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Full Length Research Paper

Parameters of immune and antioxidative systems can be considered as surgical stress markers for gastric cancer patients

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Cytokines, haematological indices, lipid peroxidation product and antioxidative system parameters were investigated for surgically treated and red blood cells transfused stages 3 and 4 gastric cancer patients. Serum of 58 patients was analysed at pre-surgical and post-surgical periods (after 7 and 14 days). TGF-beta1 and TNF-alpha were tested by ELISA. Haematological indices were analyzed by standard methods. Malondialdehyde level and antioxidative system parameters were analysed by specified methods spectrophotometrically. Level of TGF-beta1 increased and lymphocyte index decreased after surgery and the transfusion for both stages cancer patients. Level of malondialdehyde decreased at early post-surgical period for non-transfused both stages cancer patients while the parameter decreased only at late post-surgical period for transfused patients. Total glutathione concentration decreased during post-surgical period only for transfused stage 3 cancer patients. Pearson correlations were determined between cytokine and malondialdehyde levels and between cytokine levels and activities of catalase and glutathione S-transferase at early post-surgical period. Decreased lymphocyte count, increased TGF-beta1 level and lower total glutathione concentration after surgery were related to red blood cells transfusion. Malondialdehyde level was not significantly influenced by gastric cancer stage while it was also affected by the transfusion. All altered parameters mentioned showed the additional transfusion-dependant stress to develop surgical stress. The parameters can be considered as possible surgical stress markers.

Key words: Cytokines, lipid peroxidation, antioxidative system, surgical treatment, RBC transfusion.

INTRODUCTION

Gastric cancer is one of the most prevalent malignancies world-wide. Surgery is predominant treatment for gastric cancer patients although complications associated with long post-surgical period and red blood cells (RBC) transfusion usually follow (Mohandas and Aledort, 1995).

Perioperative blood transfusion is known to unbalance immune system of cancer patients (Landers et al., 1996) and the transfusion-related immune dysfunction can result in clinical complications by the mechanism not completely understood (Chen et al., 2007).Immuno-suppressive cytokine TGF-beta and pro-inflammatory cytokine TNF-alpha (cachectin) are crucial in cancer progress but the role of allogenic RBC transfusion for the parameters of gastric cancer patients is not reported. Controversial discussion if the additional stress triggers an immunosuppressive effect for the transfused patients is still the subject.

Surgical stress can be assumed as condition of varied parameters of the treated organism both at clinical, antioxidative and immune system levels. This stress actually has not valuable quantitative markers although alterations of parameters of antioxidative system and of lipid peroxidation markers are discussed possibly to be related to the condition (Sugawara et al., 2002). The oxidative stress is involved in tumour pathophysiology (Hristozov et al., 2001) and various parameters of the antioxida-

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tive system compose the protection frame against the stress. Oxidative stress can also deepen surgical stress and determine worse effect of the surgical treatment for cancer patients. Lipid peroxidation marker malondialdehyde (MDA), essential antioxidant glutathione in both reduced and oxidized forms (GSH and GSSG, respectively), antioxidative enzyme catalase (CAT) and detoxifying enzyme glutathione S-transferase (GST) are among most important parameters to be evaluated for cancer patients.

Relation of changes of immune and antioxidative system parameters that indicate status of two essential protective systems of the organism suffering from the cancer progression is also essential subject. Oxidative stress is shown to enhance level of TNF-alpha and this cytokine lowers GSH level (Powell et al., 2003). Therefore, disturbance of GSH metabolism and enhanced TNF-alpha release can unbalance immune system of cancer patients (Rahman et al., 1999).

It is hypothesized changes of TGF-beta1 and TNF-alpha levels after surgery and RBC transfusion to show negative effect on the immune system of gastric cancer patients. Those parameters also should be related to dysfunction of antioxidative system of the patients. Search for markers of surgical stress is complex process although discovery of such markers could help to predict clinical recovery of cancer patients and to schedule the post-surgical period crucial for the effect of the treatment.

