

The anti-apoptotic impact of immunonutrition in pancreatic cancer patients is questionable

Antyapoptotyczne działanie żywienia immunomodulującego u chorych z rakiem trzustki jest wątpliwe

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Abstract

Aim: To investigate whether preoperative enteral immunonutrition possesses some anti-apoptotic properties and can influence the lymphocyte apoptotic signalling pathways in patients with pancreatic cancer.

Material and methods: The studies were performed in 48 patients operated on for pancreatic cancer. Thirty-four malnourished patients were randomized to receive either the enteral preoperative standard diet (group I) or the immune-enhancing enteral diet (immunonutrition) (group II). Fourteen patients (group III) of normal nutritional status did not receive the preoperative nutrition. The control group comprised 30 healthy volunteers. The expression of Bcl-2, Bax, caspase-3 and -9, NF-κB, PARP-1/89 kDa, and TNFR1/CD120a in peripheral blood lymphocytes was assessed by Western blot analysis before and after preoperative nutrition and after surgery.

Results: In malnourished patients before and after surgery (group I, II) the expression of Bcl-2, Bax, NF-κB, and PARP-1 was significantly lower, whereas the expression of caspases and TNFR1 was significantly higher as 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. The differences between groups I and II in all proteins' expression were not statistically significant both before and after pancreatic surgery.

Conclusions: Our findings suggest the down-regulation of anti-apoptotic and up-regulation of pro-apoptotic signalling

Streszczenie

Cel: Zbadanie antyapoptotycznego wpływu przedoperacyjnego dojelitowego żywienia wspomagającego odporność (*immunonutrition*) na szlaki sygnałowe związane z apoptozą limfocytów u chorych z rakiem trzustki.

Materiał i metody: Badania wykonano w grupie 48 chorych operowanych z powodu raka trzustki. Trzydziestu czterech niedożywionych chorych na zasadzie randomizacji zakwalifikowano do przedoperacyjnego dojelitowego żywienia standardowego (grupa I) lub dojelitowego *immunonutrition* (grupa II). U 14 chorych (grupa III) z prawidłowym stanem odżywienia nie stosowano żywienia przedoperacyjnego. Grupę kontrolną stanowiło 30 zdrowych ochotników. Ekspresję Bcl-2, Bax, kaspazy-3, 9, NF-κB, PARP-1/89 kDa i TNFR1/CD120a w limfocytach krwi obwodowej badano techniką *Western blot* przed żywieniem przedoperacyjnym i po nim oraz po operacji.

Wyniki: U niedożywionych chorych przed operacją oraz po jej przeprowadzeniu (grupa I, II) ekspresja Bcl-2, Bax, NF-κB, PARP-1 była istotnie obniżona, natomiast ekspresja kaspaz i TNFR1 istotnie podwyższona w porównaniu z grupą kontrolną. Przed operacją nie stwierdzono istotnych różnic w ekspresji Bcl-2, Bax i PARP-1 między grupą kontrolną a chorymi z prawidłowym stanem odżywienia (grupa III). Zarówno przed operacją raka trzustki, jak i po niej różnice w ekspresji badanych białek między grupą I i II były nieistotne statystycznie.

Wnioski: Wyniki naszych badań sugerują obniżenie ekspresji białek antyapoptotycznych szlaków sygnałowych i podwyższenie ekspresji białek proapoptotycznych w limfocytach

systems in lymphocytes of malnourished patients with pancreatic cancer and a switch to apoptosis. Preoperative enteral immunonutrition has no significant effect on the apoptotic signalling pathways and the anti-apoptotic impact of such nutrition in pancreatic cancer patients is questionable.

Introduction

Severe surgical trauma increases immune system suppression and deepens malnutrition in patients with digestive tract cancers [1, 2]. The immune disorders and malnutrition are worse in the early postoperative period, which considerably affects the process of wound healing, intestinal barrier function and the number of postoperative complications [3-6]. Pancreaticoduodenectomy is one of the most invasive operations in upper abdominal surgery, with a high incidence of postoperative complications [7-9]. One of the ways to improve the immunity and to lower the number of postoperative complications in oncological patients after an extensive surgical trauma was the introduction of immunonutrition. The results of a meta-analysis of perioperative immunonutrition aimed at enhancing immunity in patients operated on for tumours of the digestive tract has shown a decrease in the number of postoperative complications, reducing the length of hospital stay and improving selected immune parameters such as: increase in the total number of lymphocytes, the sub-population of CD4 lymphocytes and the concentration of IgG, and decrease in the concentration of IL-6 [10]. In the group of patients after extensive surgical trauma adding glutamine has always resulted in a decrease of post-surgical complications, reduction in the length of hospital stay and even a reduction of the mortality rate in seriously ill patients [11]. Multi-centre studies have shown that the administration of high doses of glutamine associated with antioxidants in seriously ill patients hospitalized in intensive care units results in a significant increase in morbidity and mortality [12]. The majority of studies that have been carried out to date show that unsaturated fatty acids (N-3 PUFAs) have a significant regulative impact on immune response and outcomes in patients after surgery with serious infections including acute respiratory distress syndrome (ARDS) [13, 14].

