Arch. Immunol. Ther. Exp. (2011) 59:385–397
DOI 10.1007/s00005-011-0140-3

ORIGINAL ARTICLE

Apoptosis in Lymphocytes of Pancreatic Cancer Patients: Influence of Preoperative Enteral Immunonutrition and Extensive Surgery

Robert Słotwiński · Waldemar Olszewski · Maciej Słodkowski · Gustaw Lech · Marzanna Zaleska · Sylwia Kędziora · Anna Włuka · Anna Domaszewska · Sylwia Słotwińska · Wojciech Krasnodębski · Zdzisław Wójcik

Received: 6 August 2010 / Accepted: 8 April 2011 / Published online: 5 August 2011

Abstract The mechanisms of correcting immune disorders in patients with pancreatic cancer requiring major surgery procedures by introducing perioperative immune-enhancing diet (immunonutrition) are still unclear. The purpose of our study was to investigate the effect of pancreatic cancer, extensive surgery and immunonutrition versus enteral standard nutrition on the apoptotic signaling pathways. The randomized studies were performed in 72 patients before and after pancreatic cancer resection with preoperative standard (Group I) or enteral immunonutrition (Group II). The expressions of Bcl-2, Bax, caspases-3, -9, NF-κB, PARP-1/89 kDa, TNFR1/CD120a and Fas/CD95 in peripheral blood lymphocytes were assessed by western blot analysis and flow cytometry before and on day 1, 3 and 7 after surgery. In malnourished patients before and after surgery, the expression of Bcl-2, Bax, NF-κB, PARP-1 was significantly lower, whereas the expression of caspases, as well as the percentage of cells with death receptors were significantly higher when compared with the control group. There was no difference in Bcl-2, Bax and PARP-1 expression between the control group and the patients with normal nutritional status (Group III) before surgery. In comparison to the standard nutrition, the preoperative immunonutrition increased the Bcl-2 and Bax expression inconsiderably but significantly increased the percentage of CD95- and CD120a-positive lymphocytes after surgery. In malnourished patients with pancreatic cancer, the overwhelming expression of caspases and the decrease expression of anti-apoptotic proteins may lead to inappropriate lymphocyte apoptosis and higher cell depletion. The preoperative enteral immunonutrition prevents the postoperative decrease in lymphocyte subsets, but a higher level of lymphocyte susceptibility to undergo accelerated apoptosis can also be considered.

Keywords Pancreatic cancer · Immunonutrition · Apoptosis

R. Słotwiński (✉) · W. Olszewski · M. Zaleska · A. Włuka · A. Domaszewska
Department of Surgical Research and Transplantology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Pawińskiego 5, 02-106 Warsaw, Poland
e-mail: robert_slotwinski@yahoo.com; slotwinski@cmdik.pan.pl

R. Słotwiński · S. Kędziora
Department of Immunology and Nutrition, Medical University of Warsaw, Warsaw, Poland

M. Słodkowski · G. Lech · W. Krasnodębski
Department of General, Gastroenterological and Oncological Surgery, Medical University of Warsaw, Warsaw, Poland

W. Olszewski
The Norwegian Radium Hospital, Oslo, Norway

S. Słotwińska
Department of Conservative Dentistry, Medical University of Warsaw, Warsaw, Poland

Z. Wójcik
Department of Human Nutrition, Medical University of Warsaw, Warsaw, Poland

Introduction

The malnutrition occurring in approximately 50% of patients with digestive tract cancers (Bruun et al. 1999; Edington et al. 2000) considerably affects the process of wound healing, intestinal barrier function and the number of post-operative complications (Detsky et al. 1987; Farreras et al. 2005; Haydock and Hill 1986; Reynolds

et al. 1996). Extensive surgical trauma increases both immunosuppression and malnutrition. The early diagnosis of malnutrition and the use of immunosuppression-decreasing perioperative enteral or parenteral nutritional support are one of the treatment solutions allowing us to obtain better outcome in the group of oncological patients requiring major surgery. Despite the advantage of positive clinical effects of immunonutrition on the treatment of surgical patients, the impact of this nutrition on immune system remains still unclear (Heyland et al. 2006; Novak et al. 2002; Zheng et al. 2007). The most controversial is the effect of immunonutrition on the pre- or post-operative immune system function, which is very important for the adequate host response to surgical trauma, intra-operative infection and may also influence the early formation of multiple metastases.

The extensive resection of the pancreas in patients with pancreatic adenocarcinoma is still the best treatment determining outcome (Wagner et al. 2004). Pancreaticoduodenectomy is one of the most invasive operations in upper abdominal surgery with a very high incidence of post-operative complications (Alexakis et al. 2004; Halloran et al. 2002; Yeo et al. 1997). The frequency of malnutrition in patients with pancreatic cancer ranges from 80 to 85% (von Meyenfeldt 2005) and is connected with systemic and local immunosuppression (Greco et al 2007; Poch et al. 2007; von Bernstorff et al. 2001). The attempt to correct the immune disorders by introducing pre-operative or post-operative immunonutrition is a promising way to improve outcome after pancreatic surgery, however, the mechanisms underlying the immune system suppression (e.g. decrease in lymphocyte numbers) and the mechanisms of correcting postoperative immune disorders (e.g. by regulation the lymphocyte apoptosis) using this type of nutrition, are still unclear.

Apoptosis (programmed cell death) is one of the processes important for the proper functioning of the immune system especially in oncological patients requiring major surgery. There are two major pathways of apoptotic cell death induction: extrinsic signaling through death receptors that leads to the formation of the death-inducing signaling complex, and intrinsic signaling mainly through mitochondria leading to the formation of the apoptosome and activation of caspases-3, which initiates apoptosis. The extrinsic pathway of apoptosis can be induced through the members of the tumor necrosis factor (TNF)/TNFR superfamily such as for example: receptor 1 for TNF-α (TNFR1) (Ankersmit et al. 2002). Whether activated by membrane-bound death receptors (Walczak and Krammer 2000) or by stress-induced mitochondrial perturbation (intrinsic pathway through mitochondria interaction between Bax and Bcl-2 leads to the formation of pores in the mitochondria) with a subsequent cytochrome c release (Loeffler and Kroemer 2000), the activation of downstream caspases leads to stepwise cellular destruction by disrupting the cytoskeleton, shutting down DNA replication and repair, degrading chromosomal DNA, and, finally, disintegrating the cell into apoptotic bodies (Nagata 2000). The central component of apoptosis is a family of caspases. The caspase 3 is also responsible for the proteolytic cleavage of nuclear enzyme poly-(ADP-ribose) (PARP), a polymerase that catalyzes the addition of ADP-ribose units to DNA. This affects many different cellular processes as diverse as transcription, DNA replication, differentiation, gene regulation, protein degradation and spindle maintenance. The interactions between the anti-apoptotic members like Bcl-2 protein and pro-apoptotic members like Bax protein provide a mechanistic basis in modulating the apoptotic cell death (Marzo et al. 2001; Wei et al. 2001). Bcl-2 directly or indirectly prevents the release of cytochrome c from mitochondria in a variety of tissues (Green and Reed 1998). The Bax protein is a pro-apoptotic member of the Bcl-2 family that resides in the cytosol and translocates to mitochondria upon induction of apoptosis (Hsu et al. 1997). The last stage of apoptosis both in the extrinsic pathway and in the intrinsic pathway (mitochondrial) is the so-called executor phase. It begins with a caspase cascade that activates cytoplasmic endonucleases degrading the genetic material of the cell as well as nuclear and cytoskeletal protein degrading proteases. Among other apototosis pathways, authors described also the mechanisms connected with granzyme A and B, sphingomyelin-ceramide pathway and stress-induced pathway (Lieberman and Fan 2003; Lin et al. 2006; MacDonald et al. 1999; Shiraishi et al. 2006).

The purpose of our study was to investigate the influence of pancreatic cancer, surgical trauma and enteral immunonutrition versus enteral standard nutrition on the changes of apoptotic signaling pathways. The expression of Bcl-2, Bax, caspases-3, -9, NF-κB, PARP-1/89 kDa[poly-(ADP-ribose) polymerase], TNFR1/CD120a and Fas/CD95 was measured in peripheral blood lymphocytes. These proteins were found in our previous studies to be the most sensitive markers of apoptosis in surgical patients (Kędziora et al. 2010). We have also tested the hypothesis that preoperative enteral immunonutrition possesses some anti-apoptotic properties and can influence the lymphocytes apoptotic signaling pathways in patients before and after extended pancreatic cancer surgery.