The aim of the present study was to estimate if surgical treatment-based and allogenic RBC transfusion-based fluctuation of two essential cytokine levels and dynamics of intrinsic antioxidative system parameters in peripheral blood of gastric cancer patients are in correlation to consider those parameters as possible surgical stress markers.

PATIENTS AND METHODS

58 patients (30 men and 28 women) diagnosed for primary gastric cancer in Clinic of Surgery, Department of General and Abdominal Surgery and Oncology, Institute of Oncology, Vilnius University, Lithuania were involved in the study. The study was approved by Lithuanian Bioethics Committee (protocol No 2; date 14 January, 2004) also providing written informed consent from all patients. Gastric cancer patients were included in the study according to the following criteria: age of 45–67 years; weight over 60 kg; histologically confirmed stages 3 and 4 gastric adenocarcinoma; not previous blood transfusions; essential haematological parameters prior the surgery – Hb level exceeded 100 g/L, haematocrit index was over 35%, platelet count was higher than 150×10^9/L. Patients were excluded from the study in the case of active infections, chronic active hepatitis, chronic pulmonary and ischemic cardiac diseases, chronic anticoagulation and also if they were consuming immunosuppressive drugs prior surgical treatment.

Venous blood of cancer patients was collected and tested prior the surgery (1st analysis) and also 7 and 14 days after the surgery (2nd and 3rd analysis, respectively). Groups of patients were composed considering cancer stage and blood transfusion during the surgery. Group of stage 3 cancer patients consisted of 30 individuals including 15 transfused and 15 non-transfused patients.

Surgical treatment by gastrectomy was provided to 4 transfused and 5 non-transfused patients while subtotal gastric resection was performed to 11 transfused and 10 non-transfused patients. Group of stage 4 cancer patients involved 28 individuals including 14 transfused and 14 non-transfused patients. Both transfused and non-transfused gastric cancer patients of this group were treated as follows: 4 persons by gastrectomy and 10 persons by subtotal gastric resection.

Transfused patients received 1–2 packets of RBC intra-surgically (blood loss over 500 ml) or up to 5 days after the treatment if Hb dropped to 70 g/L and haematocrit index decreased to 35%. RBC packets were prepared by removing plasma and aliquots of RBC were suspended in saline-adenine-glucose-mannitol (SAGM) (leukocytes concentration was more than $1.2\cdot10^9$/ L) in National Blood Center of Lithuania according to the recommendations of the Council of Europe.

Analysis of haematological parameters

Total erythrocytes, total leukocytes, granulocytes, lymphocytes and platelets count (absolute number) and Hb concentration was determined by Abbott Cell Dyn 1700 haematology analyzer. Percent of lymphocytes, granulocytes was determined by microscopic visualization of smears in peripheral blood stained by Pappenheim method (May–Grunwald–Giemsa).

Analysis of cytokine levels

Blood serum was separated from collected peripheral venous blood of patients. The samples were deep-frozen and stored at –70°C until analysed. Level of cytokines TGF-beta1 and TNF-alpha was determined by ELISA method with standard Biosource kits (Niveles, Belgium) in accordance to instructions. Sensitivity of the assays was 15.6 pg/ml for TGF-beta1 and 3 pg/ml for TNF-alpha. Optical density at 450 nm was detected with ELISA reader (Multiskan EX, Labsystem Oy).

Analysis of lipid peroxidation and antioxidative system parameters

Level of lipid peroxidation product MDA, concentration of antioxidant glutathione (GSH+GSSG) and catalytic activities of enzymes CAT and GST were determined spectrophotometrically. MDA was tested by thiobarbituric acid (TBA) assay based on TBA reaction with MDA resulting in the release of colour complex as described (Surinenaite et al., 2006). Total glutathione was analysed by the method of recycling system with 5,5'-dithiobis-(2-nitrobenzoic acid) and GSSG reductase (Baker et al., 1990). CAT activity was defined as the rate of formation of a hydrogen peroxide/ammonium molybdate complex. GST activity was measured as formation of GSH/1-chloro-2,4-dinitrobenzene conjugate (Galli et al., 1999).

