High levels of soluble TNF receptors are related with deficiency of GSH and severity of sepsis in patients with septic infectious endocarditis

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Abstract

Background: *GSH is a major intracellular antioxidant playing a critical role in the deactivation of toxic compounds as well as in the maintenance of normal immunoreactivity. To reveal interrelations between TNF and GSH, and possible significance of soluble TNF receptors (sTNFRs) as markers of sepsis severity was the aim of present study.*

Material and methods: *24 patients with septic infectious endocarditis (Simplified Acute Physiology Score (SAPS) – 7.66±0.52) were monitored before and after surgery, and intensive care procedures. Lymphocyte subsets were studied by flow-cytometry. TNF, sTNFR1 and sTNFR2 were determined in blood plasma samples. Total glutathione and GSH were determined in the whole blood.*

Results: *Lymphocytopenia with decrease of CD4, CD8 T cells, NK cells, high levels of TNF, sTNFR1 and sTNFR2, low levels of total glutathione and especially GSH, and high levels of oxidized glutathione were detected in septic patients. SAPS indices correlated positively with levels of sTNFR1 and sTNFR2. High negative correlations between GSH levels and sTNFR1, sTNFR2 and TNF were evident. Positive correlations between GSH versus NK cells before and after operation, and between GSH and CD19 cells after operation were found. Valvular surgery and intensive care procedures resulted in improvements of cell counts and their relative percentage.*

Conclusion: *Septic patients may acquire severe GSH deficiency in part due to the excessive activity of TNF. Restoration of GSH balance may be promising prognostic factor in patients with severe sepsis. Soluble TNF-Rs may reflect the severity of clinical septic cases.*

Key words: *GSH, TNF, soluble TNF-receptors, sepsis*

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Background

Sepsis is a generalized inflammatory response, which involves organs remote from the site of initial infectious insult, accompanied by the release of excessive amounts of proinflammatory cytokines and the subsequent formation of reactive oxygen and nitrogen species.

GSH (γ-gluthamyl-cysteinyl-glycine) is present in all mammalian cells. It is involved in a broad range of biological functions and plays a key role in a cell physiology. GSH is important at protein and DNA biosynthesis, protein tertiary structure completion, co-enzyme function, DNA repair, detoxification and scavenging of free radicals [1]. In septic patients, aerobic cells are exposed to variety of oxygen reactive species originating from mitochondrial respiratory chain as well as from detoxification of various endogenous toxic compounds

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as a result of septicemia. The GSH system is a protective mechanism used by mammalian cells to minimize these danasing events. Prognosis and outcome of the septic patients are determined by a possible development of multiple organ failure. There is an exhaustion of GSH system in septic patients which is related with increased release of TNF-α [2]. The lethal outcome of septic shock is brought by activation of inflammatory mediators which alter the homeostasis folloved by hemodynamic disturbances and organ failure [3]. TNF can directly or indirectly, mediate the release of soluble TNF receptors (TNF-Rs) to peripheral blood. Cardiac (surgical) patients with APACHE-II score greater than 24 expressed considerably elevated TNF levels that could be used to predict an adverse outcome [4]. The goal of the present study was the investigation of relationship between soluble TNF receptors (sTNF-R1, p55 and sTNF-R2, p75) and clinical status, and GSH in septic patients with infectious endocarditis. Possible compensatory and protective role of GSH and TNF-Rs in neutralization and attenuation of destructive tissue effects is manifested by elevated levels of sTNF-Rs which can be markers of severity of sepsis.

Methods

Patients

24 patients, 18 men and 6 women, aged between 15-45 years (mean age ±SEM = 33.1±2.1) with septic infectious endocarditis (survivors) were investigated before and after valvular surgical operation and intensive care. Simplified Acute Physiology Score (SAPS) was used for estimation of severity of clinical status of patients. Mean SAPS of observed patients was 7.66±0.52. *Staphylococcus aureus* was the predominant micro-organism. Venous blood samples were examined before and 24 hours after operation. 20 healthy blood donors, 11 men and 9 women, aged between 22-45 years (mean age ±SEM=32.3±1.9) served as the control group. The relevant Local Ethical Committee has given approval to the study.