Materials and Methods

Patients

Eighty-eight patients with pancreatic cancer were admitted to the study. Sixteen patients were excluded according to

the criteria of investigation. Fifty out of the 72 patients operated on for pancreatic cancer were randomized (by using numbered sealed envelopes stratified by the surgeon) to receive either the enteral standard diet (Group I: 24 patients, mean age 62.5 ± 9.2) or the immune-enhancing enteral diet (Group II: 26 patients, mean age 61.8 ± 8.7). Twenty-two patients (Group III, mean age 62.0 ± 9.3) after pancreatic cancer surgery of normal nutritional status did not receive the pre-operative nutrition. After completing a full set of clinical diagnostic procedures (imaging and laboratory tests), all patients were subjected to pancreatic head resection (Whipple's pancreatoduodenectomy with lymphadenectomy). A histopathological examination confirmed the diagnosis (the results of pathologic examination for the whole group of patients detected adenocarcinoma in resected specimens). The characteristics of the groups of patients requiring pre-operative enteral nutrition are shown in Table 1. All patients received preventive antibiotics (1.2 g augmentin and 2.0 g of cefoperazone), low-particle heparin and were intravenously given crystalline fluids as well as electrolytes depending on actual demand.

The investigation did not include patients treated with early enteral or parenteral post-operative nutrition, patients showing early serious post-operative infectious complications, the ones with unresectable pancreatic cancer or inflammatory tumor, and those who had organ transplants, patients treated with chemo- or radiotherapy or immunosuppressors, patients suffering from autoimmune diseases, with diabetes type 1 (insulin-dependent), chronic respiratory insufficiency (chronic obstructive pulmonary disease), cardiovascular insufficiency, kidney and liver diseases (biopsy-proven cirrhosis or a serum total bilirubin higher than 3.0 mg/dL). The control group comprised 30 healthy sex- and age-matched volunteers (mean age 58.2 ± 8.9).

Table 1 Patient characteristics and surgical parameters of the two groups of patients with pre-operative enteral supplemented (immunonutrition) and standard diet

Characteristics	Supplemented diet ($n = 26$)	Standard diet ($n = 24$)
Age (years)	61.8 ± 8.7	62.5 ± 9.2
Gender (M:F)	15 (57.7%)/11 (42.3%)	12 (50%)/12 (50%)
Tumor staging (TNM classification)		
I	0	0
II	17 (65.4%)	16 (66.7%)
III	9 (34.6%)	8 (33.3%)
IV	0	0
Duration of surgery (min)	318 ± 58	323 ± 57
Operative blood loss (mL)	540 ± 300	500 ± 350
Transfused patients	8 (30.7%)	7 (29.2%)

Data are reported as mean ± SD or frequencies (for all parameters $p > 0.05$)

Enteral Nutrition

In the preoperative period, standard enteral nutrition or immunonutrition were used for 5 days as a supplementary diet. The indication for pre-operative enteral nutrition treatment was the loss of body mass (higher than 5% within 2 months) and the extent of surgery (including the advancement of the tumor) (Weimann et al. 2006). Except for the moderately undernourished patients (28 patients), in the group of patients treated with preoperative nutrition, 22 seriously malnourished persons were detected (including >10–15% weight loss within 6 months in 7 persons from the group receiving standard nutrition and 9 in the group with immunonutrition as well >15% weight loss within 6 months in 6 patients). Two enteral diets were used: a standard diet (Nutridrink®, Nutricia Export BV, Zoetermeer, Holland) and an immune-enhancing diet (FortiCare®, Nutricia Export BV, Zoetermeer, Holland and Glutamine Plus®, Fresenius Kabi). Each patient received two sachets (Glutamine Plus, $n = 17, 160$ kcal/day) and three containers or two bottles (FortiCare $n = 9, 600$ kcal/day or Nutridrink, $n = 24, 600$ kcal/day) of diets per day according to manufacturer's instructions. The immune-enhancing diets contain 20 g of glutamine, over 2 g of eicosapentaenoic acid and 1.2 g of docosahexaenoic acid. Table 2 shows the composition of the diets.

Table 2 Composition of diets

Variables	Composition of the diets/100 mL		
	Supplemented diet ($n = 26$)		Standard diet ($n = 24$)
	Glutamine plus (in 1 sachet: 22.4 g)	FortiCare	Nutridrink
Calories (kcal)	80	160	150
Protein (g)	10	9.0	6.0
Glutamine	10	–	–
Carbohydrate (g)	10	19.1	18.4
Sugar (g)	0.36	13.6	6.7
Fat (g)	–	5.3	5.8
EPA/DHA (g)	–	0.597/0.3	–
Na (mg)	3	110	90
K (mg)	10	215	159
Zn (mg)	3.3	2.0	1.8
Se (μg)	50	14	8.6
Vit. A (μg)	–	130	123
Vit. E (mg)	83	4.1	1.9
Vit. C (mg)	250	30	15

EPA eicosapentaenoic acid, *DHA* docosahexaenoic acid

Lymphocyte Isolation

Blood samples were collected from a peripheral vein before (−1) and after (day 0) pre-operative nutrition and on post-operative days 1, 3 and 7. Lymphocytes were separated from heparinized blood by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) according to manufacturer's instructions (Boyum 1968). Isolated lymphocytes were suspended in phosphate-buffered saline (PBS).

Assessment of Proapoptotic and Antiapoptotic Proteins

Western Blot Analyses

Lymphocytes suspended in PBS were mixed with an equal amount of Laemmli sample buffer containing 0.5% β-mercaptoethanol (Biorad, CA, USA) and boiled for 5 min. Fifty μg of cell lysate was resolved by using 12% SDS-PAGE (Amersham Bioscience, Buckinghamshire, UK) and transferred onto polyvinylidene difluoride membranes (Porablot PVDF–PVDF membrane, Macherey-Nagel, Düren, Germany) by using TRANS-BLOT SD, SEMI DRY TRANSFER CELL (Biorad). As a marker of protein size Novex Sharp Protein Standard (Invitrogen, Carlsbad, CA, USA) was used. The membranes were saturated with 1% blocking solution (Western Blocking Reagent Solution, Roche, Basel, Switzerland) for 2 h at room temperature and probed with a specific antibody (diluted in 0.5% blocking solution 1:500) to Bcl-2 (sc-783, rabbit polyclonal), Bax (sc-526, rabbit polyclonal), PARP-1 (sc-1561, goat polyclonal), NF-κB (sc-7151, rabbit polyclonal), Cas3 (sc-7148, rabbit polyclonal), Cas9 (sc-7885, rabbit polyclonal), TNFR1 (sc-1068, goat polyclonal) and GAPDH (sc-25778, rabbit polyclonal) (as an internal control) (Santa Cruz Biotechnology, Santa Cruz, USA) for 1.5 h at room temperature. The step was followed by washing with TBS-T (Tris-buffered saline containing 0.01% Tween-20) for 10 min × 2 and TBS for 10 min × 2 at room temperature. Then, the membranes were probed with a secondary antibody conjugated with alkaline phosphatase (goat anti-rabbit AP sc-2034 or bovine anti-goat AP sc-2381, Santa Cruz Biotechnology) diluted in 0.5% blocking solution 1:5,000 for 1 h at room temperature. The step was followed by washing it with TBS-T (Tris-buffered saline containing 0.01% Tween-20) for 2 × 10 min and TBS (Tris-buffered saline) for 2 × 10 min at room temperature. Protein–antibody binding was detected by using Alkaline Phosphatase Conjugate Substrate Kit (Biorad). Values are expressed as a percentage of patients or controls with the positive expressions of pro- and antiapoptotic proteins.