Statistical analysis

Statistical analysis of parameters was performed by Mann Whitney U-test. Correlation analysis was provided by Pearson's rank correlation test. The statistical program SPSS Version 14.0 was used. Differences in values were considered as significant when $p\leq0.05$.

RESULTS

Stage 3 gastric cancer patients

Level of cytokine TGF-beta1 increased significantly (p=

Table 1. Lymphocyte index and level of cytokines TGF-beta1 and TNF-alpha in blood serum of surgically treated and RBC transfused stages 3 and 4 gastric cancer patients.

Stage	Parameters	Period of analysis		
		Prior surgery (1st analysis)	7 days after surgery (2nd analysis)	14 days after surgery (3rd analysis)
3	LYM %	24.00±2.00■*	14.00±1.60■	12.00±1.30*
	LYM 10^9/L	1.90±0.10■*	1.20±0.07■	1.20±0.07*
	TGF-[beta1] (ng/ml)	11.10±1.40*	11.60±2.30	13.80±1.80*
	TNF-[alpha] (pg/ml)	22.00±1.80	23.60±2.90	22.90±2.60
4	LYM %	19.00±1.60■*	12.00±2.20■	11.00±1.40*
	LYM 10^9/L	1.50±0.20*	1.40±0.30	1.00±0.20*
	TGF-[beta1] (ng/ml)	11.10±1.90■*	14.40±1.60■	16.40±1.60*
	TNF-[alpha] (pg/ml)	21.70±2.70	24.20±4.70	24.20±3.00

Significant differences (p≤0.05). Each parameter presented in the following comparison: ■ pre-surgical versus post-surgical periods of 7 days; * pre-surgical versus post-surgical periods of 14 days.

Table 2. Parameters of lipid peroxidation and antioxidative system in serum of surgically treated and RBC transfused or non-transfused stage 3 gastric cancer patients.

Parameters	Period of analysis		
	Prior surgery (1st analysis)	7 days after surgery (2nd analysis)	14 days after surgery (3rd analysis)
With RBC transfusion			
MDA (nmol/ml)	16.55±1.03*	17.02±1.40▲	12.41±1.25*▲
CAT (nmol/l/min)	29.81±2.42	32.63±2.51▲	26.89±2.04▲
GSH+GSSG (mkmol/ml)	0.17±0.02■*	0.14±0.02■	0.15±0.03*
GST (nmol/ml/min)	671.51±99.71	515.77±96.13	688.13±83.05
Without RBC transfusion			
MDA (nmol/ml)	18.27±1.02■*	13.30±1.22■▲	11.89±0.96*▲
CAT (nmol/l/min)	33.38±2.74	32.23±2,11	29.47±1.80
GSH+GSSG (mkmol/ml)	0.24±0.03	0.19±0.02	0.21±0.02
GST (nmol/ml/min)	333.98±59.29	384.88±72.09	432.58±72.24

Significant differences (p≤0.05). Each parameter presented in the following comparison: ■ pre-surgical versus post-surgical periods of 7 days; * pre-surgical versus post-surgical periods of 14 days; ▲ post-surgical periods of 7 and 14 days.

0.01) 14 days after surgical treatment and RBC transfusion for stage 3 gastric cancer patients comparing with the level prior to the treatment. Level of cytokine TNF-alpha did not change significantly. Both parameters did not change significantly in any post-surgical period for non-transfused patients. Lymphocyte index (percent and absolute number of cells count) was found to decrease significantly in the group of transfused patients at post-surgical periods of 7 and 14 days: percent (p=0.005 and p=0.001, respectively), absolute number (p=0.002 and p=0.006, respectively) (Table 1). Other haematological parameters determined did not change significantly in this group.