Determination of glutathione in the whole blood

The wole blood (0.2 ml) samples were mixed with equal volumes of 10% sulfosalicylic acid (SSA), and centrifuged for 1 min at 10,000 g. The SSA-extract was collected and used for glutathione assays. Total glutathione was determined by enzymatic method as previously described [5] with minor modifications. Briefly, whole blood SSA-extract was diluted 1:200 in deionized water and 50 µl of diluted SSA-extract was mixed with 200 µl glutathione reductase-DTNB solution (1 µM 5.5'dithiobis (2-nitrobenzoic acid), 1 U/ml yeast glutathione reductase (Sigma)) in assay buffer (0.1 M PBS pH 7.4, 10 mM EDTA), and with 50 µl 0.2 µM β-NADPH solution in assay buffer. An absorbance increase per 1 min at 414 nm was recorded by Multiskan MCC/340 reader (Labsystems Oy, Finland) using kinetic measurement with 1 min intervals for total 6 min. The reduced glutathione (GSH) was determined by non-enzymatic method in undiluted SSA-extracts with DTNB reagent as previously described [6]. The oxidized glutathione (GSSH) was calculated by subtraction of GSH values from the total glutathione values in each sample. Pure GSH (Sigma) diluted in deionized water in a concentration range of 0.5-10 µM (for total glutathione assay) or 50-1000 µM (for GSH assay) was used as the standard.

Flow cytometry of lymphocyte subsets

Lymphocyte subsets were identified by three-color flow cytometry using erythrocyte-lysins the whole blood method [7] of lymphocyte staining by FITC-, PE- or PerCP-conjugated monoclonal antibodies (mAbs) to the following human lymphocyte antigens: CD3, CD3/HLA-DR, CD3/CD56, CD3/CD4, CD3/CD8, CD19, CD3-/CD16+CD56 (Becton Dickinson, USA). Stained test samples were analysed by FACScan flow cytometer using FACScan Research and Lysis software (Becton Dickinson, USA).

TNF and TNF-Rs determination

Solid-phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) was used for determination of TNF, sTNF-R1 (p55) and sTNF-R2 (p75) (Pharmingen, USA and Biosource, Belgium) in peripheral blood plasma.

Statistical analysis

For description of the distribution of the data we used mean and SEM. Statistical analysis of differences between groups was carried out by non-parametric Mann-Whitney U-test in unpaired analyses and Wilcoxon ranking test in paired analysis using GraphPad InStat (GraphPad Software, Inc., San Diego, California, USA).

Results

Before the operation, elevated levels of leucocytes and total numbers of granulocytes and monocytes were evaluated in patients with septic infectious endocarditis and compared to healthy controls (Table 1). In contrast to controls, the significantly decreased lymphocyte numbers were found in septic patients. Additionally, the low total counts of CD3+, CD3+CD4+, CD3+CD8+, CD19+ lymphocyte subsets and considerably low total numbers of NK-cells (CD3-CD16+56) and NKT-cells (CD3+CD56+) were observed. It is noteworthy that the reduction in CD4+ T cells number was much more pronounced than that observed in CD8+ T cells. As a consequence, the CD4/CD8 ratio was little lower in septic patients than in controls. When flow cytometry data were expressed as cell proportions in septic patients and compared to controls thus exhibited a significant increase in the percentage of granulocytes and decrease in the percentage of lymphocytes to a considerable extent (Table 2). After operation and intensive medical care, some improvements in leukocyte and

Table 1. Blood cell counts (10^9 cells/L) in patients with septic infectious endocarditis before and after operation (Mean±SEM)

Parameters	Controls (x10^9/L)	Septic patients (x10^9/L)	
		before operation	after operation
leukocytes	6.92±0.43	11.067±1.68*	10.31±2.28
monocytes	0.36±0.02	0.58±0.09*	0.50±0.07
granulocytes	4.15±0.43	9.48±1.62**	8.31±2.13
lymphocytes	2.4±0.16	0.99±0.17****	1.49±0.23**#
CD3+	1.66±0.14	0.70±0.14****	1.08±0.18*#
CD3+HLA-DR+	0.27±0.04	0.16±0.06	0.22±0.07
CD3+CD56+	0.14±0.03	0.046±0.008**	0.06±0.016*
CD3+CD4+	0.95±0.05	0.41±0.07****	0.61±0.08**#
CD3+CD8+	0.63±0.10	0.26±0.07**	0.41±0.09 #
CD19+	0.26±0.02	0.16±0.03*	0.21±0.06
CD3-CD16+CD56+	0.42±0.07	0.08±0.01****	0.12±0.01***#