Flow Cytometry

The peripheral blood of each patient was collected before and after surgery simultaneously to western blot analyses. To stain surface proteins, the 100 μl heparinized blood samples were incubated with 20 μl of the following monoclonal antibodies: CD3 PE, CD95 FITC (Becton-Dickinson Immunocytometry Systems; Becton-Dickinson and Co., San Jose, CA, USA), CD120a PE (Immunotech, a Beckman Coulter Company 13009 Marseille, France) and their appropriate isotype-matched control antibodies. After 30 min of incubation at room temperature, red cells were lysed by adding FACS Lysing Solution (Becton-Dickinson Biosciences) to the tubes. After another 10 min, samples were washed in PBS with 1% bovine serum albumin (BSA; Sigma Chemical Co., Balcatta, WA) and 0.1% sodium azide (NaN_3) and fixed with 1% paraformaldehyde (Sigma Chemical Co., Balcatta, WA).

To evaluate the intracellular expression of Bcl-2, the 100 μl heparinized blood samples were lysed and permeabilized with BD FACS Permeabilizing Solution 2 (Becton-Dickinson and Co.) for 10 min. Then, samples were washed with PBS–BSA–NaN_3 and incubated using 20 μl of Bcl-2 PE (Becton-Dickinson and Co.) for 30 min at room temperature. Then, after washing with PBS–BSA–NaN_3, the samples were fixed with 1% paraformaldehyde.

The stained cells were acquired using the LSR instrument (Becton-Dickinson) to detect two fluorescence parameters and two scatter parameters. The data were analyzed using CellQuest ProSoftware version 9.2 (Becton-Dickinson) and expressed as a percentage of lymphocytes with positive surfaces or intracellular pro- and anti-apoptotic protein expressions (mean ± SD).

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.

Statistical Analysis

Statistical analysis was performed using the Statsoft Statistca v.7.0 program. To evaluate the statistical significance of differences in apoptotic proteins expressions between groups the Fisher's exact test with the Bonferroni correction was used. To compare the differences in lymphocyte percentage between groups and preoperative versus post-operative results, the Mann–Whitney or Wilcoxon signed-rank test with the Bonferroni correction was used. Patient characteristics and surgical parameters of the two groups (standard vs. supplemented) were compared by using

one-way ANOVA, Chi-squared and Fisher's exact tests. $P < 0.05$ was defined as significant.

Results

Western Blotting

In the whole group of pancreatic cancer patients, the frequency of Bcl-2, Bax, NF-κB and PARP-1 expression in lymphocytes was significantly decreased when compared with the control group (Bcl-2 $p = 0.02$ and for other proteins $p < 0.01$; Fig. 1a, b). The frequency of caspase-3, -9 and TNFR1 expression was significantly elevated when compared with the control group (all $p < 0.01$). In the group of healthy volunteers caspase-3, -9 and TNFR1 expressions were not detected. The analysis of the statistical significance of differences in the expression of measured proteins inside the whole group of patients with pancreatic cancer showed a significantly higher expression of Bcl-2 in comparison with TNFR1, NF-κB, PARP-1 and caspase-9 expression (all $p < 0.05$), whereas caspases-3 expression was significantly higher when compared with NF-κB, PARP-1 and TNFR1 (all $p < 0.05$). In the whole group of pancreatic cancer patients, the differences between the pre-operative and post-operative expressions of pro- and anti-apoptotic proteins were not significant.

The similar changes of the expression of pro- and anti-apoptotic proteins were observed in the pancreatic cancer patients receiving pre-operative standard enteral nutrition (Group I; Fig. 2a, b). In comparison to the standard nutrition, the pre-operative enteral immunonutrition increased Bcl-2 and Bax expression (day 0 before surgery) resulted in insignificant differences between Group II and controls both before and in the early post-operative period (day 1 after surgery). The pre-operative standard nutrition and enteral immunonutrition did not significantly alter the expression of caspases-3, -9, NF-κB, PARP-1 and TNFR1. Still, in both groups of patients (Group I and II) an elevated expression of caspases-3, -9, TNFR1 and a decreased expression of NF-κB and PARP-1 were detected. The differences between the Group I and II in all protein expressions were not statistically significant both before and after pancreatic surgery.

The frequency of Bcl-2, Bax and PARP-1 expression in patients with normal nutritional status (Group III) without preoperative enteral nutrition did not differ from the control group (Fig. 3). However, the decreased expression of

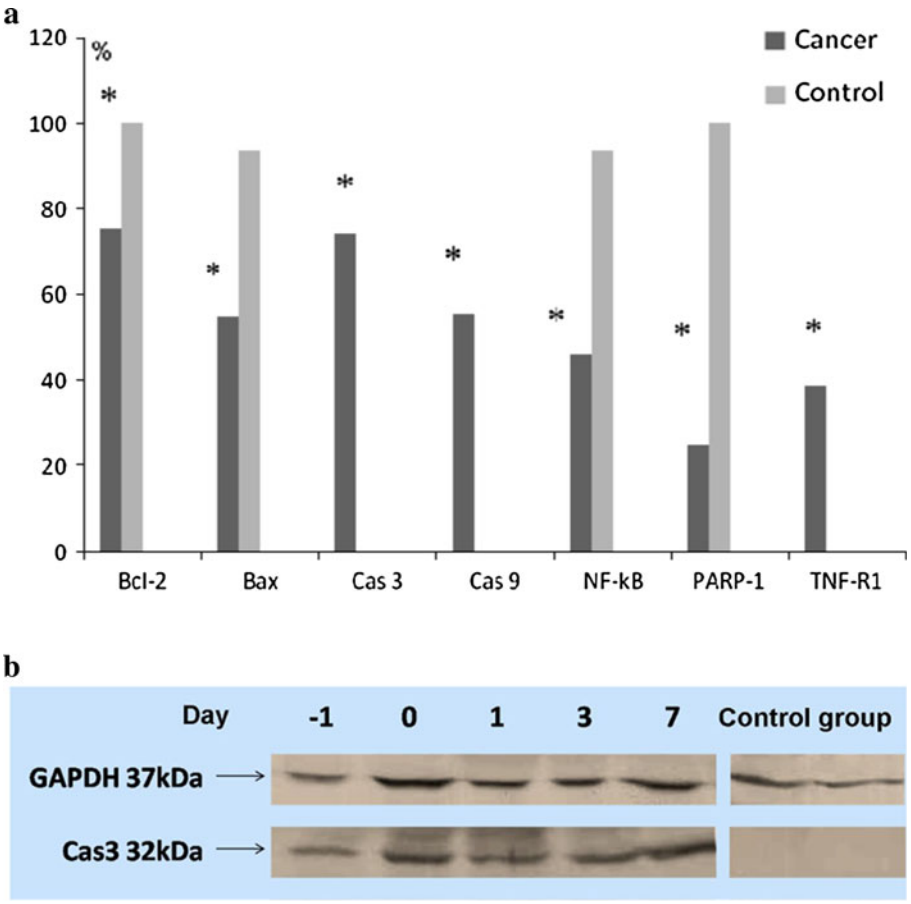

Fig. 1 **a** Percentage of patients with pro- and anti-apoptotic proteins expressions in the whole group of pancreatic cancer patients when compared with control group (pre-operative day −1). Caspases and TNFR1 expression in control group was not detected. *Asterisk* indicates $p < 0.05$. **b** Caspase-3 expression in a pancreatic cancer patient before and after extended surgery when compared with a healthy subject (in control group caspases-3 expression was not detected)

