Snapshot assessment of RNA-expression in severely burned patients using the PAXgene™ Blood RNA System: A pilot study

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Abstract

Severe burn induces destabilization of the immune system and the likelihood of multiple organ dysfunction syndrome. Current studies focus on RNA-expression analyses of immune system cells, however, the present methods of analysis are complex, potentially altered by artefacts and therefore not feasible for routine analyses. The new PAXgene™ Blood RNA System provides "snapshot" analysis of RNA by immediate cell lysis and prevention of RNA-degradation. Using this system the aim of this study was to analyse intracellular cytokine RNA-expression under clinical conditions.

Whole blood samples (PAXgene™ tubes) of nine severely burned patients were drawn at admission and 6, 12, 24, 48 and 72 h after trauma during routine treatment. Four healthy individuals served as control. Analysis of RNA-expression of TNF-alpha as pro-inflammatory and IL-10 as anti-inflammatory mediator was performed by RT-PCR.

The RNA-expression of TNF-alpha was increased at 72 h after burn. The increase occurred mainly in surviving patients. In contrast, RNA-expression of IL-10 was elevated already at 24 h and the difference between surviving and deceased patients occurred earlier.

We demonstrate for the first time a "snapshot" analysis of cytokine RNA-expression in severely burned patients under routine conditions. The results correspond well to current hypothesis of posttraumatic MODS development.

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1. Introduction

The majority of burn related deaths are associated with the development of systemic inflammatory response syndrome and severe multiple organ failure (MOF) [1–3]. In this context, destabilization of the human immune system has been identified as one of the critical activators associated with the development of MOF [4]. Although several experimental and clinical studies demonstrated the role of mediators, e.g. TNF-alpha, IL-1 [5], the initial mechanisms leading to the inhomogeneous destabilizing immune system response remain incompletely characterized, so far. Since RNA is the biological messenger of protein acting as the above described mediators, many research activities were directed to potential clarification of similar activating mechanisms after severe burn injury using various techniques, such as microarray analysis of...
systemic immune system cells [6-8]. Although these efforts provide completely new insights into the potential mechanisms responsible for immune system destabilization, they were carried out in cell populations isolated from whole blood. However, these isolation techniques are not only suspicious for artificial induction of further activation [9,10] but also require complex laboratory working conditions for isolation counteracting routine clinical use.

In contrast, for this setting the recently developed PAXgene™ Blood RNA System (PreAnalytiX, Hombrechtikon, Switzerland) allows for whole blood RNA-expression analysis with markedly reduced laboratory complexity and less cause of artificial activation due to immediate cell lysis and RNA stabilization [11].

This analysis system comprises PAXgene™ tubes based on commonly used BD Vacutainer™ technology for blood collection, which contain a proprietary solution providing immediate cell lysis and stabilization of RNA. Furthermore a specific kit for RNA-isolation is adapted to the PAXgene™ tube sample size for standardized isolation procedure. By that, the system proved to allow for “snapshot” analysis of intracellular RNA-expression under experimental conditions [11,12]. However, it is not clear whether application of this system may provide valid and reproducible information about alteration of RNA-expression of specific cytokines in whole blood samples of severely burned patients. Hence, to test the applicability of this system under routine conditions the aim of this investigation was (i) to analyse if measurement of RNA encoding for specific pro- and anti-inflammatory cytokine out of PAXgene™ blood samples provides reliable information under control and stimulation conditions, (ii) to test, whether measurement of specific cytokine RNA is feasible under routine conditions and (iii) compare the obtained results to clinical data, such as secondary survival or development of multiple organ failure.

2. Patients and methods

2.1. Study design and collectives

Approval to conduct this study was granted by the local Ethics committee, the study was performed according to Good Clinical Practice at a level 1 trauma center. Signed informed consent was obtained from the patient or from legal representatives.

2.2. Control group

Blood samples from healthy volunteers served as a control group and were enrolled to establish a negative and positive control group, respectively. Therefore, at each sampling time point, two identical EDTA-tubes were drawn. One sample was left native as negative control, the other probe was incubated with LPS as described below. For repetitive measurement double analysis of each was performed.

2.3. Patient collective

Severely burned patients older than 18 years of age were included if they had been admitted initially to the burn center within 90 min after trauma and without previous treatment in another hospital. Further inclusion criteria were total body surface area (TBSA) of burns at least 20%, burn depth at least second degree, no collateral trauma and no obvious immunological disease. All patients were admitted to the burn intensive care unit after the initial resuscitation. They were treated according to Parkland formula guidelines [13] and underwent surgery (escharotomy, debridement) in a multi-phase strategy. All data including demographical and clinical properties of patients were collected prospectively.

