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Cytokine gene expression in monocytes of patients undergoing cardiopulmonary bypass surgery evaluated by real-time PCR

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Abstract

Cardiopulmonary bypass (CPB) surgery induces systemic release of proinflammatory cytokines causing unspecific inflammatory reactions. This study deals with the development of a sensitive technique for detecting changes at the mRNA level in monocytes of patients undergoing CPB surgery, by using real-time PCR. Blood samples from patients undergoing elective coronary artery bypass grafting were obtained at six different time points. RNA was extracted from isolated monocytes and cDNA was synthesized by reverse transcriptase. CPB surgery induced gene expression of IL-1β, IL-6, IL-8, and TNF-alpha, followed by a decrease below the preoperative expression values 6 h post CPB. High significant increases in gene expression for IL-8 at the end of surgery ($p = 0.001$) were detected. Real-time PCR is a powerful tool for getting simultaneously numerous sensitive, accurate, and reliable results from small amounts of biological material. This method avoids time-consuming and hazardous post-PCR manipulations and decreases the potential risk of PCR contamination.

Keywords: cardiopulmonary bypass - cytokines - real-time PCR - housekeeping gene

Introduction

Cardiac surgery with cardiopulmonary bypass (CPB) induces the so-called "postpump syndrome", which results mainly from the blood's contact with the various artificial surfaces of the extracorporeal devices during the actual surgery. This reaction can lead to the systemic inflammatory response syndrome (SIRS) and may contribute to the development of postoperative complications, including myocardial dysfunction, acute lung failure (ARDS, adult respiratory distress syndrome), renal and neurological dysfunction, bleeding disorders, altered liver functions, and ultimately multiple organ failure (MOF) [1-3].

The present study deals with the verification of a highly sensitive and quantitative technique for

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detecting changes at the mRNA level in circulating monocytes of patients undergoing cardiac surgery, by using a novel real-time PCR technique. It has been shown that blood contacting artificial surfaces during extracorporeal circulation procedures activate blood monocytes. Activated monocytes play an important role in the so called "host versus graft" mechanisms, which includes activation of coagulation factors and cytokines, stress response, and phagocytosis [4]. Increased levels of pro-inflammatory cytokines have generally been associated with negative outcomes after cardiac surgery. Therefore, measurement of specific markers in these blood cells subserve for the ideal evaluation of the hemocompatibility of biomaterials. Important mediators of the inflammatory response are interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), both of them cause a series of biologic effects on several other blood and vascular cells including induction of endothelial apoptosis [5]. IL-1β and TNF-α may be distributed systematically throughout the circulatory system and induce other cytokines, e.g. IL-6, CSF, IF [4], initiating the inflammatory pathway against the supposed invader.

Investigations of gene expressions require a sensitive, reproducible and precise technique. Quantitative real-time PCR represents a sensitive method for detection of gene expressions on the mRNA level. This technique enables quantification of rare transcripts and produces reliable as well as rapid results [6]. A key factor of real-time PCR is measuring the product of the target gene within the linear range of the amplification reaction. During this linear range the amount of amplified target is directly proportional to the input amount of the target [7]. A well established method for detection of newly synthesized PCR products in real-time is the use of SYBR®Green I fluorescence dye that binds specifically to the minor groove double-stranded DNA [8]. Detection during the linear range of amplification eliminates the need of wasteful post-PCR analysis and specialized software simplifies the evaluation of the results.

For the most frequently used semi-quantitative approach an endogenous standard is used as reference. Therefore, reference genes or housekeeping genes like glyceraldehydes-3-phosphate dehydrogenase (GAPDH), β-actin or 18S rRNA were applicable [9], these genes show stability under attempt conditions. These housekeeping genes are present in all nucleated cell types since they are necessary for basic cell survival. The mRNA synthesis of these genes is considered to be extremely stable, even under experimental treatment [9,10]. However, several studies have already shown that the housekeeping genes are regulated and vary under experimental conditions [11,12]. So, a reliable and accurate housekeeping gene is needed for each real-time PCR study [13].

Materials and methods

Housekeeping genes

The identification of a valid reference for data normalization remains a problem and none of the solutions proposed is ideal [14]. It is especially difficult when dealing with *in vivo* samples, such as peripheral blood mononuclear cells (PBMCs), and comparing gene expression patterns between different individuals [14].

For comparing several reference genes (β-actin, GAPDH and 18S rRNA) total RNA was isolated from whole blood using the Quiagen Rneasy Mini kit (Quiagen, Hilden, Deutschland) following manufacturer's instructions and treated with RNase free DNase I (Boehringer, Mannheim, Germany). All samples were electrophoretically verified by ethidiumbromid staining and by OD_{260}/OD_{280} nm absorption ratio > 1.95. One µg of every RNA sample was reversed transcribed with 100 U of SuperScript II RNase H^- Reverse (Invitrogen, Karlsruhe, Germany) using 500 ng Oligo $(dT)_{15}$ primer (Roche, Mannheim, Germany) according to the manufacturer's instructions. The RNA of each separate individual blood sample before (control) and after treatment (with plastic or metal) was always reversed transcribed in the same run.

Patients and operation procedures

The protocol for this study was approved by the internal review board of the University of Tuebingen, according the guidelines of the Declaration of Helsinki. We studied five male patients undergoing elective aorta-coronary bypass grafting with the use of a heart-lung-machine. These patients consented to removal of additional blood

Table 1 Characteristics of primer sequences for housekeeping and target genes used in the real-time PCR.