After pancreaticoduodenectomy the established nutritional goal can be obtained by enteral feeding and the immunonutrition seems to improve outcome [15]. The rate of postoperative complications was lower in the immunonutrition-treated group than in the group treated with standard enteral formula or after total parenteral nutrition. Early postoperative enteral feeding may safe-

ly and effectively replace parenteral nutrition in patients undergoing pancreaticoduodenectomy. Other authors [16] included patients with oesophageal, gastric and peri-pancreatic, or bile duct cancer undergoing resections and receiving early postoperative enteral feeding with an immune-enhancing formula and the results showed that there were no significant differences in the number of minor, major or infectious wound complications, mortality or length of hospital stay between the groups. According to these data, early enteral feeding with an immune-enhancing formula is not beneficial and should not be used in a routine fashion after surgery for upper gastrointestinal malignancies.

Despite the advantage of positive clinical effects of immunonutrition for the treatment of surgical patients, the impact of this nutrition on the immune system still remains unclear. The most controversial is the effect of immunonutrition on postoperative immune system function, which is very important for an adequate host response to surgical trauma and intra-operative infection. There is a large body of evidence which points to the fact that apoptosis (programmed cell death) plays a positive and negative immune regulatory role in postoperative immunosuppression [17-19]. The phase of immunosuppression after severe trauma or major surgery is characterized by increased apoptosis in monocyte, lymphocyte and dendritic cell subsets [20-22]. These changes may contribute to the overwhelming inflammatory response (SIRS) to trauma and infection. The mechanism used by immunonutrients to protect the cells against apoptosis is still unclear. Glutamine could modulate apoptosis-related cellular mechanisms and can protect human T cells from apoptosis by up-regulating glutathione, Bcl-2 and CD45RO anti-apoptotic protein expression in lymphocytes and down-regulation of the expression of caspase-3, Fas (CD95) and Fas ligand pro-apoptotic proteins [23-25].

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Aim

In this study, we therefore aimed to investigate the hypothesis that preoperative enteral immunonutrition possesses some anti-apoptotic properties and can influence the lymphocyte apoptotic signalling pathways in patients before and after extended pancreatic cancer surgery.

Material and methods

Thirty-four out of the 48 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 – 15 patients, mean age 62.4 ±10) or the immune-enhancing enteral diet (group II – 19 patients, mean age 61.4 ±8). Fourteen patients (group III – mean age 65.2 ±8) with pancreatic cancer of normal nutritional status did not receive the preoperative nutrition. After full clinical diagnostic procedures (imaging and laboratory tests), all patients were subjected to pancreatic head resection (Whipple's pancreaticoduodenectomy). A histopathological examination confirmed the diagnosis.

The present investigation did not include patients: treated with early enteral or parenteral postoperative nutrition; showing early serious postoperative infectious complications; with unresectable pancreatic cancer; who had organ transplants; treated with chemo- or radiotherapy or immunosuppressors; suffering from autoimmune diseases; or with diabetes type 1 (insulin-dependant), chronic respiratory insufficiency (chronic obstructive pulmonary disease), cardiovascular insufficiency, or 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).

Enteral nutrition

In the preoperative period standard enteral nutrition or immunonutrition was used as a supplementary diet for 5 days. The indication for preoperative enteral nutrition treatment was the loss of body mass (more than 5% within 2 months) and the extent of surgery [26]. 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 2 sachets (Glutamine Plus, $n = 11$) or 3 containers (FortiCare, $n = 8$ or Nutridrink, $n = 15$) of diets per day according to the manufacturer's instructions. The immune-enhancing diets contained 20 g of glutamine, over 2 g of eicosapentaenoic acid (EPA) and 1.2 g of docosahexaenoic acid (DHA).

Assessment of pro-apoptotic and anti-apoptotic proteins

Lymphocyte isolation

Blood samples were collected from a peripheral vein before (–1) and after (day 0) preoperative nutrition and on postoperative day 1. Lymphocytes were separated

from heparinized blood by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions [27]. Isolated lymphocytes were suspended in phosphate-buffered saline (PBS).