2.4. Blood sampling and quantitative mRNA-expression analysis

In the control group whole blood samples (2× 2.5 mL in PAXgene™ blood RNA tubes) served as negative controls. As a positive control, whole blood samples collected in EDTA tubes were stimulated with 10 μg of a 100 ng/mL LPS solution (Sigma–Aldrich, Munich, Germany) for induction and were then incubated for 4 h at 37°C and 5% CO2 in culture medium consisting of RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 25 mM HEPES buffer (Vitromex, Vilshofen, Germany), L-glutamine, 10% fetal calf serum, and 0.1 mg/mL gentamycin (Merck, Darmstadt, Germany). Afterwards these samples were transferred to PAXgene™ tubes.

In patients whole blood samples (2× 2.5 mL in PAXgene™ blood RNA tubes, PreAnalytiX, Hombrechtikon, Switzerland) were drawn in a standardized manner at admission time at the burn center less than 90 min after trauma as well as 6, 12, 24, 48 and 72 h after the traumatic event.

All samples were incubated for 2 h at room temperature, followed by +4°C for the time of further storage and transported to the laboratory for further processing and quantitative analyses.

For PAXgene™ tubes, total cellular RNA was extracted according to the manufacturer’s protocol using PAXgene™ blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland) as described before [10]. Afterwards, nucleic acids specifically adsorbed to columns were digested with DNase (RNase-Free DNase Set; QIAGEN, Hilden, Germany), and the column was washed with buffers supplied in the reagent set. RNA was eluted from the column twice with 40 μL of BR5 buffer, and eluates were incubated for 5 min at 65°C in a heating block followed by immediate chilling on ice. Extracted RNA from all samples was stored at minus 80°C.

The concentration of the isolated RNA was determined by spectrophotometric measurement of absorbance at 260 nm (Biophotometer, Eppendorf, Hamburg, Germany).

An aliquot of 8.2 μL RNA was reverse transcribed using avian myeloblastosis virus-reverse transcriptase (RT) and oligo(dT) as primer (First Strand cDNA Synthesis kit; Roche, Mannheim, Germany). The obtained cDNA was diluted 1/25 with water and 10 μL were used for amplification. The quantitative analysis of target gene expression was performed on a LightCycler by real time-PCR using the LightCycler FastStart DNA SYBR Green I kit (Roche, Mannheim, Germany) according to the manufacturer’s manual and as described previously [14]. More precisely, the RT-PCR reaction was carried out in a 20 μL final volume containing: 2 μL Primerset, 2 μL Sybr Green I Premix and RNase free water up to 20 μL. Additional to the samples of patients a positive
control, a negative control and a standard dilution series (1:1–
1:10,000 with known concentration) were provided for analysis.
The procedure of RT-PCR analysis was performed according to
the following steps: denaturation and polymerase activation
(95 °C, 10 min), amplification of RT-PCR products in a 45 cycle
one-step PCR including denaturation (95 °C, 10 s)/annealing (68–
58 with –0.5 °C each cycle)/extension (72 °C, 16 s) for each cycle,
analysis of melting curve including denaturation (95 °C, 1 s)/
cooling (58 °C/10 s)/melting from 58 to 95 °C at 0.1 °C/s and
quantitative fluorescent analysis, final cooling of rotor and
chamber (40 °C, 30 s).

For specificity control of the amplification products, a
melting curve analysis was performed. No amplification of
unspecific products was observed. For calculation, data
processing and documentation the LightCycler analysis soft-
ware (Version 2.0) was used.

Furthermore, agarose gel-electrophoretic analysis was
performed to exclude significant DNA contamination and
check specificity of RT-PCR products.

2.5. Study parameters

We analysed RNA-expression of TNF-alpha as one of the most
extensively described pro-inflammatory cytokines [15,16]. For
that, specific primers for RT-PCR analysis were designed by
Search-LC (Heidelberg, Germany) to span at least one intron of
the gene transcript.

As representative anti-inflammatory cytokine RNA expres-
sion of IL-10 [17–19] was analysed by similar design of primers.

To answer the first question of this study, these parameters
were measured in control samples before and after stimula-
tion by LPS. Tests for intra- and inter-individual variability
were performed to ascertain if the obtained results were
reproducible and valid (see Section 2.6).