Significant changes of MDA level, CAT activity and concentration of GSH+GSSG were determined for RBC-transfused stage 3 gastric cancer patients. MDA level was lower (p=0.015) 14 days after the surgery than prior the treatment, although it slightly increased 7 days after the treatment. It was shown to decline (p=0.025) during post-surgical period if comparing the period of 7 and 14 days. CAT activity also decreased significantly (p=0.006) during post-surgical period. The drop of GSH+GSSG concentration was determined at both post-surgical periods comparing to pre-surgical period (p=0.018 and p=0.043, respectively). Significantly lower MDA level was determined for non-transfused cancer patients in all three analyses (p=0.002, p=0.0001 and p=0.047, respectively). No significant alterations of GST activity were determined in any group of stage 3 cancer patients (Table 2).

Stage 4 gastric cancer patients

Level of cytokine TGF-beta1 increased significantly (p= 0.04) for RBC transfused stage 4 gastric cancer patients

Table 3. Parameters of lipid peroxidation and antioxidative system in serum of surgically treated and RBC transfused or non-transfused stage 4 gastric cancer patients.

Parameters	Period of analysis		
	Prior surgery (1st analysis)	7 days after surgery (2nd analysis)	14 days after surgery (3rd analysis)
With RBC transfusion:			
MDA (nmol/ml)	14.16±1.13*	16.80±2.54▲	10.97±0.67*▲
CAT (nmol/l/min)	25.43±2.24	25.22±2.50	23.20±1.88
GSH+GSSG (mkmol/ml)	0.23±0.02	0.25±0.03	0.24±0.01
GST (nmol/ml/min)	360.96±48.44	302.16±71.83	305.36±82.25
Without RBC transfusion			
MDA (nmol/ml)	17.92±1.37*	14.73±1.78	14.57±1.56*
CAT (nmol/l/min)	29.29±2.64	31.82±2.57	29.24±3.66
GSH+GSSG (mkmol/ml)	0.21±0.04	0.19±0.03	0.20±0.05
GST (nmol/ml/min)	302.18±29.59	472.32±29.58	267.75±54.03

Significant differences (p≤0.05). Each parameter presented in the following comparison: ▪ pre-surgical versus post-surgical periods of 7 days; * pre-surgical versus post-surgical periods of 14 days; ▲ post-surgical periods of 7 days and 14 days.

patients already at post-surgical period of 7 days and enhancement of the parameter continued at post-surgical period of 14 days (p=0.008). Level of cytokine TNF-alpha did not change significantly after RBC transfusion for the patients. Lymphocyte index in percent decreased for RBC transfused patients at both post-surgical periods (p=0.01 and p=0.008, respectively) but significantly changed in absolute number only at the later period (p=0.05) (Table 1). Other haematological parameters did not change significantly for transfused patients. Level of cytokines and any haematological parameter also did not change significantly post-surgically for non-transfused patients.

Stage 4 gastric cancer most probably resulted in complicated defence of the organism against the malignancy as only MDA level significantly changed both for transfused and non-transfused patients. It also was slightly increased 7 days after the treatment for RBC-transfused stage 4 cancer patients similarly to stage 3 cancer patients. The parameter was found to be lower 14 days after surgery than at pre-surgical period (p=0.019 for transfused patients and p=0.011 for non-transfused patients, respectively). MDA level also diminished (p=0.039) comparing two post-surgical periods for transfused patients but did not change significantly for non-transfused patients. No significant alterations of antioxidative system parameters were determined for stage 4 gastric cancer patients (Table 3).

Correlation of parameters

Pearson's correlation between analysed parameters of antioxidative system, MDA and cytokines TGF-beta1 and TNF-alpha was calculated both at pre-surgical period (1st analysis) and at post-surgical periods of 7 and 14 days (2nd and 3rd analyses), also considering RBC transfusion. The single significant correlation in pair of (GSH+GSSG) 2/GST2 was found in the groups of transfused stages 3 and 4 cancer patients. The correlation was positive for stage 3 cancer patients (r=0.893, p=0.001) and negative for stage 4 cancer patients (r=-0.894, p=0.016). A number of significant positive correlations in pairs of cytokines (TGF2/TNF2 - r=0.608, p=0.036; TGF3/TNF3 - r=0.837, p=0.001), of cytokines and lipid peroxidation marker or antioxidative system parameters (TGF2/CAT2 - r=0.611, p=0.046; TGF2/GST2 - r=0.845, p=0.034; TNF2/MDA2 - r=0.934, p=0.000; TNF3/MDA3 - r=0.590, p=0.044; TGF3 /MDA3 - r=0.618, p=0.032) and of antioxidative enzyme activities (CAT3/GST3 - r=0.936, p=0.006) were found in the group of non-transfused stage 3 cancer patients. No any correlation was determined in analogous group of stage 4 cancer patients.

