Gene Expression Changes in Leukocytes During Cardiopulmonary Bypass Are Dependent on Circuit Coating

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Background—Cardiopulmonary bypass (CPB) results in a systemic inflammatory response. Leukocytes play a crucial role in inflammatory reactions. Their gene expression profile in the context of CPB is unknown.

Methods and Results—In a prospective, randomized, and double-blind clinical trial, 12 male patients underwent elective coronary artery bypass grafting with either heparin-coated (group H) or protein-coated (group P) CPB circuits. Oligonucleotide microarray analyses of 22,283 genes were performed on circulating leukocytes, collected immediately before surgery and 6 hours after CPB. Microarray results were validated with real-time polymerase chain reaction. All patients had uneventful surgery, and no significant differences between groups were observed during the clinical course. Multiple statistical analyses with different methods were performed. Compared with preoperative expression at a threshold value of \( P < 0.01 \), postoperative expression revealed 814 upregulated and 1187 downregulated genes in group H compared with 99 upregulated and 231 downregulated in group P (\( P < 0.001 \)). Fifty genes exhibited a >4-fold increase and 27 exhibited a >4-fold decrease in group H, whereas only 7 genes exhibited upregulation and 7 revealed downregulation in group P. Microarray-pathway-profile-finder analyses determined 1405 upregulated and 1454 downregulated pathways in group H compared with 552 upregulated and 818 downregulated pathways in group P (\( P < 0.01 \)). Pathways related to inflammatory response exhibited highest \( z \) scores in group H, reflecting cellular inflammatory activation.

Conclusions—Heparin coating resulted in a more profound alteration in leukocyte gene expression when compared with protein coating. Microarray analyses present an innovative approach for the evaluation and understanding of inflammatory reactions associated with CPB. (Circulation. 2005;112[suppl I]:I-224–I-228.)

Key Words: cardiopulmonary bypass • genes • inflammation • leukocytes

Cardiopulmonary bypass (CPB) is associated with a profound systemic inflammatory response.1–2 The interaction of blood with artificial surfaces activates numerous plasma protein systems and blood cells, causing multiple organ disturbances, edema, decreased myocardial contractility, and thorough changes in vascular resistance.3–5 Surface modification of CPB circuits has been pursued to reduce clinical complications and improve patient outcome.6–7 Gene expression analyses using high-density oligonucleotide probe arrays enable profiling of gene expression in tens of thousand of genes.8 In the present study, we evaluated gene expression in circulating leukocytes after CPB with different circuit coatings using microarray analyses.

Methods

Experimental Setting

This prospective, randomized, and double-blind clinical trial was approved by the Institutional Ethics Committee, and written informed consent was obtained from all of the patients. Twelve male White individuals undergoing elective coronary artery bypass grafting (CABG) were randomized to group H (heparin-coated circuits) and group P (protein-coated circuits). Exclusion criteria were ejection fraction <0.30; left ventricular end-diastolic pressure >20 mm Hg; pulmonary, renal, or hepatic failure; insulin-dependent diabetes; acute infections; obesity (body mass index ≥30); emergency and reoperations; and use of antiinflammatory drugs including aspirin during the 5 days preceding surgery. All of the patients received the same routine intraoperative and perioperative treatment. CPB consisted of a Type S III pump (Stoeckert), the QUADROX HMO 1010 oxygenator, the QUART arterial filter, the VHk 4201 reservoir, and a standard tube setting (all Maquet). In group H, all of the circuit components were coated with covalent-bounded heparin (Bioline-Coating, Maquet). In group P, the total CPB surface was modified with recombinant albumin (Safeline-Treatment, Maquet). Anesthesia was performed using Propofol (6 to 12 mg/kg), Sufentanyl (0.15 to 0.7 \( \mu \)g/kg), Rocuronium (0.3 to 0.6 mg/kg), and Sevofluran at low flow. Activated clotting time was kept at >400 s. Mild systemic hypothermia and cold blood cardioplegia were applied. Arterial blood samples were drawn after the induction of anesthesia but before surgical incision and 6 hours after weaning from CPB. Patients were followed until discharge from the hospital.

