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Glutamine abolishes the *TLR-4* gene expression levels in pancreatic cancer patients: a preliminary study

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Abstract

Aim of the study: *The purpose of the study was to investigate the effect of pancreatic cancer and preoperative enteral immune-enhancing diet (immunonutirtion) on the expression of TLR4 gene in leukocytes of the malnourished patients with pancreatic cancer.*

Material and methods: *Sixteen malnourished patients with pancreatic cancer received for 5 days preoperative enteral immune-enhancing diet containing glutamine (20 g per day), antioxidative vitamins and trace elements (GlutaminePlus, Fresenius Kabi). The expression of TLR-4 gene in leukocytes of peripheral blood was measured in all patients twice before surgery (before and after immunonutrition) and ones after surgery. The control group comprised 15 healthy sex- and age-matched volunteers.*

Results: *The expression of TLR-4 gene in leukocytes of pancreatic cancer patients before surgery was significantly higher as compared to the healthy volunteers. Expression of TLR-4 gene significantly decreased after preoperative immunonutrition with glutamine. After surgery expression of TLR-4 gene in leukocytes decreased insignificantly as compared to expression at day before surgery but this decrease abolished the significant differences of TLR-4 gene expression between group of pancreatic cancer patients and control group.*

Conclusions: *The high expression level of TLR-4 gene in leukocytes of pancreatic cancer patients may reveal the up-regulation of the innate antibacterial response. This disorders may contribute to increased susceptibility to postoperative infectious and septic complications. Preoperative enteral supplementation of glutamine by decreasing TLR-4 expression may reduce of susceptibility to infectious and septic complications in pancreatic cancer patients.*

Key words: *pancreatic cancer, glutamine, Toll-like receptor, gene expression.*

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Introduction

Pancreatic cancer is one of the leading cause of cancer-related death. In majority of patients with early formation of metastases to lymph nodes or distant organs at the time of diagnosis tumor resection is impossible and prognosis is still poor [1]. The five-year survival rate is only 4% and the average survival rate is only 3-15 months. Surgical resection remains the most important therapy for pancreatic carcinoma, however only in 15-20% of pancreatic cancer patients surgical tumor removal can be performed. The average survival rate after tumor resection is 8 to 25 months and the overall five-year survival rate is about 20-25% [2]. In addition pancreatic cancers are unresponsive to most standard oncologic therapies (adjuvant chemotherapy, radiotherapy) improves the survival rate by up to same weeks or months [3]. Progress in surgical treatment of pancreatic can-

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cer has allowed reducing perioperative mortality to about 5%, but the number of complications is still very high and can reach up to 70% [4]. The main reasons of this high morbidity are immunosuppression [5-7] and malnutrition which occurs in 70-85% pancreatic cancer patients [8, 9]. Extensive surgical trauma additionally increases malnutrition and immune disorders. It contributes to the risk of severe postoperative infectious and septic complications and increases time of hospitalization, costs of treatment and mortality [10, 11].

The early diagnosis of immune disorders and nutritional treatment are the basic conditions for obtaining better treatment results in this group of patients. Immunonutrition ameliorates the immunometabolic response to major trauma and improves outcome after pancreaticoduodenectomy [12]. Multiple-centers randomized studies revealed improvement of treatment results in oncological and septic patients after immunonutrition containing glutamine, arginine and polyunsaturated fatty acids [13, 14]. Despite the advantage of positive clinical effects of immunonutrition on the treatment of surgical patients, the impact of this nutrition on innate immune system remains still unclear. It is supposed that immune-enhancing ingredients can regulate local activity of cells participating in elimination of bacterial pathogens from surgical wound, which may support the process of wound healing and decrease septic complications.