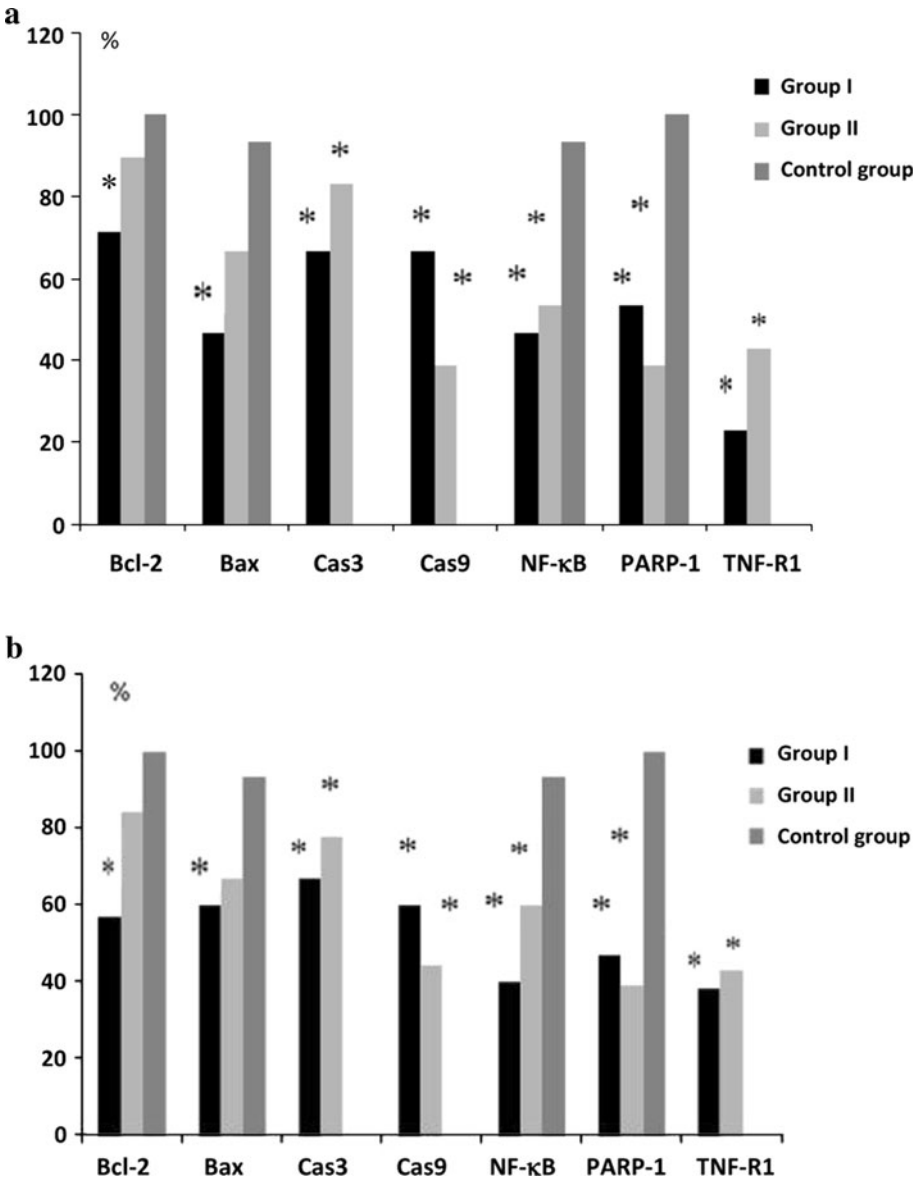

Fig. 2 **a** Percentage of malnourished pancreatic cancer patients with pro- and anti-apoptotic proteins expressions after preoperative enteral standard (Group I) and immune-enhancing nutrition (Group II) when compared with control group (day 0 before surgery). *Asterisk* indicates $p < 0.05$. **b** Percentage of malnourished pancreatic cancer patients with pro- and anti-apoptotic protein expression after pre-operative enteral standard (Group I) and immune-enhancing nutrition (Group II) when compared with the control group (day 1 after surgery). *Asterisk* indicates $p < 0.05$

NF-κB and elevated caspase or TNFR1 expressions still persisted. There were also no significant differences between the pre-operative and post-operative expressions of pro- and anti-apoptotic proteins.

Flow Cytometry

In the whole group of pancreatic cancer patients (group I, II, III), the percentage of lymphocytes with Fas ($CD95^+/CD3^-$) and TNFRI (CD120a) phenotypes were significantly higher before as well as after surgery when compared with the control group (Fig. 4). Percentage of Bcl-2-positive lymphocytes was significantly decreased on day 1 and 7 after surgery when compared with the control group (Figs. 5, 6). There were no significant differences between the pre-operative and post-operative levels of $CD95^+/CD3^-$ and CD120a-positive lymphocytes. Furthermore, no significant difference was found between the percentage of $CD95^+/CD3^+$ lymphocytes (in the whole group) in comparison with the control group (before and after surgery), whereas $CD95^-/CD3^+$-positive cells were significantly decreased on day 1 after surgery (16.38 ± 10.3 vs. $30.4 \pm 14.9\%$, $p = 0.002$).

The immune-enhancing pre-operative enteral nutrition including both glutamine and polyunsaturated fatty acids significantly increased the percentage of $CD95^+/CD3^-$ and CD120a-positive lymphocytes on day 3 after pancreatic resection when compared with the group of patients receiving standard pre-operative enteral nutrition (Figs. 6, 7, 8). There was also a tendency toward increase in the percentage of $CD95^+/CD3^-$ and CD120a-positive lymphocytes on day 7 after surgery in patients with pre-

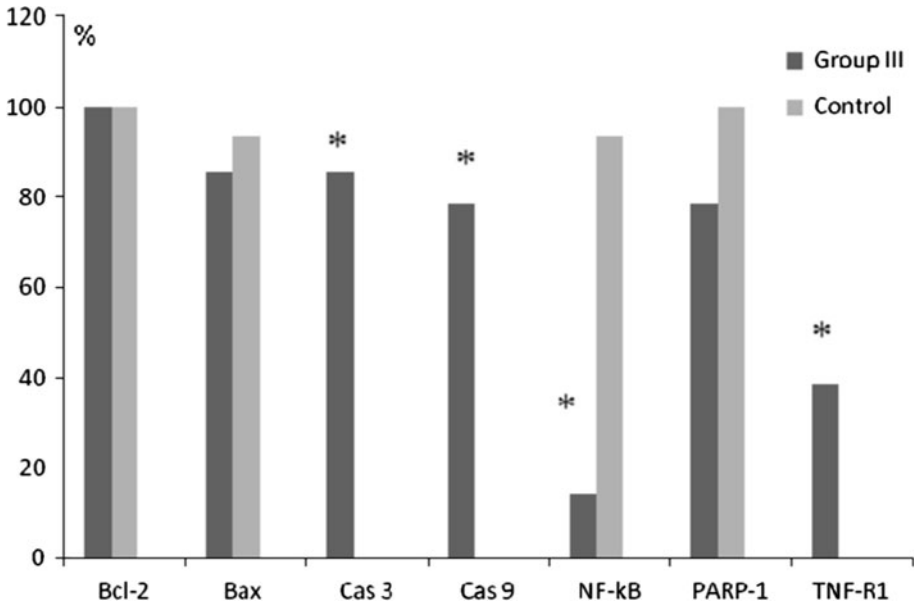

Fig. 3 Percentage of pancreatic cancer patients with pro- and anti-apoptotic proteins expressions and normal nutritional status who did not receive the preoperative enteral nutrition (Group III) when compared with control group (preoperative day −1). Caspases and TNFR1 expression in control group was not detected. *Asterisk* indicates $p < 0.05$

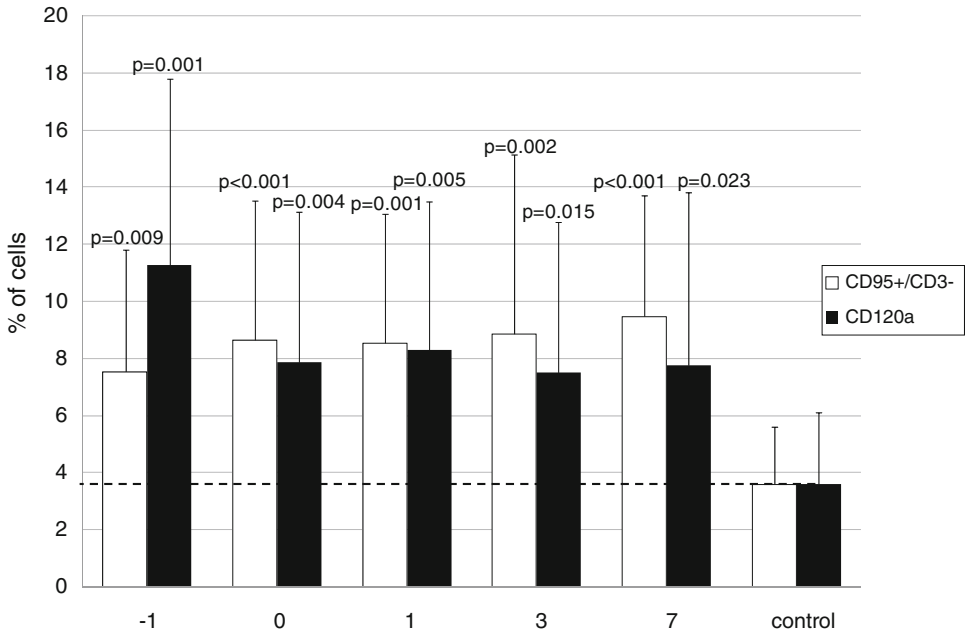

Fig. 4 Percentage of Fas ($CD95^+/CD3^-$) and TNFRI (CD120a)-positive lymphocytes in the whole group of pancreatic cancer patients before and after surgery when compared with control group. Values are expressed as mean ± SD

operative immunonutrition ($p = 0.05$). Conversely, no significant difference was found in the percentages of $CD95^+/CD3^+$, $CD95^-/CD3^+$-positive lymphocytes and the percentage of Bcl-2-posive cells between the group of patients with immunonutrition versus standard nutrition both before and after pancreatic surgery.