RNA-Isolation, cDNA Synthesis, and Biotin Labeling

Leukocytes were separated from blood by centrifugation for 25 minutes at 400 × g immediately after collecting the samples. Total
RNA extraction from leukocytes was rapidly performed (RNeasy Midi kit, Qiagen), and RNA concentration was adjusted between 500 and 1000 µg/mL, controlled by spectrophotometry (A260/A280 ratio, 1.7:2). RNA quality was controlled with routine acrylamid gel electrophoreses. Ten nanograms of RNA were used for cDNA-synthesis (Superscript II, Invitrogen). cRNA was biotin-labeled by in vitro transcription (BioArray High Yield RNA Transcript Labeling kit, Enzo Biochemicals).

Microarray Hybridization and Signal Analysis
After metal-induced fragmentation by incubation at 94°C for 35 minutes, 15 µg of the biotin-labeled cRNA was mixed with eukaryotic controls and hybridized to the Affymetrix human genome chip U133A (Affymetrix Inc.) for 16 hours at 45°C in a rotating oven (60 rpm). After washing and staining, cRNA was detected with streptavidin phycoerythrin (Molecular Probes) by the Affymetrix Fluidic Station, and analysis was completed using the Affymetrix Scanner. The Affymetrix Microarray Suite (v.5.0) was used to scan and analyze the relative abundance of each gene based on the intensity of the signal from each probe set. Data analyses were managed with the Microarray Data Mining Tool, followed by statistical analyses with JMP (SAS Institute Inc.), SPSS (SPSS Inc.), and Excel (Microsoft). Microarray analyses were performed following the “minimum information about a microarray experiment” guidelines.9

Statistical Analysis of Microarray Data
The present-call filter numerates the amount of correct hybridization and enables evaluation of the overall microarray performance.10 The signal-to-noise ratio (SNR) refers to the multiple testing problem that emerges from repeated testing of the 22 283 genes at a given probability value threshold, indicating the proportionality and reliability of statistical comparison.

Methods A and B focused on the alteration of gene expression within groups. Method A identified significant differences of probe sets using paired t test analyses, stating the preoperative measure as control. Parametric distribution of microarray data was affirmed with the Shapiro-Wilk test for normality. Three different probability value cut-off levels for upregulated and downregulated genes were chosen: 0.05, 0.01, and 0.001. Method B recovered the differentiability of probe sets preoperatively versus postoperatively by their signal intensity fold change (fc). Five different threshold values for upregulated and downregulated genes were computed: 1.2, 1.5, 2, 4, and 6. Only genes exhibiting a differential expression at a significant level (P<0.05) and a fc >4-fold were considered to be differentially expressed within groups.

Methods C and D revealed expression changes between groups. Method C assessed the relative signal intensity values of probe sets by intragroup substafraction of preoperative from postoperative signal intensity values. This was followed by an unpaired t test analysis of the relative signal intensity values in group H compared with group P, setting the threshold probability value at 0.05. Method D evaluated the differential expression (Δ expression) between groups by intergroup substrafration of relative signal intensity values. Only genes exhibiting a significant difference of relative signal intensity values and a Δ expression >0.4 were considered to be differentially expressed between groups and were reported.

Micro-array-pathway-profile-finder (MAPPFinder, Gladstone Institute) is a software tool that creates global gene expression profiles by merging microarray data with the annotations of the Gene Ontology project.11,12 It computes a z score for each pathway (Gene Ontology term), which ranks them by their relative amounts of gene expression changes. Positive z scores indicate pathways with a greater number of genes meeting the criteria than expected by chance. MAPPFinder analyses were run within groups on 2 criteria, either an increase (fc >1.2 and P<0.05) or decrease (fc <1/1.2 and P<0.05) in gene expression in leukocytes 6 hours after weaning from CPB, compared with leukocytes collected preoperatively.

Functional Classification of Genes
Genes are reported by their gene name or symbol, locus link accession number, expression ratio, Δ expression respectively, and a cut-off levels for upregulated and downregulated genes were chosen: 0.05, 0.01, and 0.001. Method B recovered the differentiability of probe sets preoperatively versus postoperatively by their signal intensity fold change (fc). Five different threshold values for upregulated and downregulated genes were computed: 1.2, 1.5, 2, 4, and 6. Only genes exhibiting a differential expression at a significant level (P<0.05) and a fc >4-fold were considered to be differentially expressed within groups.