Housekeeping gene:	Forward Primer	Reverse Primer	Amplicon size (bp)
GAPDH	5'-TCA ACA GCG ACA CCC ACT CC-3'	5'-TGA GGT CCA CCA CCC TGT TG-3'	126
β-Actin	5'-GCG TGT GTG TGT GTG TGT GT-3'	5'-CCT CCC TCC TCC CTA TGT GT-3'	151
18S rRNA	5'-AAA CGG CTA CCA CAT CCA AG-3'	5'CCT CCA ATG GAT CCT CGT TA-3'	155
Target Gene:	**Forward Primer**	**Reverse Primer**	**Amplicon size (bp)**
IL-1β	5' CCC ACA GAC CTT CCA GGA GA 3'	5' CGG AGC GTG CAG TTC AGT G 3'	138
IL-6	5' CAC ACA GAC AGC CAC TCA CCT C 3'	5' CTG CCA GTG CCT CTT TGC TG 3'	135
IL-8	5' GAC TTC CAA GCT GGC CGT G 3´	5' CTC CTT GGC AAA ACT GCA CC 3'	81
TNFα	5' CCG TCT CCT ACC AGA CCA AGG 3'	5' CTG GAA GAC CCC TCC CAG ATA G 3'	125

samples drawn from the central venous catheter at different time points: pre operative (1); after 20 minutes of ECC (2); end of ECC (3); 6 h post OP (4); 24 h post OP (5) and 72 h post OP. The first 3 ml of each blood drawing were discarded to avoid artificial activations.

Blood samples were immediately cooled on ice for 10 minutes. Afterwards, monocytes were isolated by using the monocyte isolation kit® (Dynal Biotech, Oslo, Norway) according to the manufacturer's protocols. Samples were aliquoted, shock –frozen with liquid nitrogen, and stored at –80°C until further analyses.

Monocyte mRNA was harvested from monocyte solution by using the mRNA Isolation Kit® (Dynal Biotech, Oslo, Norway).

Monocyte mRNA from all probes of one patient was always reverse transcribed in the same run with their respective control samples. The oligo$(dT)_{15}$-primed first-strand cDNA synthesis was carried out with SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a total volume of 20 µl according to the manufacturer's prescription.

Quantitative real-time RT-PCR

Primer Design and optimization in regard to primer dimers, self priming formation, miss priming and amplicon length was done with the Primer 3 (Whitehead Institute Center for Genom Reseach, Cambridge, MA, USA) [15] and Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA, USA) primer design software. All primers were optimized to an equal annealing temperature of 60°C, a similar GC-content between 57 and 63.2% and supplied by MWG Biotech (Ebersberg, Germany).

The product size was verified by electrophoresis through 1,5 % agarose gels with ethidiumbromid staining and by melt curve analysis. The sequences of the PCR primers are listed in Table 1.

PCR was performed with standard protocols using SYBR®Green as fluorescent detection dye in a real-time iCycler (Biorad, Hercules, CA, USA). All PCR reactions had a final volume of 15 µl comprised out of 2 times SYBR®Green PCR kit, 360 µM forward and reverse primer and 2 ng of reverse transcribed RNA. The whole reaction was pipetted on ice into a 96-well plate (Abgene, Rochester, NY, USA) and the well contents were collected by brief centrifugation of the plate. Cycling was started with an activation step at 95°C for 15 minutes, the amplification program repeated 45 times (denaturation: 95°C for 20 s; annealing: 60°C for 30 s; extension: 72°C for 45 s) with fluorescence measurement at 72°C. For characterization of the generated amplicons and to control the contamination by unspecific byproducts a melting curve analysis was applied between 50 – 100°C at 0.5°C intervals.

Table 2 Patients and surgery data.

Characteristics	Mean	Range
Age (yr)	58.8	46 – 74
Weight (kg)	81.4	65 – 111
Duration of surgery (min)	195	170 – 230
Duration of ECC (min)	78.2	60 – 98
Duration of aortic cross-clamping (min)	51	36 – 62
Reperfusion (min)	22	19 – 27

Standard curves for 6 two-fold-dilution steps between 4 ng and 0.125 ng of reverse transcribed RNA samples were run for all primer pairs in quinticates to determine the PCR efficacy under the experimental conditions for the different target genes and the housekeeping gene.

All PCR reactions for a given sample were pipetted in 3 replicates in order to control and adjust for the variability of the PCR amplification. After the exclusion of outliers the average of the replicates was used for statistical data processing.

Real-time PCR data analysis and statistics

For all PCR evaluations cycle threshold values (CTs) were calculated for the different products (Fig.1). The CT-value is defined as the PCR cycle where the fluorescence intensity of an amplicon crosses a threshold line. This threshold line was set in the exponential amplification phase for all the different amplicons at 1/10 of the average maximum intensity measured. The CTs obtained for the different amplicons were statistically processed by the software package REST ® applying pair wise fixed reallocation randomization test, which has particularly been designed for the evaluation of semi-quantitative real-time PCR results [16].

This evaluation procedure takes the different amplification efficiencies for the target genes (E_{target}) and the reference gene ($E_{reference}$) into consideration and determines, whether the expression relation of a reference gene to target genes of an experimental group is significantly different from a control group. The blood samples taken during

Fig. 1 Mean normalized expression (MNE), with its standard error (SE) and coefficient of variation (CV), for real-time PCR data. CT = cycle threshold, E = amplification efficiency for target or reference genes.

