Inflammatory Response After Open Heart Surgery
Release of Heat-Shock Protein 70 and Signaling Through Toll-Like Receptor-4

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Background — Coronary artery bypass grafting with the use of cardiopulmonary bypass is known to mediate an inflammatory response. The stress-inducible heat-shock protein (HSP) 70 has been detected in myocardial cells after CABG, and toll-like receptors (TLRs) are suggested as putative signaling receptors for the HSPs, mediating synthesis of inflammatory cytokines. The main aims of our study were to explore the release of HSP70 and the regulation of monocyte TLR-2 and TLR-4 expression after CABG.

Methods and Results — Twenty patients referred for elective CABG were included in this study. Using immunoassays, we detected HSP70 in plasma after CABG, with peak concentration immediately after surgery. Interleukin-6 in plasma reached peak concentration 5 hours after surgery. Monocyte CD14, TLR-2, and TLR-4 expression, as analyzed by flow cytometry, was initially downregulated. On day 1, CD14 expression normalized, whereas TLR-2 and TLR-4 expression was upregulated. TLR-4 was significantly upregulated even on postoperative day 2. Additional experiments revealed that peritoneal macrophages from control (C3H/HeN) mice responded to HSP70 with release of tumor necrosis factor, whereas macrophages from mutated TLR-4 (C3H/HeJ) mice were unresponsive. In vitro, human adherent monocytes responded to recombinant HSP70 with interleukin-6 and tumor necrosis factor release. CD14 and TLR-4 monoclonal antibodies inhibited the cytokine response.

Conclusions — In this study, we observed an immediate release of HSP70 into the circulation and a modulation of monocyte TLR-2 and TLR-4 expression after CABG. TLR-4 and CD14 appear to be involved in an HSP70-mediated activation of innate immunity. (Circulation. 2002;105:685-690.)

Key Words: heat-shock proteins ■ toll-like receptors ■ inflammation ■ cardiopulmonary bypass ■ angina

It is well recognized that CABG with the use of cardiopulmonary bypass (CPB) causes an inflammatory response.1–4 This may be related to the surgical trauma, myocardial ischemia and reperfusion, anesthesia, cardioplegia, and the heart-lung machine. It has been suggested that microbial invasion may contribute to the postoperative inflammatory response.5 Nevertheless, noninfectious, endogenous danger signals may be released during surgery, thus activating innate immunity.

Heat-shock proteins (HSPs) are abundant intracellular proteins found in both prokaryotic and eukaryotic organisms. Their main function appears to be as chaperones, involved in protein folding and transport.6 The HSP70 family, located in the cytosol and the nucleus of the cell, is the most conserved and the best-studied class of HSPs and includes the constitutive HSP73 (HSC70) and the stress-inducible HSP70 (HSP72).7 Myocardial ischemia induces HSP70 as a stress response,7 and HSP70 is increased in atrial tissue of patients with unstable angina.8 Demidov and coworkers9 found induction of HSP70 in myocardial cells in 40% of 33 patients undergoing CABG. Circulating inducible HSP70 after CABG has, to our knowledge, not been demonstrated previously.

The receptor complex of CD14, toll-like receptor (TLR)-4, and MD-2 on monocytes appears to be the principal receptor complex of lipopolysaccharide (LPS), mediating activation of nuclear factor-kappa B and synthesis of proinflammatory cytokines.10–14 It appears that TLR-2 is important for cell activation of some Gram-positive bacteria.14,15 Chen et al16 observed that HSP60 had immunostimulatory properties when added to macrophage cultures. Extracellular human HSP60 and HSP70 appear to activate innate immunity by a CD14-dependent mechanism,17,18 and TLR-4 is suggested to be involved in HSP60 signaling.19
We hypothesized that modulation of monocyte TLR-2 and TLR-4 expression was induced by heart surgery. Accordingly, the first aim of the present study was to investigate the monocyte surface expression of TLR-2 and TLR-4 in patients through the first 2 days after CABG. Our second aim was to explore the potential release of Hsp70 into plasma, hypothesizing HSP70 to be an endogenous ligand for TLRs on monocytes, thus mediating synthesis of proinflammatory cytokines.

**Methods**

### Patients

From April through June 2000, 20 patients referred for elective CABG at the Department of Cardiothoracic Surgery/St Elisabeth Heart Center, University Hospital of Trondheim, Norway, were included in the study. The patients had angiographically verified 3-vessel or in a few cases 2-vessel disease, were <75 years old, and before surgery had no signs of infection. Emergency cases and patients undergoing combined procedures or reoperations were excluded. The regional ethics committee approved the study protocol, and informed consent was obtained from each subject.