DISCUSSION

Alteration of cytokines, of haematological indices and of antioxidative system parameters can be related to surgical stress and to allogenic RBC transfusion for gastric cancer patients. Immunoreactive cell counts, levels of TGF-beta1, TNF-alpha, MDA, concentration of GSH+ GSSG and catalytic activities of CAT and GST were examined at post-surgical period of 7 days (early) and 14 days (late) and were compared to the parameters prior the treatment in order to determine their dynamics during patients hospitalization.

The present study showed that variation of TGF-beta1 level and of lymphocyte count most probably was determined by RBC transfusion. That is suggested as level of the cytokine increased and lymphocyte count decreased significantly for transfused both stages gastric cancer pa-

tients but the parameters did not change significantly for non-transfused patients. Level of cytokine TNF-alpha did not change significantly after RBC transfusion. So it could be suggested RBC transfusion rather than surgical stress determined unbalance of immune system in the organism of advanced stages gastric cancer patients. Other authors show that allogenic blood transfusion causes deep impairment of immune response for gastric cancer patients (Balkwill, 2002; Chen et al., 2007; Lin et al., 2002; Sun et al., 2001). Moreover, complex biochemical alterations and the oxidative injury trigger RBC aging in erythrocytes during storage (Kriebardis et al., 2007). Internal erythrocyte constituent can be released after RBC transfusion resulting in disturbed microcirculation and oxygen delivery that can make side-effects of the transfusion (Novotny, 2007).

Cytokines TGF-beta and TNF-alpha play an important, although not completely definite role considering interaction between tumour and the organism. The higher levels of those cytokines after RBC transfusion can predict unfavourable result of cancer treatment. TGF-beta signalling pathways play a critical role in the regulation of cell growth, differentiation and development (Blobe et al., 2000; Herpin and Cunningham, 2007; Schmierer and Hill, 2007). It is also suggested this multipotent cytokine to act as stimulating factor for tumour cell growth, to suppress immune cells (Clark et al., 2008), to stimulate the invasion of cancer cells and to promote neo-angiogenesis (Bello-De Ocampo and Tindal, 2003). Clinical studies show that over-expression of TGF-beta1 is in significant correlation with lymph node metastasis for patients with primary gastric cancer and the result is considered to show poor prognosis (Saito et al., 2000). TNF-alpha is multipotent pro-inflammatory cytokine involved in essential immunologic functions including the immune system homeostasis. Level of the cytokine is usually increased for cancer patients and it can stimulate tumour growth and invasion (Balkwill, 2002).

MDA level as marker of lipid peroxidation is important parameter that indirectly shows status of antioxidative system of cancer patients (Bakan et al., 2002). Lower MDA level at late post-surgical period for RBC transfused and non-transfused both stages gastric cancer patients should indicate surgical treatment to be favourable to prevent the accumulation of lipid peroxides. Those reactive species are primal markers of damage of essential lipids in cell membranes and can be toxic for cell functions that are also involved in cancer development (Comporti, 1998). MDA level could be related to the characterization of surgical stress as significant drop of the parameter was determined only at late post-surgical period but not at the period of 7 days. Cancer stage was shown not to cause direction of post-surgical change of MDA level. In contrast, variation of the marker was found to be dependant on RBC transfusion.

The parameter was significantly lower after 14 days from surgery while it was higher after 7 days for transfused patients but it was already lower after 7 days for non-transfused patients. The decrease of MDA level was more significant for non-transfused patients. Those results are supported by other reports concluding that MDA level is considerably affected by blood transfusion as additional invasion to the organism and the parameter can indicate deeper surgical stress (Sugawara et al., 2002).