The second question was addressed by measuring these
parameters in severely burned patients during an observation
period from admission to the hospital up to 72 h after trauma.

For answering the third question the obtained results were
grouped to clinically relevant parameters such as mortality
and MODS development assessed by the use of SOFA Score [20]
and compared to each other.

2.6. Statistical analysis

To test whether an alteration of RNA-expression occurs over
time in patients ANOVA on ranks followed by Student–
Newman–Keuls-test was calculated and significance was
accepted at p < 0.05.

Comparison of values obtained from surviving/deceasing
patients was calculated using Mann–Whitney U-test at a signi-
ficance level of p < 0.05. Statistical analysis was performed using
SPSS Version 10.0 (SPSS GmbH Software, Munich, Germany).

3. Results

3.1. Control group

Blood samples from four healthy volunteers (2 female/2 male,
23–33 years of age) were used for control group analyses. The
mRNA-expression of TNF-alpha was 257 ± 53 (copies/
50 ng mRNA) in native PAXgene™ tubes and significantly
(p < 0.05) increased to 3567 ± 763 after stimulation with LPS
(see Fig. 1). Similarly, the mRNA-expression of IL-10 was 21 ± 7
in native control samples and increased significantly to
185 ± 32 after the LPS stimulation.

3.2. Patient collective

Nine patients, including three women and six men, fulfilled
inclusion criteria and were enrolled in this pilot study.
Demographic data as well as clinical baseline characteristics
are given in Table 1. The age of the severely burned patients
ranged from 18 to 66 years (47 ± 15; mean ± S.D.). Five patients
survived their injury, four patients died due to multiple organ
failure. The TBSA ranged from 20 to 70% (40 ± 17.6) and was
markedly higher in deceased patients (mean 47.3%) than in
surviving patients (mean 34.6%). The abbreviated burn
severity index (ABSI) [21,22] varied from 6 to 13 points
(9.2 ± 2.3). Inhalation trauma was proven by bronchoscopy
in eight of nine patients. Concerning organ failure the
assessed SOFA score showed no significant alteration in
surviving patients during the observation period. In contrast,
the SOFA score increased significantly in patients, who did not
survive the burn trauma and differed mainly at 6 and 12 h after

Fig. 1 – These graphs demonstrate the mRNA-expression
levels obtained from healthy individuals under native
conditions (C) and after LPS-stimulation (LPS). (Data given
as mean ± S.E.M., n = 4, *p < 0.05 in Mann–Whitney U-test
vs. control.)
the trauma. Four patients died during hospital treatment due to sepsis and posttraumatic organ related failure (see Fig. 2).

Due to high interindividual variation of gene expression levels all values from 6 to 72 h were adjusted to the individual gene expression by dividing the expression level at each time point by the admission level. Thus, data of mRNA-expression are given as relationship between baseline on admission and later sampling time. The course of TNF-alpha gene expression during the first 72 h after severe burn trauma is shown in Fig. 3. There is an increase, which is not significant until 72 h after the injury.

In contrast, mRNA-expression levels of IL-10 (Fig. 4) were clearly elevated 24 h after burn \( (p < 0.05) \). They remained elevated with a slight decrease at the end of the observation period.

The time course of TNF-alpha was influenced by the clinical course expressed in survival or death of severely burned patients shown in Fig. 5, although differences were statistically not significant. Surviving patients showed a slightly higher increase during the first 24 h after trauma while gene expression level of deceased patients remained lower until 48 h. There was a markedly decrease of TNF-alpha between 24 and 72 h.

In contrast, levels of IL-10 were significantly different concerning survival at 12 and 48 h after trauma. There was an early increase of IL-10 in surviving patients until 24 h with a further decrease. In deceased patients, mRNA-expression of IL-10 remained elevated from 24 h until end of observation period (Fig. 6).

4. Discussion

The substantial new information provided by this study is, that assessment of whole blood mRNA-expression is feasible under routine conditions by using the PAXgene™ Blood RNA System. In contrast to other analysis systems it allows for RNA-expression analysis under the described clinical conditions, as (i) only a low sample volume is required and (ii) because of the low logistic cost. This is regarded a major advantage in comparison to systems, that require initial complex and immediate processing [11]. By drawing closely matched sequential blood samples standardized after a severe burn, the results of this pilot study demonstrate clearly that the initial RNA-expression of pro- and anti-inflammatory cytokines was significantly increased in the initial posttraumatic period during the first 72 h after trauma. The obtained results correspond well to some of the current hypotheses of post-burn immune system dysfunction development. Hence, this analysis system is now ready for extended clinical studies to further illuminate mechanisms of immune system destabilization after severe burn.

