphocytes infiltrating the myocardium in a canine model of ischemia-reperfusion (9). Interleukin-10 is a monocyte-deactivating cytokine that is a strong inhibitor of the synthesis of several pro-inflammatory cytokines including TNF$\alpha$, IL1$\beta$, IL6 and IL8 (16).

JACC Vol. 38, No. 4, 2001
October 2001:1216–23

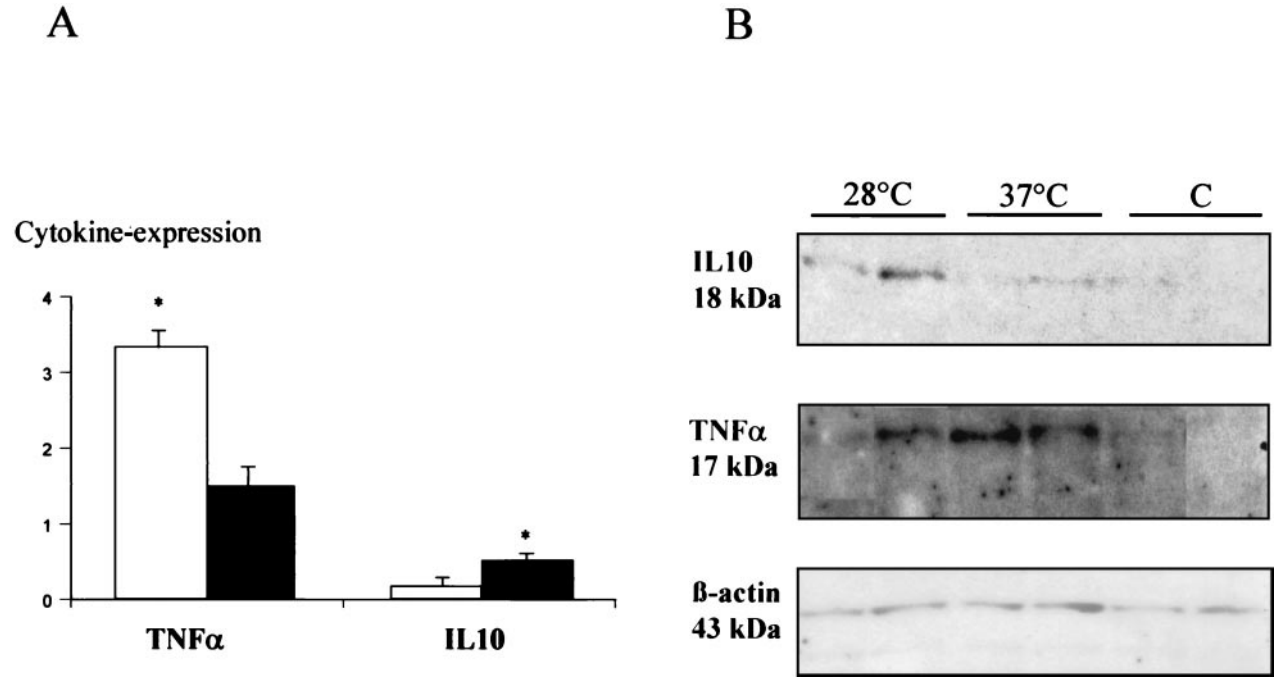


**Figure 4.** **(A)** Synthesis of tumor necrosis factor-$\alpha$ (TNF$\alpha$) and interleukin-10 (IL10) detected in the myocardium 6 h after cardiopulmonary bypass (CPB) by Western blot in pigs that were in moderate hypothermia during surgery (temperature [T°]: 28°C) **(solid bar)** and in normothermia (T°: 37°C) **(open bar)**. Band intensities for cytokines were normalized for bands of $\beta$-actin. Results are shown as mean ± SEM. *$p < 0.05$ between groups, respectively. **(B)** Exemplary gel obtained after Western blot representative for six independent experiments in each group (moderate hypothermia: T°, 28°C and normothermia: T°, 37°C) showing the effect of core T° during CPB on TNF$\alpha$ and IL10 synthesis. C = control sample before CPB.