### Anesthesia and Surgical Procedure

All patients underwent a routine procedure with median sternotomy and use of CPB. Anesthesia was induced with diazepam, fentanyl, thiopental, and pancuronium and maintained with isoflurane, fentanyl, and nitrous oxide until the start of CPB. We used cephalothin as perioperative antibiotic prophylaxis. The activated clotting time was maintained >480 seconds during CPB, and CPB was performed with nonpulsatile flow of 2.4 L/min per m² body surface area with a roller-pump. Patients were cooled down to 34 °C at the beginning of CPB, and active rewarming was started during the last distal coronary anastomosis. St Thomas cardioplegic solution No. 1 was used in 17 patients; 3 patients had cold blood cardioplegia. A membrane oxygenator with synthetic, biocompatible surfaces (Maxima Medtronic) was used for all patients. The pump prime consisted of 1800 mL Ringer solution and 7500 IU heparin. After CPB, heparin was neutralized with protamine sulfate until the preoperative level of 100 U/mL was reached. All patients were cooled to 4°C before induction of anesthesia, 30 minutes after completion of surgery, 4 hours thereafter, and at 8 AM on days 1 (19 hours) and 2 (43 hours) after surgery. VACUTAINER CPT cell preparation tubes with sodium heparin (Becton Dickinson VACUTAINER Systems) were used, and samples were centrifuged within 20 minutes of sampling. After washing, peripheral blood mononuclear cells (PBMCs) were resuspended in freezing medium. Both plasma and PBMC samples were stored at -80°C until analysis.

### Plasma Analyses

Interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β in plasma were measured with Quantikine Human Immunoassays (R&D Systems, Inc) with sensitivities 0.70 pg/mL, 4.4 pg/mL, and 1 pg/mL, respectively. HSP70 in plasma was detected with Hsp70 EIA Kit, sensitivity 200 pg/mL (StressGen Biotechnologies Corp). Plasma Analyses

### Flow Cytometry

Flow cytometric analyses were carried out with the use of an Epics XL-MCL flow cytometer and Expo 32 ADC software (Beckman Coulter Inc). All PBMC samples from each patient were analyzed consecutively, with identical flow cytometer settings and immunostaining procedure. We used PBMCs from one healthy person as an internal control each time flow cytometry was performed. Control experiments indicated that freezing of PBMC samples did not change monocyte surface CD14, TLR-2, or TLR-4 expression when compared with fresh cells (data not shown). The PBMC samples were incubated with the following monoclonal antibodies (mAbs) in different wells: Fluorescein isothiocyanate (FITC)-conjugated human CD14 mAb clone 1B12 (Diaclone) with FITC–goat anti-mouse (GAM) (Becton Dickinson) as control. We used an Alexa488-conjugated (Molecular Probes) TLR-2 mAb (TL 2.1) and an Alexa488-conjugated TLR-4 mAb (HTA125, gift of Kensuke Miyake, Saga Medical School, Japan) with an Alexa488-stained, isotype-matched irrelevant antibody as control. Gating of monocytes was done during analyses according to forward and side-scatter dot plots, and the mean fluorescence intensity (MFI) of ~5000 gated monocytes was measured. To see data interpretation, preoperative MFI values were set to 100%, and postoperative values were expressed as percentages of the initial value for each patient.

### In Vitro Studies

Peritoneal macrophages from C3H/HeN and C3H/HeJ mice (Harlan Ltd, Oxon, UK) were collected by lavage 3 days after intraperitoneal injection of 3% thiglycollate (3 mL, Difco). Cells (5x10⁶ cells/well in 24-well dishes) were adhered for 2 hours, and after washing, macrophages were stimulated in Roswell Park Memorial Institute (RPMI) 1640 medium 10% FCS for 6 hours in a total volume of 0.25 mL. Recombinant human (rhu) HSP70 (SPP-755, StressGen Biotechnologies Corp, Victoria, Canada) in various concentrations, *Escherichia coli* K235 LPS (protein <0.008%), 10 ng/mL (gift of S. Vogel, Uniformed Services University of the Health Sciences, Bethesda, Md), and the 47L synthetic lipopeptidpeptide (based on the 47-kDa Treponema pallidum lipoprotein) 10 μg/mL (gift of T. Sellati and J.D. Radolf, University of Connecticut) were used as stimuli. TNF in cell-free supernatants were measured by the WEHI 164 clone 13 bioassay.