4.1. Study design and collectives

In our study, we included nine patients after severe burn with 20–70% TBSA at least second degree and the ABSI ranged from 6 to 13 points. This is in line with other studies from larger collectives with comparable TBSA of burn, inhalation trauma and demographic parameters [23]. For assessment of early RNA levels inclusion of patients was strictly guided by the duration from trauma until admission to the burn center which did never extend 90 min. An unknown activation within this very early period till admission must be taken into account. However, a standardized preclinical sample

| Table 1 – Demographic and clinical basic parameters of patient collective |
|-------------------------|----------|----------------|----------|-----------------|-----------------|----------------|
| Patient no. | Age | Sex | % TBSA ≥ II | ABSI | Inhalation trauma | Outcome (30 days)/day of death after injury |
| 1 | 38 | m | 64 | 11 | Yes | Deceased/5 |
| 2 | 56 | f | 70 | 13 | Yes | Deceased/4 |
| 3 | 50 | | 46 | 10 | Yes | Survived |
| 4 | 51 | m | 23 | 7 | No | Survived |
| 5 | 35 | m | 40 | 8 | Yes | Survived |
| 6 | 64 | m | 20 | 7 | Yes | Deceased/9 |
| 7 | 66 | f | 35 | 11 | Yes | Deceased/5 |
| 8 | 18 | m | 25 | 6 | Yes | Survived |
| 9 | 44 | f | 40 | 10 | Yes | Survived |

Mean ± S.D. 47 ± 15 40.2 ± 17.6 9.2 ± 2.3
m, male; f, female; TBSA, total burned body surface area; ABSI, abbreviated burn severity index.

Fig. 2 – This graph depicts the quantitative assessment of SOFA score in patients according to the clinical outcome. At 6 and 12 h after burn trauma the SOFA score was significantly different between survived and deceased patients. \( (p < 0.05 \text{ in Mann–Whitney U-test survived vs. deceased.}) \)
collection seems to be not feasible due to ethic and logistic reasons.

Furthermore, none of the patients was treated anywhere else. The very early inclusion followed by an observation period of 72 h after trauma allowed the assessment of immunological activation and suppression prior to the usual occurrence of infections [24]. Multiple organ failure due to substantial alterations of the innate and the adaptive immune function remains one of the two main causes of death in severely burned patients next to respiratory failure associated with inhalation injury [25,26]. Although studies on mediators on the biological level of proteins provided substantial information about the presence of multiple organ failure after burn the measurement of RNA as biological precursor of proteins raised in the focus of research. The reason for this interest is the fact that changes of RNA-expression appear prior to systemic mediator release.

Thus, there is a need for simple and contemporary information of the immune reaction or at least of key mediator dynamics at the intracellular RNA level, which cannot be sufficiently provided by systemic analysis of serum cytokine levels. Therefore, a new analysis system was tested in this pilot study with the objective of gaining early, intracellular and upstream information under clinical conditions after severe burn injury.

4.2. Blood sampling and quantitative mRNA-expression analysis

Quantitative gene expression analyses in tissues and particularly in blood samples are increasingly used to assess the immune response to disease and injury with the opportunity to indicate the presence, dynamics and prognosis of a disease or immunological dysregulation. However, all analysis methods, such as blood sampling or further procedures for quantification of gene expression changes, are the critical steps for the evaluation of the results. In this study the PAXgene™ Blood RNA System was used for blood sampling and RNA-isolation. Since this is the first study using this system in serial whole blood analyses under routine conditions after trauma, no direct comparison to data from the literature is available. However, investigating on serum protein level Yeh et al. described an increase of TNF-alpha and IL-10 within the first days after burn with increased serum levels of TNF-alpha already at hospital admission [27] and with a peak after 2.5 days for IL-10 [28]. Hence, the precursory increase of RNA-expression level appears reasonable, although for TNF-alpha an immediate gene expression change with consecutive protein release after trauma must be assumed.
The rather small amount of whole blood (2 × 2.5 mL) did not have any influence on fluid replacement or need for RBC transfusion. Hence, samples could be drawn even if patients had instable circulation parameters or were undergoing surgical interventions. Most of the other studies investigating mRNA-expression in blood cells using densitometric centrifugation or magnetic cell sorting required markedly higher sample volumes. Using the PAXgene™ Blood RNA System provides safety during blood sampling against reflow of cell lysating substance. In this study, samples were incubated and transferred to the processing laboratory for further procedures at constant time points. This is in line with other authors, who reported about constant RNA-expression levels in PAXgene™ blood RNA tubes over time [11].