Equation 1:

$$MNE = \frac{\left(E_{reference}\right)^{CTreference,mean}}{\left(E_{target}\right)^{CTtarget,mean}}$$

Equation 2:

$$SE_{MNE} = MNE * \sqrt{\left[\ln\left(E_{target}\right) * SE_{CTtarget,mean}\right]^2 + \left[\ln\left(E_{reference}\right) * SE_{CTreference,mean}\right]^2}$$

Equation 3:

$$CV_{MNE} = \frac{MNE}{SE_{MNE} * \sqrt{3}} = \frac{1}{\sqrt{3} * \sqrt{\left[\ln\left(E_{target}\right) * SE_{CTtarget,mean}\right]^2 + \left[\ln\left(E_{reference}\right) * SE_{CTreference,mean}\right]^2}}$$

and after ECC were taken as experimental group and compared to the blood samples obtained preoperatively.

For graphical presentation of results and statistical analysis by paired Student's *t*-test and analysis of variance (ANOVA) the mean normalized expression (MNE) of the target genes were calculated according to equation 1 by using the average CT value from the three replicates for the target ($CT_{target,\ mean}$) and the average for the three replicates for the appropriate reference ($CT_{reference,\ mean}$). For the statistical comparison of the group of patients at the different time points a significant ANOVA was followed by a pair wise fixed reallocation randomization test for post-hoc analysis. In order to control for the variability of the PCR amplification step, the standard error for the MNE (SE_{MNE}) was calculated according to equation 2 by using the standard error for the target replicates ($SE_{CTtarget,\ mean}$) and the reference replicates ($SE_{CTreference,\ mean}$) (Simon, Bioinformatics, in press). The coefficient of variation (CV) was thereafter determined according to equation 3.

Results

Internal controls as reference genes

To assess gene expression by semi-quantitative real-time RT-PCR, co-amplified internal controls were established as reference genes. Therefore, the stability of mRNA levels of GAPDH, 18S RNA and β-actin were studied under three different conditions.

Fig. 2 shows the different properties of the three housekeeping genes. A) demonstrates the intra-individual comparison of three different blood activation treatments. High significant responses were noted in all tested volunteers in the gene expression of 18S RNA ($p = <0.0001$). The gene expression of β-actin under these conditions showed p-values of $p <0.05$ in individual 1, $p = <0.0001$ in individual 2 and $p <0.005$ in individual 3, implying that these two housekeeping genes are not suitable for this study. Only the expression of GAPDH was nearly unaffected (p = NS) by blood-stimulation with plastic (flexible tubes) and metal (stents), therefore GAPDH was the most convenient reference gene for this study conditions. Fig. 2B compares different individuals under the same treatment conditions. These data show that according to the applications another optimal housekeeping gene will be chosen, than in Fig. 2A. Here GAPDH showed significant differences in gene expression of varied treatments, p-values of the control, the flexible tubes and the stent samples are $p = <0.0001$. 18S RNA indicates in control and stent samples a significant p-value of $p = <0.0001$ and in the flexible tube samples p = NS. These data suggest that in case of comparison of individuals β-actin is the only gene which could potentially used as a reference gene (control and flexible tube p = NS; stent $p <0.005$).

For this study conditions a housekeeping gene was needed which exhibits an intra-individual stability.

Confirmation of primer specificity

Specificity of PCR amplification for the chosen housekeeping gene and the four target genes was confirmed by melting curve analysis and resulted in single product specific melting temperatures as follows: GAPDH – 83.0°C; IL-1β – 81.5°C; IL-6 – 81.5°C (Fig. 3A); IL-8 – 83.0°C and TNF-α – 84.0°C (Fig. 3B). In addition a gel electrophoresis was performed which resulted in single products with the desired length. No primer-dimers or unspecific by-products were generated during the applied 45 real-time PCR amplification cycles.

Real-time PCR amplification efficiencies and linearity

For the evaluation of the amplification efficiencies during PCR, dilution series were run and standard curves were generated for all primer pairs used. The correlation coefficient indicates high linearity for the different targets within the tested range of 4 to 0.125 ng of reverse transcribed RNA. For all genes studied, the average correlation coefficient of detection was 0.996 (range from 0.98 to 1.00). Furthermore, the mean slope (S) value for all genes was tested S_{GAPDH} -3.31, $S_{IL\text{-}1\beta}$ –3.68, $S_{IL\text{-}6}$ –3.21, $S_{IL\text{-}8}$ –3.64, $S_{TNF\text{-}\alpha}$ –3.90. The ideal detection is characterized as 100% efficient PCR (slope -3.3); hence the average efficiency of detection for all