One of the main bacterial antigen is lipopolysaccharide (LPS) which is found in the outer membrane of Gram-negative bacteria. It acts as endotoxins and elicits strong immune response by binding with Toll-like receptor 4 (TLR-4). Toll-like receptors (TLRs) are a family of transmembrane receptors that play a key role in the nonspecific or innate defense, particularly in inflammatory response against various invading exogenous pathogens, by recognizing receptor-specific pathogenic components for bacteria, viruses, fungi and parasites called PAMPs (pathogen-associated molecular patterns) [15]. Toll-like receptor 4, one of the best known receptor, senses Gram-negative bacteria by binding LPS which activates signaling pathways that stimulate cytokine production and other parts of the innate response [16]. Toll-like receptors are mainly expressed by immune cells and epithelial cells. Recently TLR-4 has been detected in many tumor cell lines or tumors [17, 18] also in pancreatic ductal adenocarcinoma [19]. It can promote the proliferation and inhibit the apoptosis and lead to migration, invasion and angiogenesis of tumor [20]. However, it remains unknown, what *TLR-4* gene expression is in leukocytes in pancreatic cancer patients and whether immunonutrition may impact on this expression.

The purpose of our study was to investigate the effect of pancreatic cancer and preoperative enteral immune-enhancing diet (immunonutrition) on the expression of *TLR-4* gene in leukocytes of the malnourished patients with pancreatic cancer.

Material and methods

Patients

The study was carried out in sixteen malnourished patients (weight loss > 5% for 6 months) with pancreatic cancer (9 males and 7 females, mean age 62.1 ±10.1). All patients received for 5 days preoperative enteral immune-enhancing diet containing glutamine (20 g per day), antioxidative vitamins and trace elements (GlutaminePlus, Fresenius Kabi).

After full clinical diagnostic procedures (imaging and laboratory tests) all patients were subjected to pancreaticoduodenectomy or due to irresectability of the tumor to palliative operation. Patients were classified according to UICC (TNM classification of malignant tumours): 3 patients were classified stage I, 7 patients were at stage II, 3 at stage III and 3 at stage IV [21, 22].

The present investigation did not include patients over 75, patients treated with chemo- or radiotherapy or immunosuppressors, patients with autoimmune diseases, with diabetes, chronic respiratory insufficiency, cardiovascular insufficiency, and kidney and liver diseases.

In all patients heparinised peripheral blood samples were collected twice before surgery (before immunonutrition and after immunonutrition) and ones after surgery (one day after surgery). The control group comprised 15 healthy sex- and age-matched volunteers.

Ethics

The patients gave a written consent after the details of the protocol were fully explained. The protocol of the study was approved by the Medical University Ethics Committee and conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki.

Leukocytes isolation, RNA isolation, reverse transcription and real-time PCR

Leukocytes were isolated from 10 ml of heparinised blood by density gradient centrifugation using Polymorphprep (Axis-Shield, PoC AS, Oslo, Norway) according to manufacturer's instruction [23].

The total RNA was isolated from leukocytes using Total RNA and Protein Isolation Kit (Macherey Nagel, GmbH & Co, Dueren, Germany) according to manufacturer's instruction. Isolated RNA was reversed to cDNA by using VerteK-IT (Novazym, Poznan, Poland) with oligo(dT)15 primers according to manufacturer's instruction. The concentration of cDNA was analyzed by NanoDrop spectrophotometer (Thermo Fisher Scientific Inc. Waltham MA, USA).

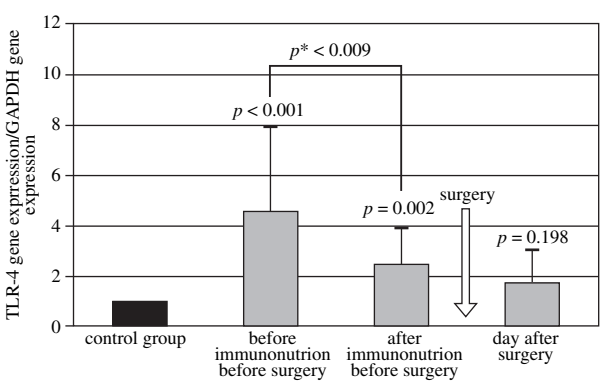
Real-time PCR was performed using a LightCycler 2.0 Instrument and LightCycler® FastStart DNA Master SYBR Green I detection kit (Roche Applied Science, Basel, Switzerland Cat. No. 12 239 264 001) as per the manufacturer's protocol. The final 20 µl real-time PCR reaction included

1000 µg RT product, 2 µl of primers, 0.8 µl $MgCl_2$ and 2 µl of LightCycler® FastStart DNA Master SYBR Green I. To reach a total volume of 20 µl per capillary, DNase-RNase-free distilled water was added. The reaction was run online at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s for denaturation, 61°C for 10 s for annealing and 72°C for 15 s for extension. After 45 cycles, a melting curve was generated by slowly increasing (0.2 C/s) the temperature from 70°C to 99°C, while the fluorescence was measured.