For quantitative assessment of specific cytokine expression the accurate preparation is crucial to minimize DNA contamination and other influencing factors [29]. Therefore, in our study we routinely used the DNase procedure to avoid DNA contamination. As a quality control gel electrophoresis assays were performed.

However, evaluation of gene expression in whole blood as well as in cellular subtypes depends on different RNA-isolation protocols and is difficult to compare [30]. Furthermore, for all analyses of mRNA-expression has to be taken into account that in this study quantitative changes of whole blood circulating cells were analysed. Due to the inhomogeneous shifts between PBL subpopulations and the unknown quantitative relation of these subsets in RNA transcription, normalization to differential blood cell counts appears difficult.

The PAXgene™ Blood RNA System does not allow for investigations of specific pathways in subpopulations of PBL because a separation of different blood cells is not possible before cell lysis and RNA-stabilization. This fact also includes other possible origin of RNA than from PBL such as from platelets. This aspect can be hardly clarified under clinical conditions but might requires alternative experimental approach. In previous studies on proteins, the prevalence of these mediators has not been classified in detail. Moreover, the discrepancy between obvious cellular cytokine release paralysis after trauma and systemic increase might be the reason mediators are released by other cellular components, such as endothelial cells. However, the aim of this study was to test whether the PAXgene™ Blood RNA System is feasible for routine studies and this target is supported by the given data.

4.3. Study parameters

4.3.1. Expression of TNF-alpha

TNF-alpha is known to be one of the central mediators participating in the posttraumatic immune response [31–33]. In this study we found a significant increase of mRNA levels of TNF-alpha 72 h after trauma. Since these results are the first ones assessed in human whole blood samples after burn trauma, no direct comparison to literature data is applicable. However, several studies demonstrated significant serum level elevation of TNF-alpha on protein level associated with higher mortality when detected in an early phase [34,35]. The reason for non-significant difference in our study might be due to still low patient number in this pilot study.

Other analyses were performed either in isolated T cell or monocyte cell populations but not in whole blood assays including all circulating immune cells, respectively.

There is no obvious reason for apparently lower levels of RNA-expression of TNF-alpha in all patients 48 h after trauma. It may be speculated that recurrently elevated levels after 72 h may be generated by routinely performed surgical procedure 2 days after trauma. No association to the significantly altered SOFA score was present in our study (Fig. 2).

4.3.2. Expression of IL-10

IL-10 is described as a major immune-regulatory cytokine, mainly produced by T cells and monocytes. Because of its inhibitory effects on the release of mediators such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-1, -6, and -8, it has originally been called "cytokine synthesis inhibitory factor" (CSIF) [36,37]. We found significantly increased of IL-10 in the early course after burn trauma with persistently elevated levels in deceased patients. These results are in line with others, who reported on a higher incidence of septic events in patients with elevated levels of IL-10 [38,39]. However, the role of IL-10 in the initiation of immune depression after major injuries is discussed controversially. Looking at different expression in surviving patients compared to deceased patients, IL-10 expression may indicate a substantial impact of the anti-inflammatory response concerning MODS development and outcome.

In conclusion, the present study demonstrated for the first time that gene expression levels of cytokines after severe burn trauma can be reliably analysed using PAXgene™ Blood RNA System. In the future, this method may allow a more detailed monitoring of early immune reaction after severe trauma contributing to a better understanding of pro- or anti-inflammatory impact of certain cytokine production. Although this approach seems to be promising and opens
new perspectives, some limitations still remain. First, there were a small number of burn victims included as yet in this pilot study and the cellular origin of the specific mediator expression and information (e.g. in subpopulations of PBL) cannot be identified by these analyses and therefore we interpreted these results carefully with respect to the whole of immunologically competent cells.

Furthermore, the correlation between intra- and extracellular parameters of the immune response might be required for estimation of the importance of RNA gene expression analysis for the prognosis and the treatment of severely burned patients. Now, larger trials will clarify the impact of intracellular mRNA-expression changes on systemic mediator effects and immune reaction in burn patients.

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