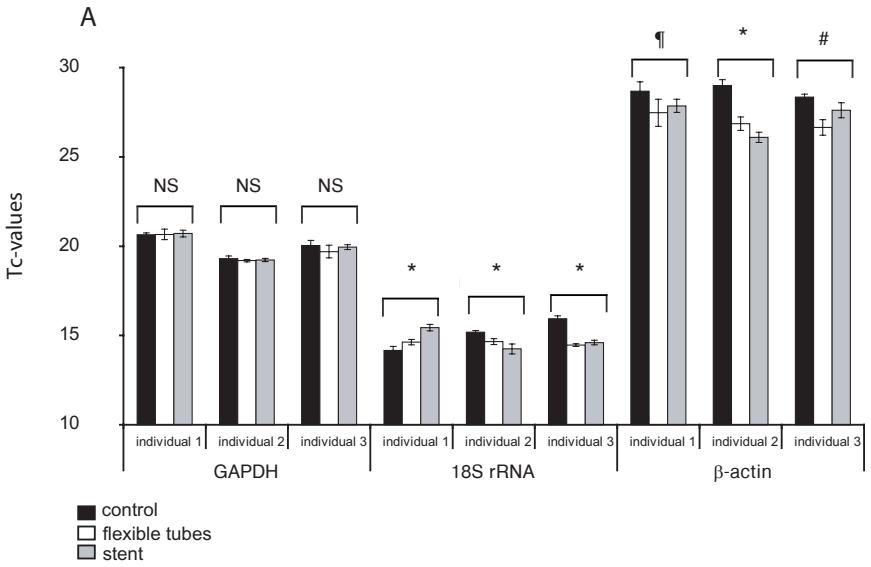
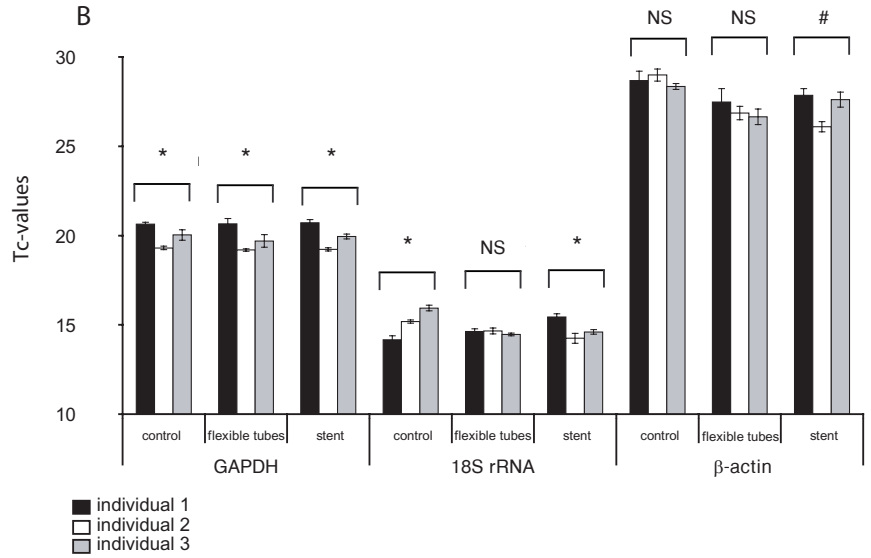


Fig. 2
A) Housekeeping Gene Individual. Differences of gene expression of three housekeeping genes (GAPDH, 18S RNA, β-actin) regarding intra-individual changes under different treatments.



B) Housekeeping Gene Treatment. Differences of gene expression of three housekeeping genes (GAPDH, 18S RNA, β-actin) concerning the treatments and its changes between different individuals.

Levels of significance:
* = p≤0.0001
# = p≤0.005
¶ = p≤0.05
NS = not significant

primer pairs in the present study was 107.5% (E_{GAPDH} 100.3%, $E_{IL\text{-}1\beta}$ 111.5%, $E_{IL\text{-}6}$ 97.3%, $E_{IL\text{-}8}$ 110.3%, $E_{TNF\text{-}\alpha}$ 118.2%).

Changes in gene expression

During the whole investigation period no significant changes in gene expression was observed in IL-1β, neither by REST ® calculation (Table 3) nor by group ANOVA of the normalized expression values (Fig. 4). In the process of the operation period IL-6 and IL-8 show same tendencies. Comparison of samples taken preoperatively and 20 minutes after beginning of CPB, both genes display a weak up-regulation, and further observations indicated a strong up-regulation at the end of surgery. In case of IL-8 significant up-regulation at 20 minutes after beginning of ECC by the factor 10.683 ($p < 0.05$), and after the end of ECC by the factor 236.313 ($p = 0.001$) was detected. 24 h post operatively the gene expressions dropped down to the preoperative values. IL-6 showed a significant down-regulation at 24 h after ECC by the factor 77.758 ($p < 0.01$). Further, IL-8 indicated a low down-regulation by the factor 4.155 ($p < 0.05$) at 72 h after ECC.