Significantly lower activity of primal antioxidative enzyme CAT was determined only at late post-surgical period for RBC transfused stage 3 cancer patients. It should be suggested the catalytic action of the enzyme to be affected by various factors and could not be discussed as possible universal marker of oxidative or surgical stress for advanced stages gastric cancer patients.

Glutathione is multifunctional agent involved in main pool of antioxidative system, in detoxication reactions and in modulation of immune system. It is important parameter to evaluate status of antioxidative system of gastrointestinal cancer patients (Skrzydlewska et al., 2003). In this study the drop of total glutathione concentration for RBC transfused stage 3 gastric cancer patients at both post-surgical periods could be supposed to be not favourrable result. According to other authors (Fiashi et al., 2005) it may show cancer cells to accumulate the antioxidant to become less susceptible to oxidative stress when total glutathione concentration is lower in blood serum. The parameter did not change significantly for non-transfused stage 3 cancer patients and also in both groups of stage 4 cancer patients. Consequently, that change should be related to gastric cancer stage and to RBC transfusion for stage 3 cancer patients. The result most probably was not associated to increased enzymatic activity of GST. The parameter did not change signifycantly in the present analysis. More experiments are necessary to provide well-founded conclusions about significance of GST as possible biomarker of surgical stress. It was suggested by other authors (Kim et al., 2005; Nomani et al., 2005) that fluctuations of GST activity should be important marker of cancer risk and predictive biomarker of cancer progression.

Correlation between lipid peroxidation markers, antioxidative system parameters and multipotent cytokines is important for cancer patients. It shows the status of two essential protective systems of the organism and it also should help to search for surgical stress markers. Correlation in pair of GSH2/GST2 for RBC transfused both cancer stage patients was of high strength and showed relation of enzyme activity/substrate consumption 7 days after surgery. It was positive for stage 3 cancer patients and negative for stage 4 cancer patients. This result possibly indicated unbalance of glutathione metabolism for advanced stage 4 cancer patients.

Few positive correlations in pairs of cytokine levels, of lipid peroxidation marker and of antioxidative system parameters were found in the group of non-transfused stage 3 cancer patients. The most significant links of highest strength were found in pairs of TNF2/MDA2, TGF

3/TNF3 and CAT3/GST3. The result showed that certain relations between parameters of each protective system should help to predict markers of surgical stress if RBC transfusion did not impair integrated action of antioxidative and immune systems.

It is shown that antioxidants diminish blood serum level of TNF-alpha. Glutathione concentration is a substantial factor for the immune response and regulation of this factor is related to cells sensitivity to the apoptosis induced by TNF-alpha (Fiashi et al., 2005). The cytokine impairs GSH production (Meurette et al., 2005). Oxidative stress usually increases release of TNF-alpha (Mantovani et al., 2003). Correlation between activities of two antioxidative enzymes at post-surgical period is the fact guiding search for other multiple correlations in order to find possible organism markers of the surgical treatment prognosis and of surgical stress that still lacks universal characterization. The fact that no correlation was determined for stage 4 gastric cancer patients should lead to suggest that immune and antioxidative systems were unbalanced with gastric tumour progression.

Conclusions

Decreased lymphocyte count and increased TGF-beta1 level after surgery was related to RBC transfusion for both stages gastric cancer patients. Malondialdehyde level was not significantly influenced by gastric cancer stage while it was also affected by the transfusion. Longer post-surgical period was found to be necessary to lower the parameter significantly for RBC transfused patients than for non-transfused patients of both stages cancer. Lower total glutathione concentration after surgery was also related to the transfusion. All those altered parameters showed the additional red blood cells transfusion-dependant stress to develop surgical stress and can be considered as possible surgical stress markers. Total oxidative, surgical and suppressed immune system stress can be supposed to determine worse effect of surgical treatment for gastric cancer pa-tients.

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