Discussion

The attempt to correct the immune system dysfunction using the influence of preoperative enteral immunonutrition on lymphocyte apoptotic signaling pathways protein expressions is a new promising way of improving outcomes after extended pancreatic cancer surgery. Further progress in this field in the first place requires determining what changes in pro- and anti-apoptotic expression occur in patients with pancreatic cancer, then, assessing the actual potentials of using immunonutrition to influence the expression of those proteins both in the pre- and postoperative period. In selecting parameters to be investigated we used the results of previous studies performed on a smaller group of patients suffering from pancreatic cancer that showed a high sensitivity of the proteins assessment (Kędziora et al. 2010). Also some results of studies performed by other authors suggesting the possibility to regulate

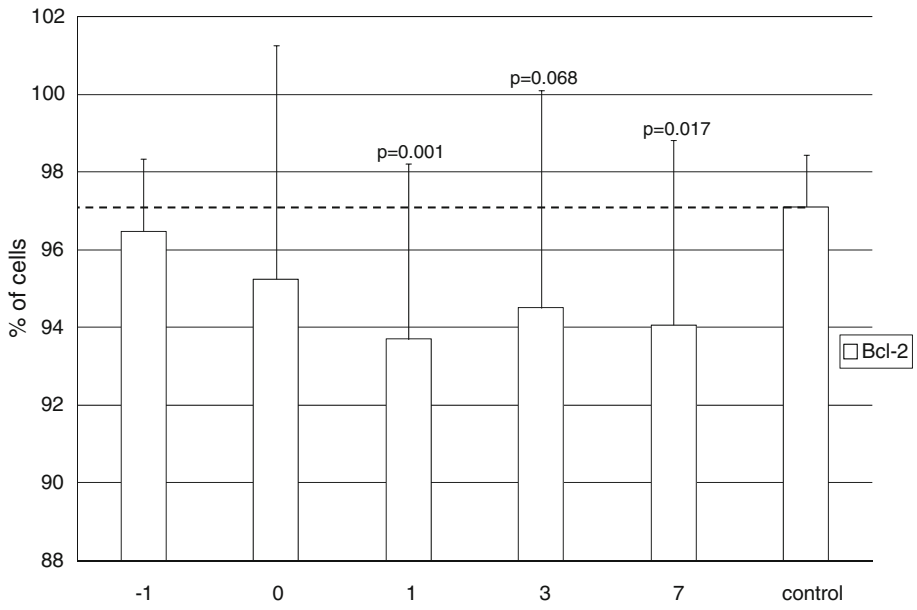

Fig. 5 Percentage of Bcl-2-positive lymphocytes in the whole group of pancreatic cancer patients before and after surgery when compared with control group. Values are expressed as mean ± SD

the process of lymphocyte apoptosis with immunonutrition affecting the Bcl-2, caspase-3 or Fas (CD95) expression (Chang et al. 2002; Mates et al. 2006). Available literature does not describe any similar studies regarding the influence of pre-operative immunonutrition on apoptotic signaling pathway in patients with pancreatic cancer.

Our study revealed (i) an increase in the frequency of pro-apoptotic proteins expression, and (ii) a significant decrease in the expression of anti-apoptotic proteins in peripheral blood lymphocytes, especially in malnourished patients with pancreatic cancer when compared with the control group. These pathological alterations in apoptotic signaling pathway proteins may increase the lymphocyte dysfunction resulting in immune system suppression and higher lymphocyte sensibility to inappropriate apoptosis. Such immune disorders before major surgery may influence the pancreatic cancer patients' susceptibility to infectious complications as well as to the tumor metastasis in the early postoperative period. The survival rate after pancreatic cancer resection is still very low (10–30% 5-year survival rate) and the morbidity even in high-volume centers still remains ranging from 30 to 50% (Conlon et al. 1996; Strasberg et al. 1997; Trede et al. 2001). Why the peripheral blood lymphocytes switch to the activation of cell-intrinsic suicide program remains still unclear. One of the possible explanations of inappropriate changes in the apoptotic signaling pathway proteins is related to immune system cells malnutrition in pancreatic cancer patients. In our study disease-related malnutrition detected in 70% of patients was the indication for pre-operative enteral nutrition treatment (including 44% of severely malnourished patients with >10% weight loss within 6 months).

Moreover, our findings suggest that the down-regulation of anti-apoptotic signaling systems in the lymphocytes of pancreatic cancer patients is very frequent. A decreased frequency of Bcl-2, NF-κB and PARP-1 expression in peripheral blood lymphocytes was closely related with the extremely high expression of caspase-3, which was also significantly higher when compared with NF-κB, PARP-1 and TNFR1. While the enhancement of NF-κB and PARP-1 expressions in lymphocytes prevents apoptosis, low levels of these proteins may trigger different pathways of cell death. Among the pro-apoptotic proteins being investigated within the whole group of patients, the expression of only one protein (Bax) when compared with the control groups was decreased. It may suggest that malnutrition in pancreatic cancer patients affects the production of this protein, too. Interestingly, no caspase and TNFR1 expressions were observed in the group of healthy volunteers. A release of certain proteins from the mitochondrial intermembrane space due to membrane permeabilization triggers a cascade of caspase activation that results in irreversible events culminating in apoptosis. In our study the significantly elevated frequency of caspase-3 expression was a characteristic "marker" in each group of pancreatic cancer patients (I, II, III). There are some data that show an overexpression of caspase-3 and caspase-9 in patients with type 1 diabetes mellitus (Vendrame et al. 2005), HIV infected (Zaunders et al. 2001), the ones suffering from Alzheimer's disease (Tacconi et al. 2004), patients in septic shock (Delogu et al. 2008) and gastric cancer patients (Takahashi et al. 2001). Unfortunately, the