Western blot analyses

Lymphocytes suspended in PBS were mixed with an equal amount of Laemmli sample buffer with 0.5% β-mercaptoethanol (Bio-Rad, California, USA) and boiled for 5 min. 50 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 (Bio-Rad, California, USA). As a marker of protein size Novex Sharp Protein Standard (Invitrogen, Carlsbad, California, 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, Santa Cruz, USA) diluted in 0.5% blocking solution 1 : 5000 for 1 h at room temperature. The step was followed by washing 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 (Bio-Rad, California, USA). Values are expressed as a percentage of patients or controls with the positive expression of pro- and anti-apoptotic proteins.

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.

Results

In the preoperative period similar changes of the expression of pro- and anti-apoptotic proteins were

observed in the pancreatic cancer patients receiving preoperative standard enteral nutrition (group I) or enteral immunonutrition (group II) (fig. 1). The frequency of Bcl-2, Bax, NF- κ B and PARP-1 expression in lymphocytes was significantly decreased before as well as after preoperative enteral standard nutrition as compared with the control group (all $P < 0.01$). Similar changes of the expression of NF- κ B and PARP-1 were observed in patients receiving preoperative immunonutrition. In both groups (I and II) the frequency of caspase-3, -9 and TNF-R1 expression was significantly elevated in comparison with the control group before and after surgery (all $P < 0.01$). 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. However, the decreased expression of NF- κ B and elevated caspase or TNF-R1 expression still persisted (fig. 2).

In comparison to the standard nutrition, the preoperative enteral immunonutrition maintained Bcl-2 and Bax expression resulted in insignificant differences between group II and controls both before the operation and in the early postoperative period (fig. 3). The preoperative standard nutrition and enteral immunonutrition did not significantly alter the expression of caspase-3 and -9, NF- κ B, PARP-1 and TNF-R1. Still, in both groups of patients (groups I and II) elevated expression of caspase-3, -9, and TNF-R1, and decreased expression of NF- κ B and PARP-1 were detected. The differences

between groups I and II in all proteins' expression were not statistically significant both before and after pancreatic surgery. There were no significant differences between the preoperative and postoperative expression of pro- and anti-apoptotic proteins. In the group of healthy volunteers caspase-3, -9 and TNF-R1 expression were not detected.

Discussion

Trauma-activated patient T cells may undergo higher levels of apoptosis during their trauma recovery period, but this apoptosis represents an appropriate immunoregulatory response which eliminates the no longer needed, yet activated, T cell population. Highly elevated levels of T cell apoptosis might represent the development of inappropriate apoptosis that then leads to subsequent T cell energy development [17]. The increased levels of apoptosis are not directly associated with negative trauma patient outcome nor the immediate cause of T cell energy. However, unusually high levels of apoptosis and development of severe T cell depletion occurring before complete activation and expansion of the post-trauma T cell response may presage energy and subsequent organ failure [18]. 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 [19, 28]. In the ear-

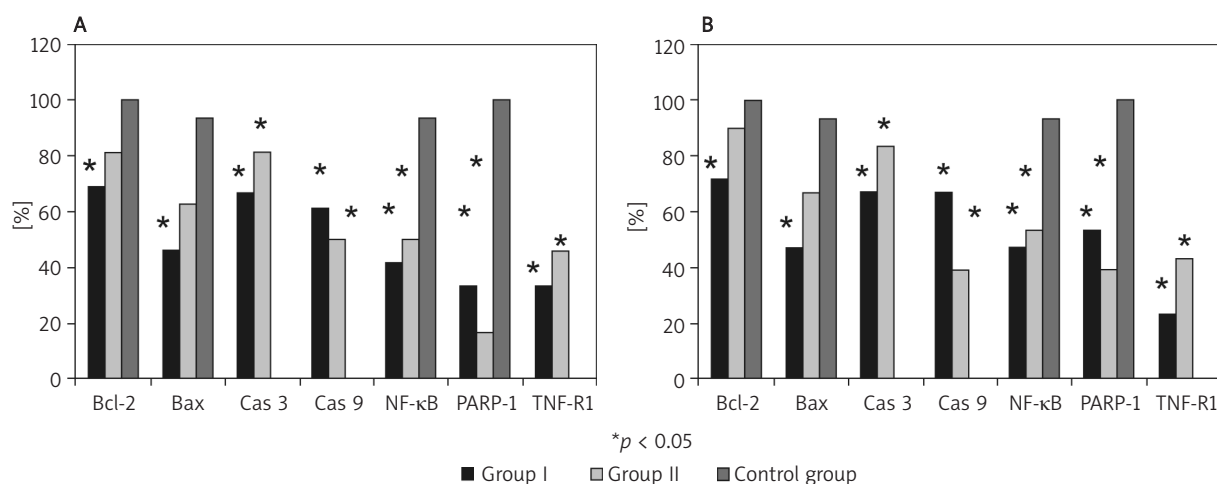


Fig. 1. The percentage of malnourished patients with pro- and anti-apoptotic protein expression before (A, day 0) and after (B, day -1) preoperative enteral standard (group I) and immune-enhancing nutrition (group II) in the group of pancreatic cancer patients as compared with the control group (before surgery). Caspase and TNF-R1 expression in the control group was not detected