Interleukin-10 is thought to control the systemic inflammatory reaction related to cardiac surgery with CPB (17) by down-regulating the expression of the above-mentioned cytokines at the transcriptional level, mainly by inhibiting NF$\kappa$B (18). In a mouse model of ischemia-reperfusion injury, endogenous IL10 production has recently been shown to inhibit the production of TNF$\alpha$ and NO and to protect the myocardium by down-regulating intercellular adhesion molecule-1, thus reducing endothelial-neutrophil interactions and recruitment of activated neutrophils into the myocardium (19).

**Anti-inflammatory and cardioprotective effects of moderate hypothermia.** We showed in a previous experimental study that moderate hypothermia during CPB increases IL10 blood concentrations and blunts TNF$\alpha$ production (2). We demonstrate here that systemic moderate hypothermia leads to increased gene expression and synthesis of IL10 in the myocardium after CPB and that this is related to myocardial protection. Despite the short observational period of 6 h, which did not allow us to extrapolate the outcome of the animals investigated, a substantial clinical benefit could be noticed, wherein the need for inotropic support to maintain stable hemodynamics was less in animals that were in moderate hypothermia during surgery. This cardioprotective effect of moderate hypothermia related to anti-inflammatory cytokine balance shown here could justify its use in clinical practice, especially in patients with severe preoperative heart failure in whom pro-inflammatory cytokine synthesis in the myocardium is thought to contribute to myocardial dysfunction (4).

The mechanisms by which core hypothermia influences cytokine-mRNA expression in the myocardium are presently unknown. The fact that myocardial T° was significantly different between both groups only before aortic clamping, but not during and after CPB, suggests that a systemic rather than a local process is responsible. This is in line with our previous report showing systemic anti-inflammatory cytokine preponderance with reduced leukocyte mobilization in animals that were in moderate hypothermia during surgery (2). It is unclear, however, whether a systemic circulating factor induced by moderate hypothermia could activate transcription factors of the STAT-family, which are recognized transcription factors of IL10 gene expression (20).

In our series, moderate hypothermia, in comparison with normothermia, was associated with significantly lower myocardial cell necrosis and total cell death with an increased apoptosis/necrosis ratio, indicating privileged induction of apoptosis of damaged myocardial cells. Because apoptosis is a noninflammatory and self-limiting process (21), this could be interpreted as an additional protective mechanism of moderate hypothermia. Moderate hypothermia has been reported to be associated with apoptosis in many cell types (22), but the mechanisms of its induction are unclear. In our series, anti-inflammatory cytokine balance with increased IL10 production could have favored apoptosis (23). Indeed,

IL10 has been shown to limit local inflammation in a model of pneumonia (24) and to play a protective role in sepsis by enhancing neutrophil apoptosis (25).

In this study, the effect of myocardial cell damage and the loss of cTnI itself also could have contributed to the balance between apoptosis and necrosis. Indeed, the balance between cTnI degradation and formation of stable covalent complexes after ischemia-reperfusion might determine whether myocardial cells will die by necrosis (induced by higher cTnI degradation) or apoptosis (induced by stable covalent complexes) (26).

**Conclusions.** Our results suggest that moderate hypothermia during CPB protects the myocardium by modifying intra-myocardial cytokine balance with increased synthesis of IL10 and decreased production of TNF$\alpha$. This net anti-inflammatory cytokine response could be responsible for myocardial protection during cardiac surgery by inhibiting necrosis and causing privileged induction of apoptosis of damaged myocardial cells.

**Reprint requests and correspondence:** Dr. Jaime F. Vazquez-Jimenez, Department of Thoracic and Cardiovascular Surgery, University Hospital, Aachen University of Technology, Pauwelsstrasse 30. D-52057 Aachen, Germany. E-mail: jvazquez-jimenez@post.klinikum.rwth-aachen.de.