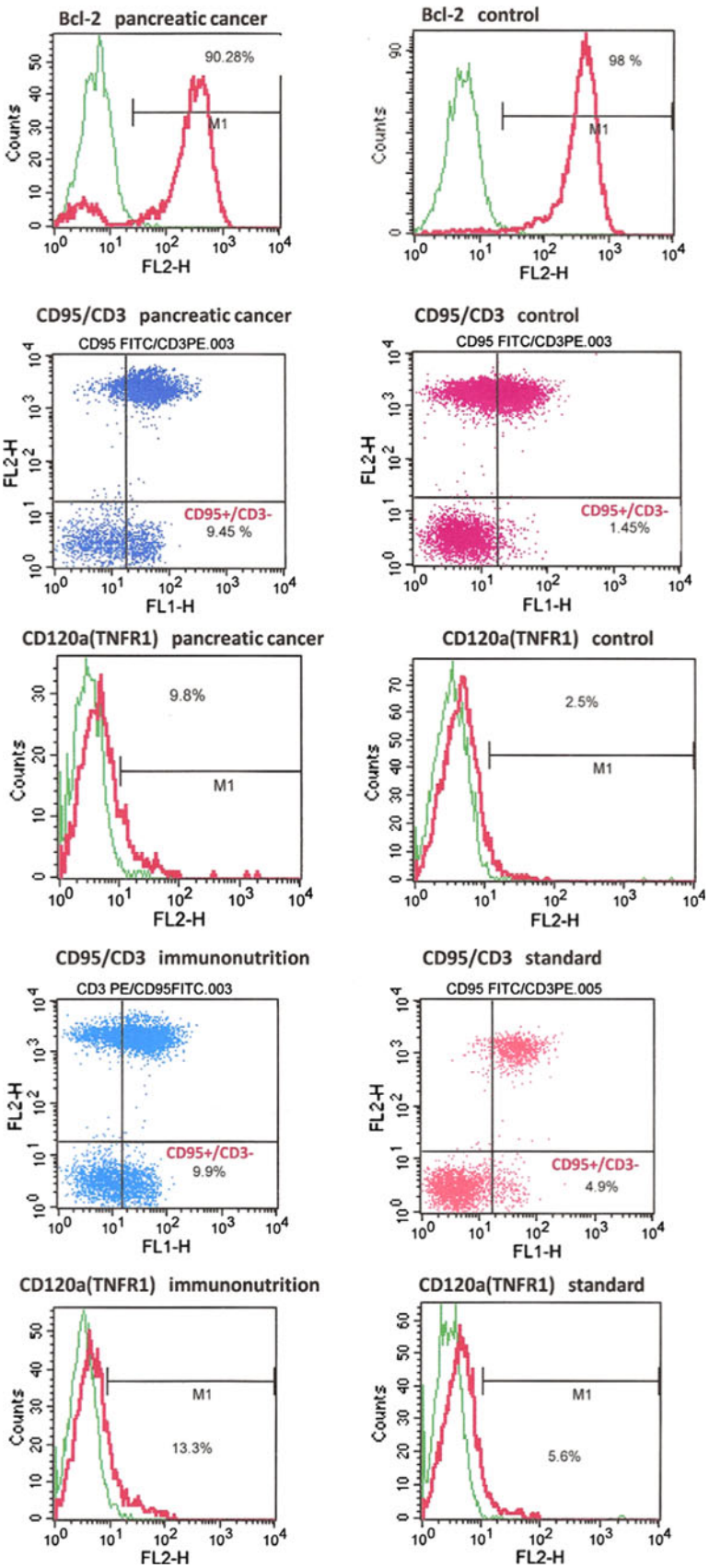

Fig. 6 Percentage of Bcl-2, Fas ($CD95^{+}/CD3^{-}$) and TNFRI (CD120a)-positive lymphocytes in patients with pancreatic cancer (day 1 after surgery) and in patients receiving preoperative enteral standard (Group I) or immune-enhancing nutrition (Group II) (day 3 after surgery)

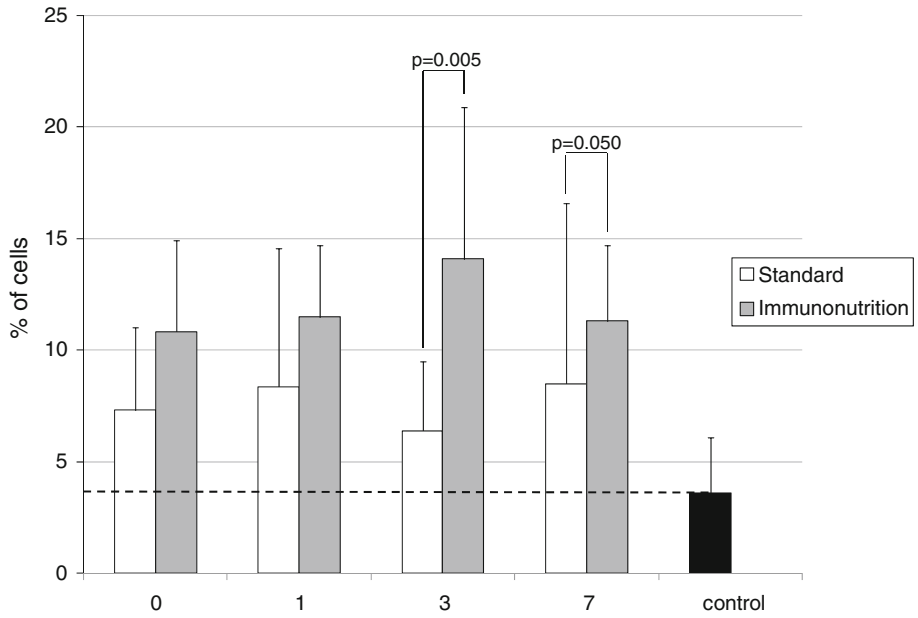

Fig. 7 Percentage of TNFRI (CD120a)-positive lymphocytes before and after surgery in pancreatic cancer patients receiving preoperative enteral standard (Group I) and immune-enhancing nutrition (Group II). Values are expressed as mean ± SD

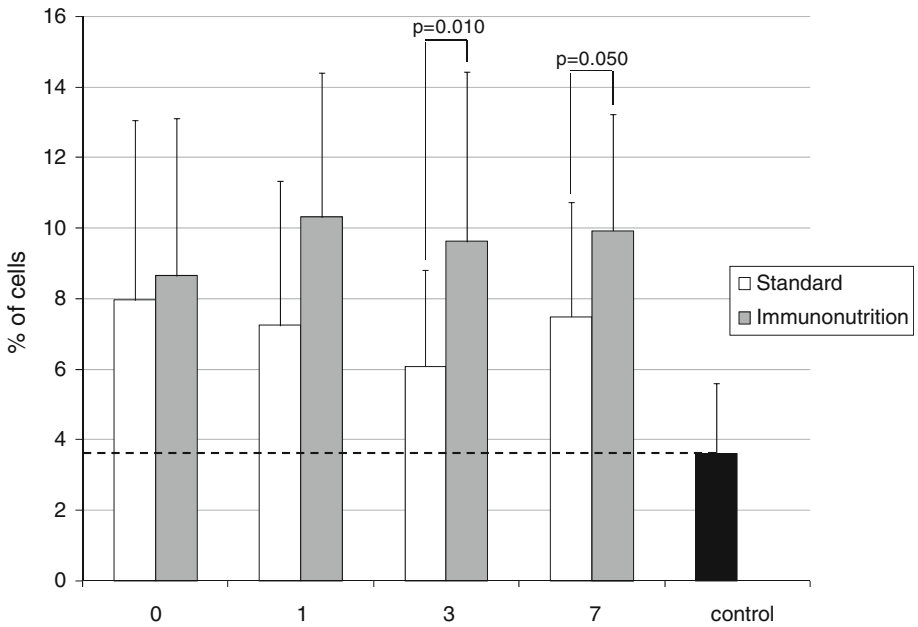

Fig. 8 Percentage of Fas ($CD95^{+}/CD3^{-}$)-positive lymphocytes before and after surgery in pancreatic cancer patients receiving pre-operative enteral standard (Group I) and immune-enhancing nutrition (Group II). Values are expressed as mean ± SD

pre-operative enteral nutrition has no significant effect on the frequency of caspase expressions.

Furthermore, a decreased expression of PARP-1 (connected with a high expression of caspase-3) suggested increasing the susceptibility of lymphocytes in pancreatic cancer patients to DNA damage. The caspase-3 is responsible for the proteolytic cleavage of PARP. PARP has a well-established role in DNA repair processes. The activation of PARP-1 by genotoxic stimuli facilitates cell survival. The lymphocytes of cancer patients show a lower expression of PARP-1, which may indicate a higher level of DNA damaged (Rajaee-Behbahani et al. 2002). This molecule has been found a potential target for the development of pharmacological strategies to increase the antitumor efficacy of chemotherapeutic agents that induce DNA damage in cancer cells (Peralta-Leal et al. 2008).

In our study, we also measured the expression of apoptotic proteins in patients showing normal nutritional status (Group III). There were no significant differences in the frequency of Bcl-2 Bax and PARP-1 expressions between pancreatic cancer patients and healthy individuals. The expression frequencies of remaining apoptotic proteins were similar to the changes detected in malnourished patients with pancreatic cancer (Group I, II). Therefore, we

may suggest that malnutrition is associated especially with Bcl-2, Bax and PARP-1 deficiency, whereas high caspase and lower NF-κB expressions may reflect pancreatic cancer progression. The results of western blot require confirmation in further studies, because at the beginning of the study in the small group of patients we did not use a semi-quantitative assay, which had actually been practiced for many years. The method we used in our study measured the frequency of non-existence (0) or presence (1) of pro- and anti-apoptotic protein bands in comparison with GAPDH and control group in each group of patients.