**p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (in comparison to control group); #p<0.05 (comparisons before – after operation)*

lymphocyte cell counts were found. There was observed tendency to diminish leukocyte, granulocyte and monocyte levels. This tendency was clear enough that did not exhibit the significant change when compared to normal controls. The surgical operation and intensive care unit procedures resulted in a significant increase of lymphocyte total numbers. As a consequence, these changes were associated with significant extension of CD3+, CD3+CD4, CD3+CD8+ and NK cell total counts. A tendency to normalize CD4/CD8 ratio was also detected (Table 1). When flow cytometry data were expressed as percentages the obtained data before operation and after intensive care resulted in significant diminishing of granulocyte and increase in lymphocyte proportions. No significant changes but tendencies were noted in proportions between lymphocyttic subsets (Table 2).

The concentrations of TNF, sTNF-R1 and sTNF-R2 in blood plasma samples, the whole blood total glutathione,

Table 2. Proportions of blood cell subsets in patients with septic infectious endocarditis before and after operation (Mean±SEM)

Cell subsets	Controls %	Septic patients	
		before operation %	after operation %
leukocytes			
monocytes	5.26±0.24	5.62±0.90	5.95±0.78
granulocytes	58.3±2.96	84.29±2.36****	76.13±3.59***##
lymphocytes	36.45±2.88	10.08±1.85****	22.41±4.76*##
CD3+	68.37±1.59	66.77±3.57	70.93±3.04
CD3+HLA-DR+	15.91±1.46	20.47±2.94	20.09±4.28
CD3+CD56+	7.92±1.14	8.02±1.54	8.52±2.17
CD3+CD4+	40.57±1.61	40.45±2.50	42.22±2.29
CD3+CD8+	24.68±1.88	24.64±1.94	26.12±2.64
ratio	1.83±0.15	1.73±0.14	1.80±0.18
CD19+	10.99±0.89	18.4±2.68*	13.83±2.15
CD3-CD16+CD56+	17.56±2.09	9.58±1.835**	10.8±2.27*

**p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (in comparison with control group); ##p<0.01 (comparisons before – after operation)*

Table 3. Glutathione and TNF factors levels (TNF, TNF-R1, TNF-R2) and SAPS in patients with septic infectious endocarditis before and after operation (Mean ±SEM)

Studied parameters	Control group (n=20)	Before operation (n=24)	After operation (n=24)
sTNF R55 (RI) ng/ml	2.88±0.09	5.29±1.4*	4.2±0.9
sTNF R75 (RII) ng/ml	3.66±0.17	10.61±1.8****	9.09±1.3****
TNF pg/ml	2.34±0.1	16.27±4.2****	11.38±2.7****
whole blood total glutathione (mcM)	1129±42	935.0 ±86.8*	996.96±72.3
whole blood GSH (mcM)	877±37	570.6±47.2****	598.7±37.2****
whole blood GSSG (mcM)	251±17	364.1±42.5*	399.8±38.3**
% GSH of total glutathione	77.6±1.4	62.9±1.6****	61.6±1.3****
SAPS		7.66±0.52	6.08±0.62#

**p<0.05, **p<0.01, ****p<0.0001 (in comparison with control group); #p<0.05 (comparisons before – after operation)*

the whole blood GSH and SAPS in evaluated patients are presental in Table 3. In blood samples collected before operation, the levels of both receptors (TNF-R1 and TNF-R2) and TNF were significantly higher than in healthy controls. Detected levels of the whole blood total glutathione and the whole blood GSH were decreased and levels of the whole blood GSSG were increased in septic patients before operation. After operation and intensive medical care procedures some improvement was observed. SAPS Score was significantly decreased after operation. Total glutathione and sTNF-R1 levels have not been significantly different between the control group and patients after operation.

Negative correlations of the whole blood total glutathione and the whole blood GSH with sTNF-R1 and sTNF-R2 but not with TNF were found before the operation. After the operation, the negative correlations of both total glutathione and GSH with TNF and sTNF-R1 and sTNF-R2 were aalso found (Table 4). Positive Spearman correlations between GSH and NK cell counts were observed before operation ($r=0.58$, $p<0.05$). After operation and intensive medical care procedures this correlation became even stronger ($r=0.62$, $p<0.05$), and additionally a correlation between GSH and CD19 cell count was also detected.