### Statistics

The SPSS Version 10.05 and GraphPad Prism Version 3.0 were used. Statistical comparisons were made by means of the Friedman test for repeated measures on nonparametric data and Dunn’s multiple comparison test as post hoc test. Differences were considered statistically significant at level of *P* < 0.05. For correlation analysis, we used Pearson correlation (2-tailed).

### Results

### Patients

Demographic data and postoperative adverse events are summarized (Tables 1 and 2, respectively). Blood samples were collected at 5 different time points in 18 patients. From the remaining 2 patients, we obtained only 4 samples. Fifteen patients were diagnosed as having stable angina pectoris, whereas 5 had unstable angina before surgery. The surgical procedure was uncomplicated in all of the patients, but one was receiving CPB for a second time before skin closure. None of the patients received blood transfusions during the operation, but 3 were transfused after surgery (Table 1).
Three patients had clinical signs of pneumonia after blood sampling was completed (Table 2). Our study population showed an increase in white cell count and a decrease in hemoglobin level and platelet count during the first 2 postoperative days (Table 3).

### Plasma Concentration of HSP70 and IL-6
Median IL-6 concentrations measured before surgery and at different time points through the first 2 days after surgery were 0, 43, 107, 93, and 118 pg/mL, respectively (Figure 1A). Correspondingly, median inducible HSP70 concentrations in plasma were 0, 1809, 1879, 0, and 0 pg/mL, respectively (Figure 1B). The quantity of IL-6 (area under curve the first day after surgery) for each patient was correlated \( r = 0.57; P < 0.01 \) to the quantity of HSP70. IL-1\( \beta \) and TNF-\( \alpha \) were not detected in any samples.

### Regulation of the Monocyte Surface Expression of CD14, TLR-2, and TLR-4
As measured by flow cytometry, median CD14 MFI values at 0, 5, 19, and 43 hours after surgery were 75% \( (P < 0.001) \), 93% (NS), 111% (NS), and 101% (NS), compared with preoperative values, respectively (Figure 2A). The corresponding median TLR-2 MFI values were 73% \( (P < 0.01) \), 95% (NS), 123% \( (P < 0.001) \), and 112% (NS), respectively (Figure 2B). The postoperative TLR-4 median MFI values were 86% (NS), 116% (NS), 144% \( (P < 0.001) \), and 153% \( (P < 0.001) \), respectively (Figure 2C). As can be expected, interindividual variations in the CD14, TLR-2, and TLR-4 MFI values were observed. Nevertheless, all the patients responded to the CABG operation in a similar manner regarding the postoperative regulation of these receptors. A representative flow cytometry histogram showing TLR-2 and TLR-4 expression on monocytes from one patient is depicted (Figure 3).

### In Vitro Studies
To explore the role of TLR-4 in HSP70 activation of cells, we used peritoneal macrophages from C3H/HeJ (tlr4 gene mutant) mice. As shown in Figure 4, macrophages from C3H/HeN (control) mice showed an HSP70 dose-dependent TNF response, whereas macrophages from the C3H/HeJ mice did not respond to HSP70.

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**Table 1.** Demographic Data and Clinical Characteristics of 20 Patients Undergoing Elective CABG Operations With CPB

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value*</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>61.4±10.0</td>
<td>46–74</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>16/4</td>
<td></td>
</tr>
<tr>
<td>CPB time, min</td>
<td>67±18</td>
<td>32–96</td>
</tr>
<tr>
<td>ACC time, min</td>
<td>39±12</td>
<td>18–60</td>
</tr>
<tr>
<td>Intubation time, h</td>
<td>4.2±2.2</td>
<td>2.0–10.3</td>
</tr>
<tr>
<td>Postoperative in-ICU stay, d</td>
<td>1±0</td>
<td>1–1</td>
</tr>
<tr>
<td>Postoperative in-hospital stay, d</td>
<td>6.7±2.4</td>
<td>3–13</td>
</tr>
<tr>
<td>Distal coronary anastomoses per patient</td>
<td>3.4±0.8</td>
<td>2–5</td>
</tr>
<tr>
<td>Blood loss, mL</td>
<td>635±444</td>
<td>235–2310</td>
</tr>
<tr>
<td>Transfusions (SAGMAN erythrocytes, IU)</td>
<td>0.35±0.88</td>
<td>0–3</td>
</tr>
</tbody>
</table>

*Values are presented as mean±SD except for the male/female ratio. ACC indicates aortic cross-clamping; ICU, intensive care unit; and SAGMAN erythrocytes, erythrocytes suspended in saline-adenine-glucose-mannitol solution.