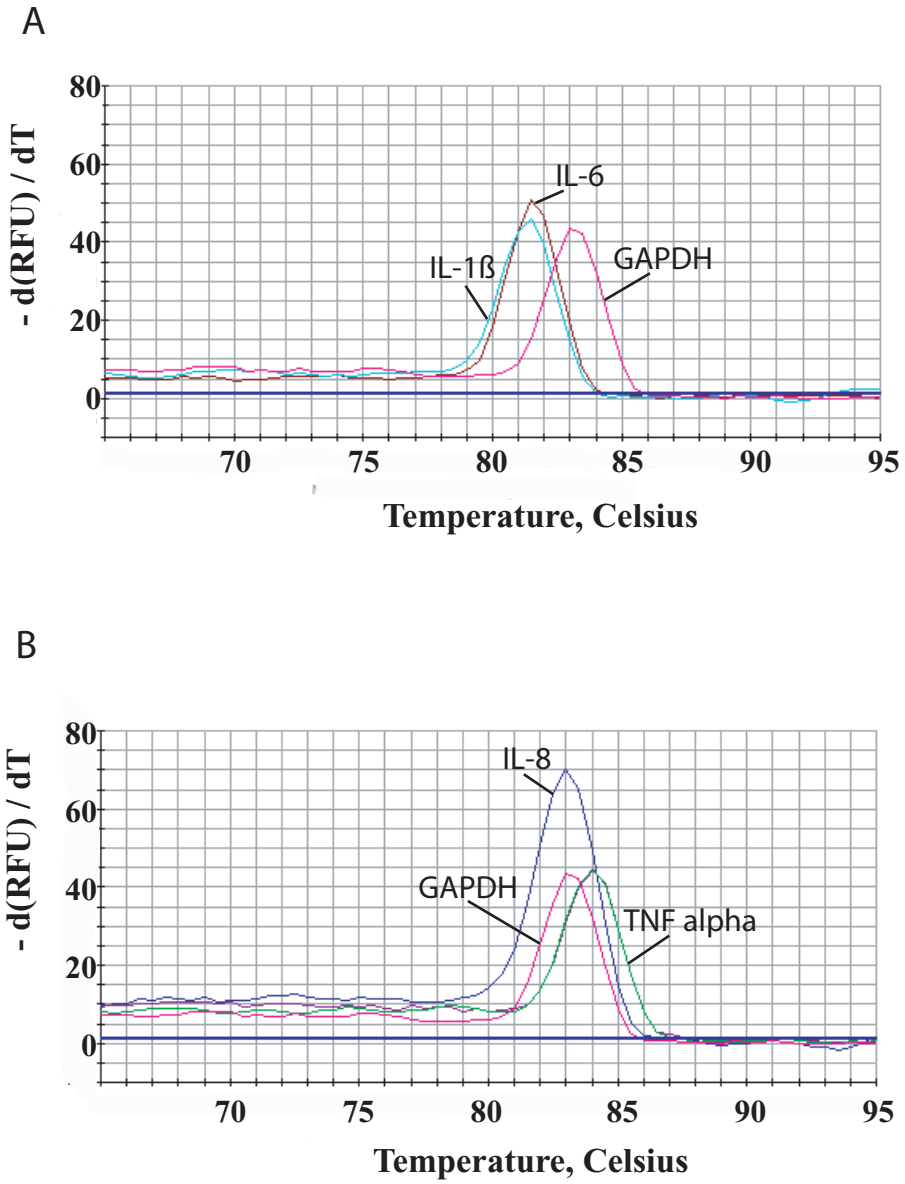


Fig. 3 Melt curve analysis of GAPDH (83°C), IL-1β (81.5°C) and IL-6 (81.5°C). Melt curve analysis of GAPDH (83°C), IL-8 (83°C) and TNF-α (84°C).

The group ANOVA shows in case of IL-6 a significant p-value of $p < 0.05$, and in case of IL-8 a high significant p-value of $p = <0.0001$. The expression curve of TNF-α indicated a low down-regulation at 20 minutes after beginning of ECC, followed by an up-regulation after the end of ECC and strong down-regulation below the preoperative value at 6 h post ECC. At 24 h and 72 h the levels of gene expression came back nearly to the preoperative value. A significant down-regulation of TNF-α at 6 h after ECC by the factor 2.635 ($p <0.005$) was observed. In all four genes the same process at 6 hours postoperatively was recognizable, the expression level dropped below the preoperative values. The cumulated results of all patients undergoing CPB are shown in Fig. 4 (normalized expression data) and Table 3 (REST ® data).

Discussion

Objective of this study was the evaluation and application of a new real-time PCR method for the expression analysis of cytokines (IL-1β, IL-6, IL-8, TNFα) in surface-activated monocytes from

Table 3 Up and down regulations and probabilities of gene expressions calculated by the software package REST ®. All samples were compared to the preoperative values. Blood drawing: (1) preoperative; (2) 20 minutes after beginning of ECC; (3) end of ECC; (4) 6 h post op; (5) 24 h post op; (6) 72 h post op.

1 / 2	**regulated**
IL-1β	down 1.169
IL-6	up 6.703
IL-8 *	up 10.683
TNF	down 1,111
1 / 3	
IL-1β	up 1.203
IL-6	up 34.559
IL-8 ***	up 236.313
TNF	up 1,842
1 / 4	
IL-1β	down 1.624
IL-6	up 2.404
IL-8	up 3.041
TNF **	down 2.635
1 / 5	
IL-1β	up 1.258
IL-6 **	down 77.578
IL-8	down 1.022
TNF	down 1.14
1 / 6	
IL-1β	down 1.236
IL-6	up 1.807
IL-8 *	down 4.155
TNF	down 1.477

Levels of significance:
* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

patients undergoing coronary artery bypass grafting (CABG).

In order to reduce complications caused by extracorporeal circulation procedures, modification of surface chemistry is a viable approach for improving the hemocompatibility of artificial surfaces [3]. In this report, we investigated commonly used oxygenators by their capacity to induce mRNAs of pro-inflammatory cytokines in human monocytes.

According to the research problem it is necessary to know whether the housekeeping gene has the required stability. For this analysis it is important to use a housekeeping gene which shows the required intra-individual stability under different treatments. In our case we choose GAPDH, which indicates no significant changes within the different test individuals.