The primer sequences for *TLR-4* gene were as follows: 5'-GCCCTGCGTGGAGGTGGTTC-3' (forward) and 5'-GTCCAGAAAAGGCTCCCAGGGC-3' (reverse); the primer sequences for GAPDH were: 5'-GTGAAGCAGGC GTCGGAGGG-3' (forward) and 5'-GCTCTTGCTGGGG CTGGTGG-3' (reverse). The control was performed using cDNA from healthy volunteers instead of cDNA from patients. The results were analyzed by calculation formula: folds = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = [Ct TLR4 – Ct GAPDH]experiment sample – $[Ct\ TLR4 - Ct\ GAPDH]_{control\ sample}$ [24]. According to this formula $\Delta\Delta Ct$ for the control group was 0 and *TLR-4* gene expression in this group equaled 1. The results of *TLR-4* gene expression for pancreatic cancer group were expressed as mean ± SD.

Statistical analysis

Statistical analysis was performed using the StatSoft Statistica v.9.0 program. To evaluate the statistical significance in *TLR-4* gene expression between pancreatic cancer patients and healthy volunteers Mann-Whitney test was used. Wilcoxon signed-rank test for paired samples was used to compare *TLR-4* gene expression before and after immunonutrition or before and after surgery. Differences at $p < 0.05$ were considered to be statistically significant.



p – Mann-Whitney test (pancreatic cancer group in comparison with control group); p – Wilcoxon signed-rank test (pancreatic cancer group before immunonutrition in comparison with pancreatic cancer group after immunonutrition); p < 0.05 was considered as statistically significant*

Fig. 1. *TLR-4* gene expression in leukocytes of pancreatic cancer patients

Results

The results of the study show a significantly higher expression of *TLR-4* gene in leukocytes of pancreatic cancer patients before surgery (before and after immunonutrition, respectively $p < 0.001$ and $p = 0.002$) as compared to the healthy volunteers. There was a significant decrease at *TLR-4* gene expression after preoperative immunonutrition with glutamine ($p = 0.009$). After surgery (one day after surgery) expression of *TLR-4* gene in leukocytes decreased insignificantly as compared to expression at day before surgery. However this decrease abolished the significant differences of *TLR-4* gene expression between group of pancreatic cancer patients and control group ($p = 0.198$) (Fig. 1). In pancreatic cancer group the results of CRP, IL-1, IL-6 and TNF-α were in normal range.

Discussion

In patients with pancreatic cancer the expression of *TLR-4* gene in leukocytes has not been investigated. The published studies were carried out only in septic and trauma patients. Recent study indicate that monocytes from trauma patients expressed higher levels of TLR-2 and TLR-4 receptors than monocytes from the healthy control [25-27]. Other studies indicate that monocyte mRNA and cell-surface receptor expression of TLR-4 were increased in surgical intensive care unit patients compared with normal control [28]. Armstrong *et al.* show that TLR-4 mRNA was increased in septic patients than in healthy controls while there was no corresponding increase in TLR-4 protein [29]. Lendemans *et al.* show that the surface expression of TLR-2 receptors was significantly decreased on monocytes collected from trauma patients (with an Injury Severity Score above 21 points), whereas the expression of TLR-4 receptors remained unchanged in comparison with healthy controls [30].

Our study revealed significantly increased *TLR-4* gene expression in leukocytes of the malnourished pancreatic cancer patients. We suggest that overexpression of *TLR-4* gene in leukocytes may be caused by occurrence and development of pancreatic tumor and malnutrition. This hypothesis require verification by further studies explaining correlation between expression of *TLR-4* gene in leukocytes and development of pancreatic cancer. Because of the increased expression of *TLR-4* gene and protein in most data in septic and trauma patients we suggest that the significantly higher expression of *TLR-4* gene in leukocytes of the pancreatic cancer patients may reveal the up-regulation of the innate antibacterial response. This disorders may contribute to increased susceptibility to postoperative infectious and septic complications.