## REFERENCES

1. Dreyer WJ, Phillips SC, Lindsey ML, et al. Interleukin 6 induction in the canine myocardium after cardiopulmonary bypass. J Thorac Cardiovasc Surg 2000;120:256–63.
2. Qing M, Vazquez-Jimenez JF, Klosterhalfen B, et al. Influence of temperature during cardiopulmonary bypass on leukocyte activation, cytokine balance and postoperative organ damage. Shock 2001;15:372–7.
3. Tabardel Y, Duchateau J, Schmartz D, et al. Corticosteroids increase blood interleukin-10 levels during cardiopulmonary bypass in men. Surgery 1996;119:76–80.
4. Meldrum DR. Tumor necrosis factor in the heart. Am J Physiol 1998;274:R577–R595.
5. Torre-Amione G, Kapadia S, Benedict C, et al. Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). J Am Coll Cardiol 1996;27:1201–6.
6. Hennein HA, Ebba H, Rodriguez JL, et al. Relationship of the proinflammatory cytokines to myocardial ischemia and dysfunction after uncomplicated coronary revascularization. J Thorac Cardiovasc Surg 1994;108:626–35.
7. Weiler JM. Introduction. In: Whaley K, Loos M, Weiler J, editors. Complement in Health and Disease. Dordrecht, Boston, London: Kluwer Academic Publishers, 1993:1–37.
8. Meldrum DR, Meng X, Dinarello CA, et al. Human myocardial tissue TNF-alpha expression following acute global ischemia in vivo. J Mol Cell Cardiol 1998;30:1683–9.
9. Frangogiannis NG, Mendoza LH, Lindsey ML, et al. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. J Immunol 2000;165:2798–808.
10. Vazquez-Jimenez JF, Seghaye M, Qing M, et al. Cannulation of the cardiac lymphatic system in swine. Eur J Cardiothorac Surg 2000;18:228–32.
11. Gujral JS, Bucci TJ, Farhood A, et al. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? Hepatology 2001;33:397–405.
12. Kalfin RE, Engelman RM, Rousou JA, et al. Induction of interleukin-8 expression during cardiopulmonary bypass. Circulation 1993;88:II401–II406.
13. Kelly RA, Smith TW. Cytokines and cardiac contractile function (editorial). Circulation 1997;95:778–81.
14. Laster SM, Wood JG, Gooding LR. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 1988;141:2629–34.
15. Sobel BE, LeWinter MM. Ingenuous interpretation of elevated blood levels of macromolecular markers of myocardial injury: a recipe for confusion. J Am Coll Cardiol 2000;35:1355–8.
16. de Waal M, Abrams J, Bennett B, et al. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209–20.
17. Seghaye M, Duchateau J, Bruniaux J, et al. Interleukin-10 release related to cardiopulmonary bypass in infants undergoing cardiac operations. J Thorac Cardiovasc Surg 1996;111:545–53.
18. Schottelius AJ, Mayo MW, Sartor RB, et al. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. J Biol Chem 1999;274:31868–74.
19. Yang Z, Zingarelli B, Szabo C. Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. Circulation 2000;101:1019–26.
20. Benkhart EM, Siedlar M, Wedel A, et al. Role of Stat3 in lipopolysaccharide-induced IL-10 gene expression. J Immunol 2000;165:1612–7.
21. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456–62.
22. Matijasevic Z, Snyder JE, Ludlum DB. Hypothermia causes a reversible, p53-mediated cell cycle arrest in cultured fibroblasts. Oncol Res 1998;10:605–10.
23. Schmidt M, Lugering N, Pauels HG, et al. IL-10 induces apoptosis in human monocytes involving the CD95 receptor/ligand pathway. Eur J Immunol 2000;30:1769–77.
24. Cox G. IL-10 enhances resolution of pulmonary inflammation in vivo by promoting apoptosis of neutrophils. Am J Physiol 1996;271:L566–L571.
25. Keel M, Ungethum U, Steckholzer U, et al. Interleukin-10 counterregulates proinflammatory cytokine-induced inhibition of neutrophil apoptosis during severe sepsis. Blood 1997;90:3356–63.
26. McDonough JL, Arrell DK, Van Eyk JE. Troponin I degradation and covalent complex formation accompanies myocardial ischemia/reperfusion injury. Circ Res 1999;84:9–20.