In contrast to that consideration, examinations which include several individuals required a different housekeeping gene. Under these circumstances GAPDH shows significant gene expressions, whereas β-actin indicates the needed stability. These findings show the importance of selecting the appropriate housekeeping gene. Several possibilities of designing clinical studies result in fundamental differences for choosing the optimal housekeeping gene.

In this study we describe the gene monitoring in directly isolated monocytes from whole blood taken pre-, during and after bypass surgery. Rodenburg et al. [17] have shown that the immunomagnetic separation of monocytes resembles physiologic conditions much more than do other isolation methods (i.e. Ficoll gradient centrifugation or Ficoll centrifugation followed by plastic adherence) since cells may undergo stimulation during isolation and purification. In addition, only a few pipetting and no centrifugation steps are necessary; thus stresses on cellular gene expressions are avoided. Therefore we selected this method for the isolation of intact monocytes from whole blood by immunomagnetic

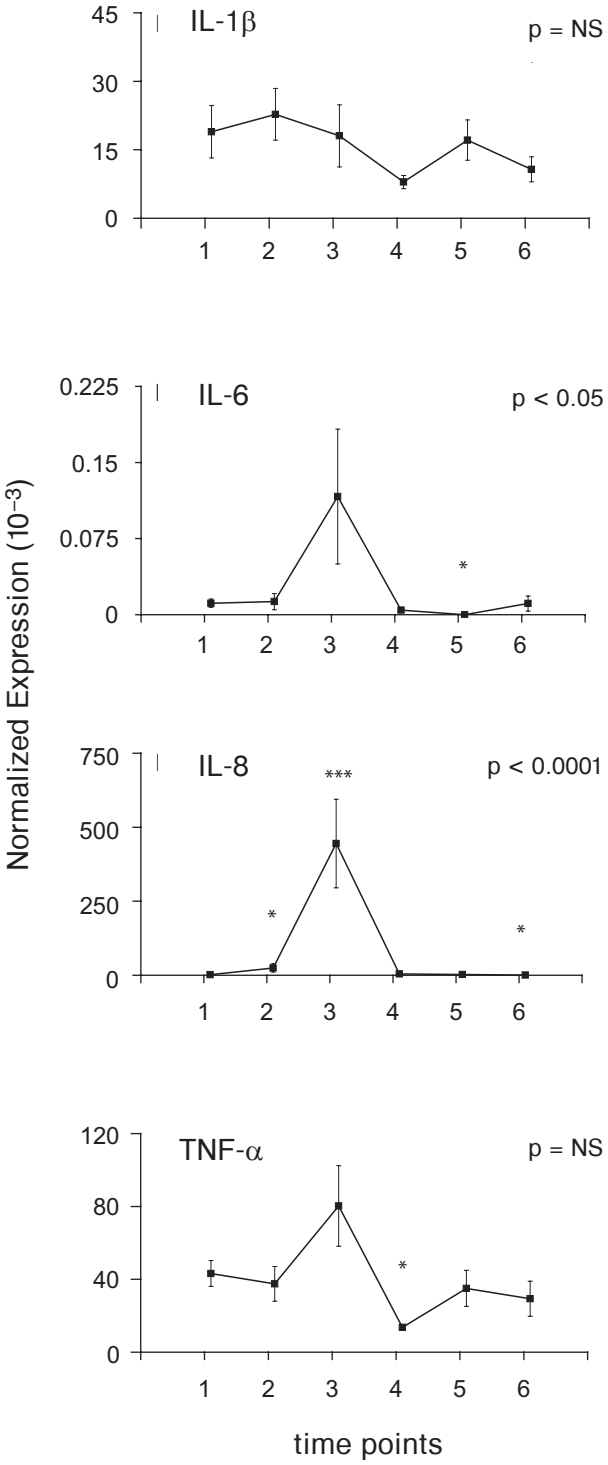


Fig. 4 Normalized gene expression for IL-1ß, IL-6, IL-8 and TNF-α (normalized by GAPDH). Time points: (1) preoperative; (2) 20 minutes after beginning of ECC; (3) end of ECC; (4) 6 h post op; (5) 24 h post op; (6) 72 h post op. Probability levels from REST ®: * p = 0.05; ** p = 0.01; *** p = 0.001. Internal group ANOVAs are marked with the abbreviation ANOVA p, significant levels are like REST ®.

separation, followed by mRNA isolation, cDNA synthesis and real-time PCR.

Since mRNA of cytokines is often expressed at very low levels and the blood samples available to be analyzed are limited, an accurate, exact, reliable and reproducible method of RNA isolation is needed. Therefore, isolation of total RNA (Qiagen) followed by DNase digestion was compared with isolation of mRNA using Dynabeads Oligo $(dT)_{25}$ (Dynal). The isolation of mRNA by Dynabeads Oligo $(dT)_{25}$ ensure intact, high purity mRNA by a strong RNase inhibiting agent together with stringent hybridization and washing buffers. An additional DNase treatment of the mRNA sample can be avoided. This is preferable because of the often low expression levels of cytokines in the sample to be analyzed, making necessary a mRNA extraction with very good efficiency. Otherwise, some of the cytokines of interest might be expressed below the detection limit [18].

This method supplied much better results for the following investigation steps, than the isolation of total RNA. Klein *et al.* [19] have shown that it is possible to isolate mRNA out of only one cell using by this method.