**Table 2.** Incidence of Postoperative Adverse Events

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>10 (50)</td>
</tr>
<tr>
<td>Intrathoracic bleeding leading to reoperation</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Sternal instability</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Stroke</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Acute myocardial infarction</td>
<td>0 (0)</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

**Table 3.** Laboratory Data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Preoperative</th>
<th>Postop Day 1</th>
<th>Postop Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>White cell count, ( \times 10^9/\text{L} )</td>
<td>7.2±1.5</td>
<td>9.8±3.1</td>
<td>11.9±3.4</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>145±12</td>
<td>105±12</td>
<td>103±9</td>
</tr>
<tr>
<td>Platelet count, ( \times 10^9/\text{L} )</td>
<td>232±49</td>
<td>156±41</td>
<td>132±33</td>
</tr>
<tr>
<td>Serum AST, U/L</td>
<td>33±19</td>
<td>73±28</td>
<td>71±32</td>
</tr>
<tr>
<td>Serum LDH, U/L</td>
<td>322±55</td>
<td>562±123</td>
<td>456±112</td>
</tr>
</tbody>
</table>

*Values given as mean±SD. AST indicates aspartate aminotransferase; and LDH, lactic dehydrogenase.

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**Figure 1.** Plasma IL-6 and HSP70 concentration (median and interquartile ranges) after CABG. A, IL-6. All postoperative IL-6 values were significantly different from the preoperative value \( P < 0.05 \) for the first postoperative concentration, \( P < 0.001 \) for the later concentrations. B, HSP70. \( P < 0.001 \) for values measured at 0 and 5 hours after surgery compared with preoperative concentration.
not respond. LPS (a recognized TLR-4 ligand) stimulation gave corresponding results, whereas the lipopeptide 47L (which is known to stimulate cells through TLR-2) activated macrophages from both mouse strains.

Human adherent monocytes stimulated with rhu HSP70 resulted in a dose-dependent release of TNF and IL-6 (Figure 5, A and B). Monoclonal antibodies against TLR-4 or CD14 inhibited the HSP70- and LPS-induced IL-6 response (Figure 5, C and D), whereas mAbs against TLR-2 did not. Polymyxin B did not inhibit the HSP70 response (Figure 5C), whereas the cytokine response to LPS was almost totally blocked (Figure 5D). In addition, RSLA blocked both HSP70- and LPS-induced cytokine production. Heat treatment (100°C for 20 minutes) almost completely abrogated HSP70-induced IL-6 production, whereas LPS still induced IL-6, although in some reduced amounts (data not shown).

Discussion
The most important finding in the present study is the release of inducible HSP70 into the circulation after CABG. Furthermore, the in vitro studies suggest that TLR-4 is involved in HSP70 signaling.

Figure 2. Monocyte surface expression of CD14, TLR-2, and TLR-4 after CABG as measured by flow cytometry. Monocytes are stained with fluorescence-conjugated mAbs; data are presented as individual MFI values in percentage of preoperative value. Horizontal bars represent median values.

Figure 3. Monocyte surface expression of TLR-2 and TLR-4 after CABG. Flow cytometry linear-log scale histograms with fluorescence intensity (FI) on the x-axis plotted against cell counts. Result of one representative patient is shown. Cells were stained with Alexa488-conjugated mAbs against TLR-2 (TL2.1) and TLR-4 (HTA125).

Figure 4. TNF in supernatants of peritoneal macrophages from TLR-4 mutant (C3H/HeJ) and control (C3H/HeN) mice. Macrophages were stimulated with rhu HSP70, the synthetic lipoprotein 47L (10 μg/mL) or LPS (10 ng/mL) for 6 hours. TNF content was determined by means of a bioassay.
It has been reported that ischemia and CABG induce HSP70 in myocardial cells.\textsuperscript{7–9} However, there are few reports on circulating HSPs in human diseases.\textsuperscript{23–25} The early peak of inducible HSP70 in plasma after CABG as demonstrated in our study is striking. Our study does not answer the question of which cells and tissues contribute to the circulating HSP70. One source could be myocardial and coronary artery endothelial cells stressed by ischemia or the surgical trauma. Another source of circulating HSP70 may be blood cells, damaged when circulating through the heart-lung machine. To elucidate this, a new study including blood samples of the coronary sinus is underway.