Flow cytometry only partially confirmed the results of western blot analyses. In this particular part of studies, the influence of underlying disease, the concomitant malnutrition, surgical trauma and pre-operative immunonutrition is significantly marked. In the whole group of pancreatic cancer patients, the percentages of lymphocytes with Fas/CD95$^+$/CD3$^-$ and TNFR1/CD120a phenotypes were significantly higher before and after surgery (when compared with the control group). Such lymphocyte phenotypes and a significantly decreased Bcl-2 intracellular expression after surgery (significant on day 1 and 7) for pancreatic cancer may lead to an inappropriate cell apoptosis. A series of studies has demonstrated that circulating lymphocytes in the early postoperative period are susceptible to apoptosis, which may cause deletion of peripheral lymphocytes after surgery (Oka et al. 1996; Sugimoto et al. 1998). In the early postoperative period, surgical trauma under general anesthesia induces an intracellular perturbation on peripheral lymphocytes, resulting in both up-regulation of death-signaling factors (Fas, FasL) and down-regulation of survival-signaling factors (Bcl-2). The increased apoptosis of CD8 lymphocytes, excluding CD4 cells, seemed to be associated with a greater risk of postsurgical infections (Delogu et al. 2000). Results of the other studies showed that in peripheral lymphocytes taken from multiple sclerosis patients there is a significant reduction in the expression ratios of pro-apoptotic to anti-apoptotic Bcl-2 members when compared with the corresponding ratios found in healthy individuals (Sharief et al. 2002). Increased level of Bcl-2 protein expression was found in T cells of patients with laryngeal carcinoma (Klatka et al. 1999).

The apoptosis-inducing receptors of the TNFR superfamily as Fas/CD95 or TNFR1 exist at the cell surface as pre-formed trimers. A ligation of the death receptors leads to apoptosis through a common transcription/translation-independent pathway. The extensive apoptotic death of lymphocytes probably contributes to profound immunosuppression after pancreatic resection. We showed that the percentages of CD95$^-$/CD3$^+$ cells and lymphocytes with Bcl-2 expressions in a response to major surgery were decreased. It has been proved that the proteins of the Bcl-2 family function in the intrinsic death pathway, playing important roles in regulating the life and death of T lymphocytes (Droin and Green 2004). In an experimental study, the deletion of Bcl-2 results in the rapid disappearance of native T cells (Wojciechowski et al. 2007). Thus, the down-regulation of survival-signaling proteins of lymphocytes may influence postsurgical outcome.

Finally, our study demonstrated that by using pre-operative enteral immunonutrition we can prevent the decrease in lymphocyte subsets (CD95$^+$/CD3$^-$ and CD120a-positive lymphocytes) after pancreatic resection when compared with the standard nutritional support. These results show the necessity of preoperative immunonutrition in pancreatic cancer patients that should be continued in the early postoperative period after extensive surgery, but the effect of such nutrition on postoperative outcome requires further studies. The changes in the percentage of T cells with death receptors family presented in our study can enhance the antitumor activity (for instance by Fas and TNFR1 mediated cytotoxicity), but a higher lymphocyte susceptibility to apoptosis after such type of immunonutrition can be also considered. Interestingly, human pancreatic cancer cells express non-functional Fas receptors and counterattack lymphocytes by expressing Fas ligand (Elnemr et al. 2001). In another study the apoptosis of T cells was correlated with high levels of FasL expression on the tumor (Reichert et al. 2002).

In the majority of patients subjected to immunonutrition a glutamine enriched diet was used. The small group of patients receiving unsaturated fatty acids (PUFAs) does not allow us to draw any separate conclusion. So, at the very initial stage of studies, the results for the group of patients treated with immunonutrition were presented in total. Considering the possibility of different that glutamine PUFAs impact on the expression of proteins under investigation, a precise assessment of the study results in a larger group of patients treated with PUFAs will require a separate analysis. It has recently become more clear that PUFAs and some of their synthesized derivatives are able to modulate the molecular pathways involved in apoptosis (Serini et al. 2009). In our study, the pre-operative enteral immune-enhancing nutrition increased the frequency of Bcl-2 and Bax expressions (but not significantly while comparing it with standard nutrition) almost up to the levels found in patients showing normal nutritional status (Group III) and healthy volunteers. In the group of patients treated with immunonutrition, the effect is clearly visible both in the pre-operative period (day 0 after immunonutrition) and after surgery (day 1) (Western-blot). The relatively weak influence of immunonutrition that affects mainly the frequency of Bcl-2 and Bax expressions and showing the lack of effect on caspase and other apoptotic protein expressions was probably connected with insufficient immunonutrients intake in the short (only 5 days)

pre-operative period and/or with the method of protein expression measurement used (semi-quantitative western blot was not used). On the other hand, it should be also expected that there are other factors (except for the underlying disease, malnutrition and immonutrition) may have influenced the expression of proteins related with peripheral blood lymphocyte apoptosis in patients with pancreatic cancer (including e.g. the change of neoplastic tissue activity at the preoperative stage influenced by nutrition received). Previous studies have already proved that e.g. conjugated linoleic acids can modulate the expression of both pro- and anti-apoptotic genes in breast or prostate cancer cells, which may result in the atrophy of neoplastic cells (Majumder et al. 2002; Ochoa et al. 2004). The age of patients, type of neoplasm, extent of surgical trauma, stage of neoplastic process, duration of surgery and the need to administer blood did not differ significantly between the groups being compared (standard vs. immunonutrition), whereas patients with serious complications (e.g. intra-abdominal abscesses, purulent fistulas, reoperations required) were excluded from those studies to allow for more accurate assessment of physiological changes in the expression of proteins under investigation. These results confirmed the immunostimulatory effect of preoperative immune-enhancing entreat nutrition when compared with standard nutrition after pancreatic tumor surgery. In an experimental study, the presence of glutamine increased lymphoproliferation as well as Bcl-2 and CD95 expression, whereas decreased CD95L and activation-induced T cell death (Chang et al. 2002). The authors of this study suggested that glutamine protects the activated human T cells against apoptosis by up-regulating glutathione and Bcl-2 levels and down-regulating both caspase-3 and caspase-8 activities.

In conclusion, our studies suggest that in malnourished patients with pancreatic cancer the overwhelming expression of caspases and the decrease of anti-apoptotic proteins may lead to inappropriate lymphocyte apoptosis. These pathological alterations in apoptotic signaling pathway proteins may increase the lymphocyte dysfunction and immune system suppression especially after pancreatic resection. The preoperative enteral immunonutrition prevents the post-operative decrease in lymphocytes subsets, but a higher level of lymphocyte susceptibility to undergo accelerated apoptosis can be also considered. Further research needs to be undertaken to examine the difference between the reactions of apoptotic signaling proteins to selected immunonutrients (for instance glutamine vs. unsaturated fatty acids).

Acknowledgments We are grateful to all patients and healthy individuals who participated in this study. This study was partially supported by the Ministry of Science and Higher Education, Poland (grant no. 2 PO5B 059 28 and 3068B P01).

References

Alexakis N, Halloran C, Raraty M et al (2004) Current standards of surgery for pancreatic cancer. Br J Surg 91:1410–1427

Ankersmit HJ, Moser B, Zuckermann A et al (2002) Activation-induced T cell death, and aberrant T cell activation via TNFR1 and CD95-CD95 ligand pathway in stable cardiac transplant recipients. Clin Exp Immunol 128:175–180

Boyum A (1968) Isolation of mononuclear cell and granulocytes from human blood. Scand J Clin Lab Invest Suppl 97:77–89

Bruun LI, Bosaeus I, Bergstad I et al (1999) Prevalence of malnutrition in surgical patients: evaluation of nutritional support and documentation. Clin Nutr 18:141–147

Chang WK, Yang KD, Chuang H et al (2002) Glutamine protects activated human T cells from apoptosis by up-regulating glutathione and Bcl-2 levels. Clin Immunol 104:151–160

Conlon KC, Klimstra DS, Brennan MF (1996) Long-term survival after curative resection for pancreatic ductal adenocarcinoma clinicopathologic analysis of 5-year survivors. Ann Surg 223:273–279

Delogu G, Famularo G, Tellan G et al (2008) Lymphocyte apoptosis, caspase activation and inflammatory response in septic shock. Infection 36:485–487

Delogu G, Moretti S, Antonucci A et al (2000) Apoptosis and surgical trauma: dysregulated expression of death and survival factors on peripheral lymphocytes. Arch Surg 135:1141–1147