Because the samples (e.g. blood) obtained to analyze are often too small to allow quantification of cytokines at the protein level, quantification at the mRNA level is increasingly used. Real-time PCR is the method of choice to quantify cytokine mRNA levels from human blood, which are often expressed at very low levels. It is a fast, sensitive and accurate quantification method [20]. This technique advantageously measures PCR product accumulation during the exponential phase of the reaction, resulting in much faster results than the previous endpoint PCR. Additionally, the real-time PCR required no post-PCR manipulations, which reduces the risk of contamination.

Compared with other methods, the PCR technique needs only a little amount of experimental material; whereas techniques such as ELISA do need target proteins in the μg/ml scale or are not sensitive enough for the analysis of cytokine expression at very low levels [18].

Cytokines are a group of low-molecular-weight polypeptides, which are responsible for the intercellular communication. They have a central role in inflammatory reactions, particularly acting on the heart, lung, liver, coagulation system, and central

nervous system, subsequently causing damaging effects [21-24]. Various reports have been published concerning CPB-caused enhancement of inflammatory cytokine production [25, 26]. Our results show that blood samples of patients taken at the end of ECC resulted in higher gene expression compared to samples obtained preoperatively, except IL-1β. IL-1β did not evince significant changes during the whole test sequence. TNF-α levels indicated a significant down-regulation, 6 h after ECC. Both, IL-1β and TNF-α productions showed no significant differences at the internal group ANOVA. Similar to our results many studies have reported that neither IL-1β nor TNF-α levels showed any significant increase during CPB [27, 28]. However, Menasche's [26] data reported that IL-1β and TNF-α levels increase during CPB and that their expression levels crest a peak 2 h after CPB. This discrepancy could be connected with factors such as the different assay methods or the length of CPB and the extent of surgical stress. Furthermore, it is possible that IL-1β and TNF-α are expressed in the late phase of the reaction, not like IL-6 and IL-8. Both of them indicate an enhancement of gene expression during the acute phase, so that the production mechanism and origin of IL-1β and TNF-α may differ from those of IL-6 and IL-8 [29].

IL-6 and IL-8 expressions indicated significant differences at the internal group ANOVA calculation. Blood samples drawn 6 h after ECC showed only moderate down regulation compared with samples taken preoperatively. In all patients the gene expression at 24 h and at 72 h after ECC resembled the preoperative samples. Several studies have reported that IL-8 levels increased during CPB and decreased after the end of CPB [28, 30]. Similar changes in levels of IL-6 have also been described by Kawamura *et al.* and Steinberg et al. [27, 30]. The results suggest that these inflammatory cytokines involved in the acute-phase reactions serve as promoter of CBP associated inflammation.

The big standard error at the time point 3, seen by IL-6, IL-8 and TNF-α can be explained by the fact that this blood samples were taken at the end of ECC. The end of ECC depends on the individual patient and its surgery. The average of duration of aortic cross-cramping is 51 minutes (+11/–15), so there is a time variation from 36 minutes to 62 minutes. The levels of these pro-inflammatory cytokines do have a positive correlation with the duration of ECC.

The present study established a reproducible, quantitative method for assessing gene expression in monocytes from patients undergoing CABG. Further clinical studies for evaluation of both, new operative techniques and devices as well as new anti-inflammatory drugs can be successfully verified by this novel method.

References

1. **Paparella D., Yau T.M., Young E.,** Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. An update, *Eur. J. Cardiothorac. Surg.,* **21:** 232-244, 2002
2. **Kerbaul F., Guidon C., Lejeune P.J., Mollo M., Mesana T., Gouin F.,** Hyperprocalcitonemia is related to noninfectious postoperative severe systemic inflammatory response syndrome associated with cardiovascular dysfunction after coronary artery bypass graft surgery, *J. Cardiothorac. Vasc. Anesth.,* **16**: 47-53, 2002
3. **Wendel H.P., Ziemer G.,** Coating-techniques to improve the hemocompatibility of artificial devices used for extracorporeal circulation, *Eur. J. Cardiothorac. Surg.,* **16**: 342-350, 1999
4. **Weber N., Wendel H.P., Ziemer G.,** Gene monitoring of surface-activated monocytes in circulating whole blood using duplex RT-PCR, *J. Biomed. Mate. Res.,* **56**: 1-8, 2001
5. **Aebert H., Kirchner S., Keyser A., Birnbaum D.E., Holler E., Andreesen R., Eissner G.,** Endothelial apoptosis is induced by serum of patients after cardiopulmonary bypass, *Eur. J. Cardiothorac. Surg.,* **18**: 589-593, 2000
6. **Walker N.J.,** Tech.Sight. A technique whose time has come, *Science,* **296**: 557-559, 2002
7. **Schmittgen T.D., Zakrajsek B.A., Mills A.G., Gorn V., Singer M.J., Reed M.W.,** Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods, *Anal. Biochem.,* **285**: 194-204, 2000
8. **Morrison T.B., Weis J.J., Wittwer C.T.,** Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification, *Biotechniques,* **24**: 954-958, 960, 962, 1998
9. **Marten N.W., Burke E.J., Hayden J.M., Straus D.S.,** Effect of amino acid limitation on the expression of 19 genes in rat hepatoma cells, *FASEB J.,* **8**: 538-544, 1994
10. **Foss D.L., Baarsch M.J., Murtaugh M.P.,** Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues, *Anim. Biotechnol.,* **9**: 67-78, 1998
11. **Chang T.J., Juan C.C., Yin P.H., Chi C.W., Tsay H.J.,** Up-regulation of beta-actin, cyclophilin and GAPDH in N1S1 rat hepatoma, *Oncol. Rep.,* **5**: 469-471, 1998