In an attempt to investigate the effect of preoperative enteral immune-enhancing diet (immunonutrition) on the expression of *TLR-4* gene in leukocytes pancreatic cancer patients received for 5 days preoperative enteral immunonutrition with glutamine (20 g per day), antioxidative vitamins

and trace elements. After immunonutrition we noted significantly decrease of this gene expression in leukocytes. Some of the most recent experimental studies show that administration of glutamine reduces the increased expression of TLR-4 on epithelial cells [31-33]. Authors suggest that this down-regulation may be a mechanism by which intestinal epithelial cells protect again dysregulated immune signaling in response to Gram-negative commensal bacteria and their products. Moreover they hypothesized that the positive effect of glutamine may be considered as a mechanism via which immunonutrition helps in the recovery of critically ill and septic patients [31]. Other authors suggest that TLR-4 might be involved in the pathogenesis of necrotizing enterocolitis and that glutamine may provide protective effects on intestine possibly through reducing the TLR-4 expression and then decreasing the apoptosis of intestinal epithelial cells [33].

In the case of critical care patients the study shows that parenteral nutrition supplemented with glutamine (0.35 g glutamine/kg/day as dipeptide Ala-Gln, Dipeptiven, Fresenius Kabi) for 5 days does not change the expression of TLR2 or TLR-4 receptors in peripheral blood monocytes in 15 septic patients [34]. Moreover expression of TLR-2 and TLR-4 receptors were similar in groups with (15 septic patients) and without (15 septic patients) glutamine supplementation. Two years later authors expanded the group with parenteral glutamine supplementation up to 23 septic patients. However they did not observe any changes in expression of TLR2 and TLR-4 proteins in peripheral blood monocytes after immunonutrition [35]. Furthermore the levels of TNF-α, IL-1β, IL-6 and IL-10 produced in response to LPS were similar in patients treated with (23 septic patients) and without (20 septic patients) glutamine pretreatment. However in these cases glutamine was supplemented parenterally unlike in our study. The results of our study show that after surgery *TLR-4* gene expression in leukocytes of pancreatic cancer patients was still decreasing, approximating to physiological value. Our unpublished studies show that *TLR-4* gene expression in leukocytes increased after surgery in pancreatic cancer patients without preoperative nutritional treatment. Therefore we suggest that decrease of *TLR-4* gene expression in patients with preoperative immune-enhancing diet is caused by glutamine, not by surgical trauma. Clinical studies have shown that preoperative administration of glutamine decrease infectious complications, morbidity and improve outcomes in septic patients and patients after major gastrointestinal surgery [13, 14]. However the mechanisms where glutamine prevents occurrence of infection are still unclear. We suggest that glutamine by decreasing of *TLR-4* gene expression in leukocytes may reduce risk of infectious postoperative complications.

Our studies may suggest that glutamine act as inhibitor of *TLR-4* gene expression in leukocytes of pancreatic cancer patients before and after surgery. Decrease of *TLR-4* gene expression level to physiological value may be beneficial in reduction of susceptibility to infectious and septic complications. We suggest that enterally supplemented glutamine by decreasing TLR-4 expression may protect pancreatic cancer patients from infectious and septic complications after severe surgical trauma. However the future research needs to be undertaken to investigate correlation between decrease *TLR-4* gene expression and occurrence of postoperative complications.

Conclusions

Our studies reveal the significantly higher expression of *TLR-4* gene in leukocytes in pancreatic cancer patients what may be caused by development of pancreatic cancer. The high expression of *TLR-4* gene in leukocytes may reveal the up-regulation of the innate antibacterial response. This disorders may contribute to increased susceptibility to postoperative infectious and septic complications. Preoperative enteral supplementation of glutamine by decreasing TLR-4 expression may reduce of susceptibility to infectious and septic complications in pancreatic cancer patients.

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The authors declare no conflict of interest.

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