Negative correlations between GSH, total glutathione and SAPS Score were found in 13 severely septic before the operation: for GSH $r=-0.58$, $p<0.04$; for total glutathione $r=-0.67$, $p<0.02$. In these patients SAPS score was 6 and higher (Mean ±SEM=8.46±0.79). Concentrations of both TNF-Rs (TNF-R1 and TNF-R2) correlated positively with indices of clinical status (SAPS Score) after the operation in 24 patients: for sTNF-R1 $r=0.42$, $p<0.04$; for sTNF-R2 $r=0.51$, $p<0.02$.

Discussion

We here confirmed that septic infectious endocarditis is associated with high levels of granulocyte counts and severe lymphocytopenia. The analysis of T-cell subsets showed that total numbers of both CD4+ and CD8+ lymphocytes were significantly lower in patients than in healthy controls. Tendency to diminish the CD4/CD8 ratio was also observed. In addition, B cell and NK cell total counts were also decreased. Nevertheless, when the results were expressed as relative percentage, they clearly indicated that suppression of lymphocytes and their subsets was generally true except of elevated proportion of B cells. It is very possible that septic process affects the generation of T cells and/or their survival in periphery. Total B cell count was less diminished. The relative increase of B cell percentage was observed due to the profound diminution of both main T cell subsets. Transient abnormalities in T-cell subsets have been reported for some infectious diseases, such as tuberculosis, hepatitis, Epstein-Barr virus infection, toxoplasmosis, pneumocystosis, where the CD4/CD8 ratio was always higher than 1.00 [8-10]. A pronounced reduction of CD4+ T cells was observed in patients with Q fever endocarditis [11]. In our study of septic patients with infectious endocarditis a *Staphylococcus aureus* was the predominant detected micro-organism. Lymphocytopenia was associated with the decrease in both CD4 and CD8 T lymphocyte subsets and NK cells. Valvular surgery and intensive medical care resulted in restoration of the cell composition although not completely. Lymphocyte total counts and subsets, and their relative percentages increased while granulocyte counts decreased after operation. On the other hand, it is evident that these improvements may be considered in close relation to improvement in clinical status of these patients.

Reactive oxygen intermediates play important role in mediating proinflammatory cytokine production (TNF, IL-6, IL1β). TNF and free radicals have been implicated in the pathogenesis of septicemia and its complications. Infections, sepsis and trauma lead to some cellular damage. The formation of nitrogen and reactive oxygen intermediates plays a central role in cellular damage. Intracellular GSH can control

Table 4. Spearman correlations (r) of GSH and TNF systems in patients with septic infectious endocarditis before and after operation (n=24)

Parameters		sTNF-p55	sTNF-p75	TNF
whole blood total glutathione	before operation	-0.6166 (p<0.002)	-0.4670 (p<0.03)	n.s.
	after operation	-0.6988 (p<0.0001)	-0.5914 (p<0.003)	-0.5730 (p<0.003)
whole blood GSH	before operation	-0.4576 (p<0.03)	-0.4267 (p<0.04)	n.s.
	after operation	-0.6497 (p<0.001)	-0.6614 (p<0.001)	-0.5424 (p<0.01)

both radical species. In its turn GSH levels are controlled by the presence of cellular growth factors. NF-kappa B/Rel transcription factors have recently emerged as crucial regulators of cell survival. NF-kappa B is activated by reactive oxygen intermediates and regulates inflammatory gene expression. Thiol compounds, such as GSH and N-acetilcysteine, scavenge hydrogen peroxide and are reported to prevent oxidative damage in various cells [12]. Activation of NF-kappa B antagonizes programmed cell death induced by TNF-Rs and several other triggers. This prosurvival activity of NF-kappa B is crucial for pathogenesis of inflammation because it often involves an antagonism of programmed cell death triggered by TNF-R-family receptors through suppression of the formation of reactive oxygen species and a control of sustained activation of the Jun-N-terminal kinase cascade. Effectors of this antagonistic activity of NF-kappa B on reactive oxygen intermediates/Jun-N-terminal kinase pathway have been recently identified [13].