12. **Bereta J., Bereta M.,** Stimulation of glyceraldehyde-3-phosphate dehydrogenase mRNA levels by endogenous nitric oxide in cytokine-activated endothelium, *Biochem. Biophys. Res. Commun,* **217**: 363-369, 1995
13. **Pfaffl M.W.,** A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic. Acids Res.,* **29**: e45, 2001
14. **Bustin S.A.,** Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.,* **29**: 23-39, 2002
15. **Rozen S., Skaletsky H.,** Primer3 on the WWW for general users and for biologist programmers, *Methods Mol. Biol.,* **132**: 365-386, 2000
16. **Pfaffl M.W., Horgan G.W., Dempfle L.,** Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, *Nucleic Acids Res.,* **30**: e36, 2002
17. **Rodenburg R.J., van den Hoogen F.H., van de Putte L.B., van Venrooij W.J.,** Peripheral blood monocytes of rheumatoid arthritis patients do not express elevated TNF alpha, IL-1beta, and IL-8 mRNA levels. A comparison of monocyte isolation procedures, *J. Immunol. Methods,* **221**: 169-175, 1998
18. **Giulietti A., Overbergh L., Valckx D., Decallonne B., Bouillon R., Mathieu C.,** An overview of real-time quantitative PCR: applications to quantify cytokine gene expression, *Methods,* **25**: 386-401, 2001
19. **Klein C.A., Seidl S., Petat-Dutter K., Offner S., Geigl J.B., Schmidt-Kittler O., Wendler N., Passlick B., Huber R.M., Schlimok G., Baeuerle P.A., Riethmuller G.,** Combined transcriptome and genome analysis of single micrometastatic cells. *Nat. Biotechnol.,* **20**: 387-392, 2002
20. **Wang T., Brown M.J.,** mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection, *Anal. Biochem.,* **269**: 198-201, 1999
21. **Marty C., Misset B., Tamion F., Fitting C., Carlet J., Cavaillon J.M.,** Circulating interleukin-8 concentrations in patients with multiple organ failure of septic and non-septic origin, *Crit. Care Med.,* **22**: 673-679, 1994
22. **Turkoz A., Cigli A., But K., Sezgin N., Turkoz R., Gulcan O., Ersoy M.O.,** The effects of aprotinin and steroids on generation of cytokines during coronary artery surgery, *J. Cardiothorac. Vasc. Anesth.,* **15**: 603-610, 2001
23. **Hennein H.A., Ebba H., Rodriguez J.L., Merrick S.H., Keith F.M., Bronstein M.H., Leung J.M., Mangano D.T., Greenfield L.J., Rankin J.S.,** Relationship of the proinflammatory cytokines to myocardial ischemia and dysfunction after uncomplicated coronary revascularization, *J. Thorac. Cardiovasc. Surg.,* **108**: 626-635, 1994
24. **Sawa Y., Ichikawa H., Kagisaki K., Ohata T., Matsuda H.,** Interleukin-6 derived from hypoxic myocytes promotes neutrophil-mediated reperfusion injury in myocardium, *J. Thorac. Cardiovasc. Surg.,* **116**: 511-517, 1998
25. **Wan S., Marchant A., DeSmet J.M., Antoine M., Zhang H., Vachiery J.L., Goldman M., Vincent J.L., LeClerc J.L.,** Human cytokine responses to cardiac transplantation and coronary artery bypass grafting, *J. Thorac. Cardiovasc. Surg.,* **111**: 469-477, 1996
26. **Menasche P., Haydar S., Peynet J., Du B.C., Merval R., Bloch G., Piwnica A., Tedgui A.,** A potential mechanism of vasodilation after warm heart surgery. The temperature-dependent release of cytokines, *J. Thorac. Cardiovasc. Surg.,* **107**: 293-299, 1994
27. **Steinberg J.B., Kapelanski D.P., Olson J.D., Weiler J.M.,** Cytokine and complement levels in patients undergoing cardiopulmonary bypass, *J. Thorac. Cardiovasc. Surg.,* **106**: 1008-1016, 1993
28. **Finn A., Naik S., Klein N., Levinsky R.J., Strobel S., Elliott M.,** Interleukin-8 release and neutrophil degranulation after pediatric cardiopulmonary bypass, *J. Thorac. Cardiovasc. Surg,*, **105**: 234-241, 1993
29. **Sawa Y., Shimazaki Y., Kadoba K., Masai T., Fukuda H., Ohata T., Taniguchi K., Matsuda H.,** Attenuation of cardiopulmonary bypass-derived inflammatory reactions reduces myocardial reperfusion injury in cardiac operations, *J. Thorac. Cardiovasc. Sur.,* **111**: 29-35, 1996
30. **Kawamura T., Wakusawa R., Okada K., Inada S.,** Elevation of cytokines during open heart surgery with cardiopulmonary bypass: participation of interleukin 8 and 6 in reperfusion injury, *Can. J. Anaesth.,* **40**: 1016-1021, 1993