Extracellular HSP may indicate cell death (necrosis) and thus represents a danger signal.\textsuperscript{26,27} Furthermore, it is now well documented that extracellular HSPs activate innate immunity, mediating an inflammatory response,\textsuperscript{16–19} and our results are consistent with these reports. The postoperative inflammatory response in the study population was evident by the rise in plasma IL-6 up to 5 hours after surgery and by the persisting high concentration through days 1 and 2. The kinetics of the IL-6 response in our study population is comparable to results from other publications,\textsuperscript{3,28} whereas the amount of IL-6 measured after surgery varies in different publications.\textsuperscript{2–4} The difference in kinetics of IL-6 and HSP70 release after surgery as well as the correlation between the amount of IL-6 and HSP70 in each patient is interesting. It is tempting to speculate that the early HSP70 release may contribute to the IL-6 peak 5 hours after surgery. In vitro, we found that rhu HSP70 indeed induced monocyte IL-6 and TNF release in a dose-dependent manner.

Because extracellular HSPs induce synthesis of proinflammatory cytokines in vitro, it is important to identify the signaling receptors involved. CD14 appears to be involved in HSP60 and HSP70 signaling,\textsuperscript{17–19} however, considered the CD14-dependent activation of monocytes by HSP70 to occur downstream of specific receptor binding and/or uptake of HSP70. Involvement of TLR-4 in HSP signaling is previously shown only for HSP60,\textsuperscript{19} and CD91 has been suggested as a receptor for HSPs, including HSP70.\textsuperscript{30,31} CD91 appears to be involved in the internalization of HSP-chaperoned peptides, necessary for the subsequent representation on the surface of antigen-presenting cells and activation of cytotoxic T cells. It is unclear whether CD91 is a signaling receptor, and HSP70 may possibly interact with other receptors mediating intracellular signaling and activation of innate immunity. Taken together, our results indicate that both TLR-4 and CD14 are involved in the HSP70-mediated proinflammatory response. Interestingly, Triantafilou et al\textsuperscript{12} recently presented evidence of a CD14-independent LPS receptor cluster that includes the constitutive forms of HSP70 and HSP90, among others. Thus, it may be that HSPs somehow must be involved in the activation of innate immunity, either in a circulating or a membrane bound form.

A major concern in this study is the possibility of LPS contamination of the rhu HSP70. According to the Limulus amebocyte lysate assay measurements, however, the LPS content is far below what is needed to obtain the amounts of IL-6 and TNF observed. Additionally, Polymyxin B did not inhibit HSP70 activation of monocytes in our experiments, in contrast to its effects on LPS. Furthermore, HSP70 shows strongly reduced activity, whereas LPS still shows a significant activity after heat treatment. Taken together, these
findings indicate that the monocyte activation observed is due to unique properties of the HSP70 protein. TLR-4 is suggested as the central lipid A–recognition protein. The inhibitory effect of the lipid A analogue RSLA on both LPS and HSP70 supports the suggestion that HSP70 is signaling through TLR-4.

To our knowledge, no previous studies on TLR regulation in postoperative patients are published. However, monocyte CD14 expression after CABG is shown to be reduced the first postoperative day. This downregulation of monocyte surface antigens probably represents a deactivation of mononuclear proinflammatory functions, and persistence of the deactivation may be associated with increasing risk of microbial invasion and infectious complications. We observed an initial downregulation of monocyte CD14, TLR-2, and TLR-4 after the CABG operation. Subsequently, CD14 normalized, whereas TLR-2 and TLR-4 were upregulated, suggesting monocyte activation by the CABG procedure. A decrease in monocyte count after initiation of CPB and monocytosis the morning after the CABG has been reported. We observed a similar variation in our flow cytometry analyses (data not shown). This must be taken into account, evaluating the significance of monocyte surface receptor regulation.

In summary, the present study shows HSP70 release and regulation of TLR-2 and TLR-4 on monocytes after CABG. Extracellular HSP70 may act as an endogenous ligand for TLR-4, thus mediating an inflammatory response. The results of this study are compatible with the suggestion of HSPs as danger signals during trauma. In a clinical perspective, circulating HSPs as markers of tissue damage and ischemia may possibly become of diagnostic and prognostic advantage. However, further studies are necessary to clarify the receptors involved in signaling mediated by HSPs as well as the tissues responsible for HSP70 release.

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References

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