Microarray Validation with Real-Time Polymerase Chain Reaction
Expression profiles of PXN, LEF1, MATN3, and NR1/2 yielded by microarrays were validated with real-time polymerase chain reaction (RT-PCR) in circulating leukocytes of both patient groups before surgery and 6 hours after CPB. GAPDH was established as housekeeping gene, and the following primers were used: PXN forward 5'-GAGCTGAGTCTGCTGTTTC-3' and reverse 5'-ATGAA-CCTCTCCCTGTCCCTG-3', LEF1 forward 5'-CTCTTGTCGT-GCAAGGTCA-3' and reverse 5'-CAGTGTGTGGGGACCAG-GGA-3', MATN3 forward 5'-TGGGCTCTAGAGAGGGTG-3' and reverse 5'-GGCACTGTTGGGTCTCAAC-3', NR1/2 forward 5'-CCATCGGAGGAAGGCTCTG-3' and reverse 5'-GGC-CTTGGTGGAATGATGTC-3'. Amplification conditions and additional analyses were performed as described previously.14

Results
Clinical Outcome
There were no significant differences between the 2 groups regarding demographic and perioperative data (Table 1). All of the patients had uneventful surgery and recovery (no reoperations and no deaths).

Present-Call Filter and SNR
Among our ≈8.5 million oligonucleotide dataset, we found a consistent percentage of present calls in group H [12 arrays, 39.1 ± 3.2% (mean ± SD)] and group P [12 arrays, 39.4% ± 2.8%]. The SNR was 4.23 at P<0.05, 8.97 at P<0.01, and 10.7 at P<0.001 in group H, whereas the SNR was 2.05 at P<0.05, 1.47 at P<0.01, and 0.65 at P<0.001 in group P.

Alteration of Single Genes
The number of differentially expressed genes was consistently greater in group H compared with group P, irrespective of listing genes by their probability value (P<0.001) (Figures 1 and 2) or fc (P<0.05) (Figure 3 and 4). According to methods A and B, 50 genes were found to be upregulated in
group H compared with 7 in group P, whereas 27 genes were downregulated in group H compared with 7 in group P. The overall greatest expression changes were observed for CRISP3, PRV1 (both 18-fold upregulation), and MAP4K1 (22-fold downregulation) in group H and for CD163 (4-fold upregulation) and, again, MAP4K1 (6-fold downregulation) in group P. Numerous genes encoding proteins crucial for innate immune response and inflammation (FCAR, JAK3, CD163, MAP4K1, CR1, and TNFRSF 12), apoptosis and cell cycle arrest (GADD45A, CHEK2, and S100A8), and angiogenesis (HGF and CEACAM1) revealed the greatest expression changes in group H.

According to methods C and D, 18 genes presented the greatest differential expression depending on circuit coating. Twelve of these were predominantly expressed in group H, encoding for proteins involved in extracellular signal transduction (MAPK12), cell-cell interaction (CDH6), and neutrophil chemotaxis (MCP2). Six genes were mainly expressed in group P, functioning in nuclear factor B activation (MINK), oxidative stress (TP53I11), and the regulation of cytochrome P450 (NR1/2) (Table 2).

**Alteration of Gene Pathways**

MAPPFinder calculations determined 1405 upregulated and 1454 downregulated pathways in group H compared with 552 upregulated and 818 downregulated pathways in group P (P<0.01) (Figure 5). The pathways small GTPase-mediated signal transduction (z=5.8, upregulated) and cytosolic ribosome (z=17.1, downregulated) showed the overall highest z score in group H and alike interleukin-1 receptor antagonist (z=8.56, upregulated) and integrase (z=5.11, downregulated) in group P. Concordant upregulation was detected for pathways involved in inflammatory processes and response to stress in group H, whereas pathways regulating protein biosynthesis and ribosomes were downregulated. Pathway analysis in group P did not exhibit coherent expression changes to a comparable extent.

**Validation**

Similar expression was observed for LEF1, NR1/2, MATN3, and PXN with microarray analysis and RT-PCR in 15 of 16 features. Expression of LEF1 detected with microarrays (group H: 2.3-fold decrease, P<0.05; group P: 2-fold decrease, P<0.05) was concordant with RT-PCR (H: 2.9-fold decrease; P: 1.3-fold decrease). Analysis of NR1/2 with microarrays (H: 2.4-fold increase, P<0.05; P: 1.4-fold decrease, P<0.05) was alike in RT-PCR (H: 5.3-fold increase, P<0.05; P: 3-fold decrease, P<0.01). MATN3 expression yielded with microarrays (H: 1.2-fold decrease; P: 4.6-fold increase, P<0.05) was similar to RT-PCR (H: 2.2-fold decrease; P: 1.4-fold increase). Microarray analysis of PXN (H: 1.5-fold increase, P<0.01; P: 1.2-fold increase) showed a discrepancy to RT-PCR in 1 feature (H: 10-fold decrease; P: 1.5-fold increase).
Our data demonstrate for the first time that gene expression in circulating leukocytes of patients undergoing on-pump CABG is directly dependent on circuit coating. Expression changes of numerous genes involved in inflammatory reactions with subsequent pathway activation were detected. A large number of genes previously not described in the context of CPB were identified.