Our study confirmed detection of the low levels of GSH in septic patients. This decrease of GSH was connected with increased production of TNF and soluble TNF receptors, and with deficiency of NK and CD19+ cell counts. It is known that surgical trauma induces the proinflammatory reactions including increase of TNF, sTNF-R1 and sTNF-R2, after cardiac surgery [14]. Soluble forms of cytokine receptors could function as anticytokines by forming high affinity complexes with the relevant cytokines. Therefore circulating soluble TNF receptors may modulate systemic and local effects of TNF [3]. In our study, there was no recovery of GSH levels in early post-operative period but relationship between GSH and TNF levels were restored, perhaps, because of improving of clinical status and homeostatic mechanisms. SAPS is an index indicating severity of clinical status of septic patients and its values point to the high possibility of mortality. Therefore negative correlation between SAPS and GSH, positive correlation between SAPS and sTNF-receptors confirm the significance of GSH and sTNF-receptors for resistance of the organism to septic shock. GSH appears as endogenous antioxidant that serves as one of the body's most important defense against the oxygen metabolites. Plasma levels of GSH are maintained by a balance between its secretion by liver and degradation in the kidney. The ability to maintain and to elevate tissue activity of GSH may be of particular importance in controlling the cytokine production in response to septicemia and injury [15-17]. Glutathione status determines the adverse effects of TNF in cardiac failure and that TNF counterbalance may be achieved by glutathione supplementation [18]. Anti-TNF monoclonal antibodies can be an effective protective agent in experimental septic models [19]. Anti-TNF antibodies have been applied in rheumatoid arthritis and Crohn's disease. However, in clinical cases of sepsis anti-TNF antibodies are not so effective, perhaps, because TNF's early release and transient appearance in serum create a narrow therapeutic window. High mobility group box 1 (HMGB1) is implicated as a late mediator of sepsis and it may widen the therapeutic window and lead to new strategies for inhibiting the deleterious effects of inflammatory cascade [20]. Currently, the treatment of septic patients is largely supportive, centering on antibiotics, fluid supplementation, hemodynamic and ventilatory support, and intensive monitoring. Activated protein A (APC), a natural anticoagulant, proposed for the treatment of severe sepsis and septic shock in selected patients, inhibited endotoxin-induced TNF production by human monocytes blocking activation of NF-κB and activator protein-1 in vitro [21]. Antioxidant therapy (ascorbic acid, glutathione, N-acetyl-L-cysteine, or vitamins A, E, and C alone or in combination) have been shown to reduce burn/sepsis mediated mortality. They seem to attenuate cellular metabolism protect microvascular circulation, reduce lipid peroxidation, improve cardiac output, and to reduce the volume of required fluid resuscitation, inhibit secretion of TNF, IL1β, IL-6 [22]. Recancostat, consisted of GSH, anthocyane and L-cysteine (Bucheli AG, Herisau, Switzerland and Integrative Therapeutics Inc., Green Bay, WI, USA) has been proved to be bioavailable by a special redox-recycling mechanism in the whole blood as well as intracellularly [23-25].

The discussed data indirectly support the hypothesis on the cellular oxidative stress which seems to be critical in sepsis and suggest that antioxidant strategies designed to either inhibit free radical formation or to scavenge free radicals may provide organ protection in septic patients. Side by side with GSH, soluble TNF receptors play an important role in organ protection by neutralization and attenuation of TNF toxic effects saving its protective inflammatory activity. In situation of GSH deficiency, the soluble TNF receptors support homeostasis by means of TNF activity control. Possible role of soluble TNF receptors was confirmed by correlating with clinical symptoms (SAPS Score) as well as with GSH levels. In the early post-operative period negative correlations

between TNF and GSH, and total glutathione appeared. It suggests regulatory and compensatory effects of soluble TNF receptors, which were insufficient before operation (due to knocked out GSH system control) while restored after operation and intensive medical care. Intensification of compensatory and regulatory role of soluble TNF receptors after operation was accompanied by tendency of lymphocytic subpopulations to normalize. Soluble TNF receptors may serve as additional markers of sepsis severity, better than TNF itself because of transient TNF presence in peripheral blood.

Conclusion

In patients with septic infectious endocarditis and Staphylococcus aureus as the predominant micro-organism, lymphocytopenia was associated with the decrease of both CD4 and CD8 T lymphocyte subsets and NK cells. Septic patients may acquire severe GSH deficiency in part due to the excessive activity of TNF. Decrease in GSH levels was connected with increased production of TNF and soluble TNF receptors, and low NK cell and CD19+ total cell counts. Possibly, soluble TNF receptors may attenuate destructive tissue effects of TNF during GSH deficiency. Treatment strategies designed to attenuate toxic effects of inflammatory mediators through restoration of GSH balance may be promising in patients with severe sepsis. Elevated levels of soluble TNF receptors may reflect the severity of sepsis being prognostic factors. Valvular surgery and intensive medical care resulted in restriction of total blood cell counts and relative cell percentages.

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