Detsky AS, Baker JP, O'Rourke K et al (1987) Predicting nutrition-associated complications for patients undergoing gastrointestinal surgery. JPEN J Parenter Enteral Nutr 11:440–446

Droin NM, Green DR (2004) Role of Bcl-2 family members in immunity and disease. Biochim Biophys Acta 1644:179–188

Edington J, Boorman J, Durrant ER et al (2000) Prevalence of malnutrition on admission to four hospitals in England. The Malnutrition Prevalence Group. Clin Nutr 19:191–195

Elnemr A, Ohta T, Yachie A et al (2001) Human pancreatic cancer cells express non-functional Fas receptors and counterattack lymphocytes by expressing Fas ligand; a potential mechanism for immune escape. Int J Oncol 18:33–39

Farreras N, Artigas V, Cardona D et al (2005) Effect of early postoperative enteral immunonutrition on wound healing in patients undergoing surgery for gastric cancer. Clin Nutr 24:55–65

Greco E, Fogar P, Mazzon C et al (2007) Pancreatic cancer pulls down lymphocyte migration. JOP 8:685–686

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281:1309–1312

Halloran CM, Ghaneh P, Bosonnet L et al (2002) Complications of pancreatic cancer resection. Dig Surg 19:138–146

Haydock DA, Hill GL (1986) Impaired wound healing in surgical patients with varying degrees of malnutrition. JPEN J Parenter Enteral Nutr 10:550–554

Heyland DK, Dhaliwal R, Day AG et al (2006) REducing Deaths due to OXidative Stress (The REDOXS Study: rationale and design for a randomizedtrial of glutamine and antioxidant supplementation in critically-ill patients. Proc Nutr Soc 65:250–263

Hsu YT, Wolter KG, Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bclxl during apoptosis. Proc Natl Acad Sci USA 94:3668–3672

Kędziora S, Słotwiński R, Olszewski WL et al (2010) Programmed lymphocytes death in pancreatic cancer patients. Centr Eur J Immunol 35:84–89

Klatka J, Rolinski J, Kupisz K et al (1999) Expression of bcl-2 protein in lymphocytes of patients with laryngeal carcinoma. Eur Arch Otorhinolaryngol 256:299–302

Lieberman J, Fan Z (2003) Nuclear war: the granzyme A-bomb. Curr Opin Immunol 15:553–559

Lin CF, Chen CL, Lin YS (2006) Ceramide in apoptotic signaling and anticancer therapy. Curr Med Chem 13:1609–1616

Loeffler M, Kroemer G (2000) The mitochondrion in cell death control: certainties and incognita. Exp Cell Res 256:19–26

MacDonald G, Shi L, Vande Velde C et al (1999) Mitochondria-dependent and -independent regulation of Granzyme B induced apoptosis. J Exp Med 189:131–144

Majumder B, Wahle KW, Moir S et al (2002) Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. FASEB J 16:1447–1449

Marzo I, Pérez-Galán P, Giraldo P et al (2001) Cladribine induces apoptosis in human leukaemia cells by caspase-dependent and -independent pathways acting on mitochondria. Biochem J 359(Pt3):537—546

Mates JM, Segura JA, Alonso FJ et al (2006) Pathways from glutamine to apoptosis. Front Biosci 11:3164–3180

Nagata S (2000) Apoptotic DNA fragmentation. Exp Cell Res 256:12–18

Novak F, Heyland DK, Avenell A et al (2002) Glutamine supplementation in serious illness: a systematic review of the evidence. Crit Care Med 30:2022–2029

Ochoa JJ, Farquharson AJ, Grant I et al (2004) Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: different molecular mechanisms for cie-9, trans-10, cis-12 isomers. Carcinogenesis 25:1185–1191

Oka M, Hirazawa K, Yamamoto K et al (1996) Induction of Fas-mediated apoptosis on circulating lymphocytes by surgical stress. Ann Surg 223:434–440

Peralta-Leal A, Rodriguez MI, Oliver FJ (2008) Poly(ADP-ribose)polymerase-1 (PARP-1) in carcinogenesis: potential role of PARP inhibitors in cancer treatment. Clin Transl Oncol 10:318–323

Poch B, Lotspeich E, Ramadani M et al (2007) Systemic immune dysfunction in pancreatic cancer patients. Langenbecks Arch Surg 392:353–358

Rajaee-Behbahani N, Schmezer P, Ramroth H et al (2002) Reduced poly(ADP-ribosyl)ation in lymphocytes of laryngeal cancer patients: results of a case control study. Int J Cancer 98:780–784

Reichert TE, Strauss L, Wagner EM et al (2002) Signaling abnormalities and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. Clin Cancer Res 8:3137–3145

Reynolds JV, O'Farrelly C, Feighery C et al (1996) Impaired gut barrier function in malnourished patients. Br J Surg 83:1288–1291

Sharief MK, Douglas M, Noori M et al (2002) The expression of pro- and anti-apoptosis Bcl-2 family proteins in lymphocytes from patients with multiple sclerosis. J Neuroimmunol 125:155–162

Shiraishi H, Okamoto H, Yoshimura A et al (2006) ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. J Cell Sci 119(Pt 19):3958–3966

Serini S, Piccioni E, Merendino N et al (2009) Dietary polyunsaturated fatty acids as inducers of apoptosis: implications for cancer. Apoptosis 14:135–152

Strasberg SM, Drebin JA, Soper NJ (1997) Evolution and current status of the Whipple procedure: an update for gastroenterologists. Gastroenterology 113:983–994

Sugimoto M, Shimaoka M, Hosotsubo K et al (1998) Up-regulation of Fas ligand (FasL)mRNA expression in peripheral blood mononuclear cells (PBMC) after major surgery. Clin Exp Immunol 112:120–125

Tacconi S, Perri R, Balestrieri E et al (2004) Increased caspase activation in peripheral blood mononuclear cells of patients with Alzheimer's disease. Exp Neurol 190:254–262

Takahashi A, Kono K, Amemiya H et al (2001) Elevated caspase-3 activity in peripheral blood T cells coexists with increased degree of T-cell apoptosis and down-regulation of TCR zeta molecules in patients with gastric cancer. Clin Cancer Res 7:74–80

Trede M, Richter A, Wendl K (2001) Personal observations, opinions and approaches to cancer of the pancreas and periampullary area. Surg Clin North Am 81:595–610

Vendrame F, Santangelo C, Misasi R et al (2005) Defective lymphocyte caspase-3 expression in type 1 diabetes mellitus. Eur J Endocrinol 152:119–125

von Bernstorff W, Voss M, Freichel S et al (2001) Systemic and local immunosuppression in pancreatic cancer patients. Clin Cancer Res 7(3 suppl):925s–932s

von Meyenfeldt M (2005) Cancer-associated malnutrition: an introduction. Eur J Oncol Nurs 9(Suppl 2):S35–S38

Wagner M, Redaelli C, Lietz M et al (2004) Curative resection in the single most important factor determining outcome in patients with pancreatic adenocarcinoma. Br J Surg 91:586–594

Walczak H, Krammer PH (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 256:58–66

Wei MC, Zong WX, Cheng EH et al (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292:727–730

Weimann A, Braga M, Harsanyi L et al (2006) ESPEN guidelines on enteral nutrition: surgery including organ transplantation. Clin Nutr 25:224–244

Wojciechowski S, Tripathi P, Bourdeau T et al (2007) Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. J Exp Med 204:1665–1675

Yeo CJ, Cameron JL, Sohn TA et al (1997) Six hundred fifty consecutive pancreaticoduodenectomies in the 1990s: pathology, complications, and outcomes. Ann Surg 226:248–260

Zaunders J, Moutouh-De Parceval L, Kitada S et al (2001) Apoptosis of CD4+ and CD8+ T lymphocytes in primary HIV-1 infection is associated with proliferation, increased Bax: Bcl-2 ratio and caspase activation. In: Program Abstr 8th Conf Retrovir Oppor Infect Conf Retrovir Oppor Infect 8th 2001 Chic Ill, vol 8, p 275 (abstract no. 761)

Zheng Y, Li F, Qi B et al (2007) Application of perioperative immunonutrition for gastrointestinal surgery: a meta-analysis of randomized controlled trials. Asia Pac J Clin Nutr 16(suppl 1): 253–257