Leukocytes are of special interest because of their crucial function in the pathogenesis of inflammation and CPB-associated inflammatory reactions.15 Microarray analyses were feasible and validated with RT-PCR. Although no standard procedure for the analysis of microarray data exists,16 we believe that highly suitable statistical analyses were performed.

It is remarkable that only the 2 genes CD163 and MAP4K1 were shared by both groups. The CD163-encoded protein is an acute-phase regulated macrophage protein functioning as a hemoglobin-haptoglobin scavenger receptor and has been proposed as a target for potential manipulation of the acute response to CPB-associated inflammation.17 MAP4K1 functions as an upstream activator of proinflammatory and proapoptotic pathways (mitogen-activated protein kinase kinase, c-jun-amino-terminal kinase, and stress-activated protein kinase) in response to stress.18 CD163 and MAP4K1 were expressed irrespective of circuit coatings, suggesting them as markers of proinflammatory and proapoptotic cellular activation, as yet described in other tissues for c-fos, c-jun, EGR-1,19–21 interleukin 6,1,14 tumor necrosis factor/H9251 and interleukin 8.14

The observed changes in leukocyte gene expression correlate to similar findings in the cardiomyocytes of atrial samples after CPB.21,22 The significant increase of MCP2 in leukocytes and of MCP1 in cardiomyocytes 21 after CPB points at induction of inflammation and cellular damage. In contrast, ischemic preconditioned leukocytes showed a decrease of the corresponding chemokine receptor, suggesting a reduction in cellular injury.23 Thus, the reported increase of MCP1 and 2 may suggest that ischemia or hypoxia are not primarily involved in CPB-induced cellular impairment. The decrease of TNFRSF 12 in leukocytes and of TNFRSF7 in
cardiomyocytes might support the suggestion that these cytokines do not contribute to CPB-associated inflammatory reaction. The \textit{CEACAM1}-encoded protein binds to endothelial E-selectin and has been reported to stimulate proliferation, chemotaxis, and angiogenesis. It was upregulated in leukocytes but downregulated in cardiomyocytes. The increased expression of \textit{CDH6}, which has similarities to \textit{CEACAM1}, may indicate pronounced leukocyte activation, as demonstrated previously during inflammation after CPB.

This holds also true for the increased expression of \textit{S100A8} in leukocytes and of \textit{S100A4} in cardiomyocytes, genes that have been reported in correlation to cerebral damage after CPB. Numerous mitogen-activated protein kinase genes were differentially expressed in leukocytes and cardiomyocytes after CPB and ischemic preconditioned leukocytes. These genes are involved in the regulation of a large variety of proteins and mechanisms and are pivotal in cellular response to stress. In addition, the induction of apoptosis in endothelial cells reported previously by our group corresponds with the present findings of \textit{GADD45A}, \textit{MAP4K1}, and \textit{CHEK2} upregulation.

Our data demonstrate that heparin coating creates much more genomic disturbances in leukocytes than protein coating. This is a novel and somewhat unexpected observation, because heparin coating is thought to imitate physiological conditions of the vessel wall, thereby ameliorating CPB-associated inflammatory reactions. Protein coating possibly inhibits genomic alteration and ameliorates CPB-induced inflammatory reactions. However, we did not find any significant differences in the clinical outcome of patients, similar to previous studies. This may be attributable to small patient numbers. Furthermore, we do not know to what extent the observed changes in gene expression translate into protein synthesis and function. Future studies will have to clarify these points.

\section*{Conclusion}
Leukocytes undergo a profound alteration of gene expression after CPB dependent on circuit coating. Inflammatory reactions after CPB are observable on gene expression level, whereas microarray analysis serves as a highly suitable tool.

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\section*{References}
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