Ryc. 1. Odsetek niedożywionych chorych z rakiem trzustki, z ekspresją pro- i antyapoptotycznych białek przed (A, dzień 0) i po (B, dzień -1) przedoperacyjnym dojelitowym żywieniu standardowym (grupa I) i wspomagającym odporność (grupa II) w porównaniu z grupą kontrolną (przed operacją). W grupie kontrolnej nie wykryto ekspresji kaspaz i TNF-R1

ly postoperative period, surgical trauma under general anaesthesia induces an intracellular perturbation in peripheral lymphocytes, resulting in both up-regulation of death-signalling factors and down-regulation of survival-signalling factors. The increased apoptosis of CD8 lymphocytes, excluding CD4 cells, seemed to be associated with a greater risk of post-surgical infections [21]. The attempt to correct the postoperative immune disorders by introducing preoperative or postoperative immunonutrition is a promising way of improving outcome after pancreatic surgery.

Our study indicated a significant decrease in the expression of anti-apoptotic proteins (Bcl-2, Bax, NF- κ B, PARP-1) and increase of caspases (3, 9) and TNF-R1 proapoptotic proteins before as well as after surgery for pancreatic cancer. Whereas the enhancement of NF- κ B and PARP-1 expression in lymphocytes prevents apoptosis, low levels of these proteins may trigger different pathways of cell death. Furthermore, decreased expression of PARP-1 suggested increasing the susceptibility of lymphocytes in pancreatic cancer patients to DNA damage. PARP has a well-established role in DNA repair processes. The activation of PARP-1 by genotoxic stim-

uli facilitates cell survival. The 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 and TNF-R1 expression (not detected in healthy volunteers) may be the most characteristic "marker" in each group of pancreatic cancer patients (I, II, III).

The results of our studies clearly revealed the down-regulation of anti-apoptotic signalling systems in lymphocytes of malnourished patients with pancreatic cancer and a switch to apoptosis. These pathological alterations in apoptotic signalling pathway proteins may increase lymphocyte dysfunction and immune system suppression especially after pancreatic resection. Unfortunately, preoperative enteral immunonutrition as compared to standard nutrition has no significant modulatory effect on changes in these apoptotic signalling pathways. In particular, preoperative enteral immunonutrition has no effect on the frequency of extremely high caspase expression. The weak influence of immunonutrition on the frequency of anti-apoptotic

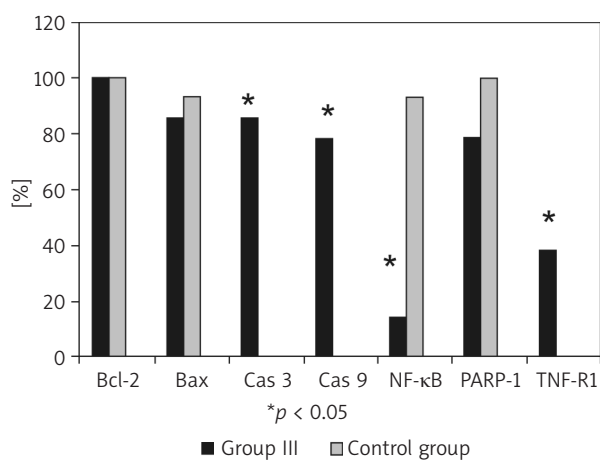


Fig. 2. The percentage of pancreatic cancer patients with pro- and anti-apoptotic protein expression and normal nutritional status who did not receive preoperative enteral nutrition (group III) as compared with the control group (before surgery). Caspase and TNF-R1 expression in the control group was not detected

Ryc. 2. Odsetek chorych z rakiem trzustki i prawidłowym stanem odżywienia, nieleczonych żywieniem przedoperacyjnym, z ekspresją pro- i antyapoptotycznych białek w porównaniu z grupą kontrolną (przed operacją). W grupie kontrolnej nie wykryto ekspresji kaspaz i TNF-R1

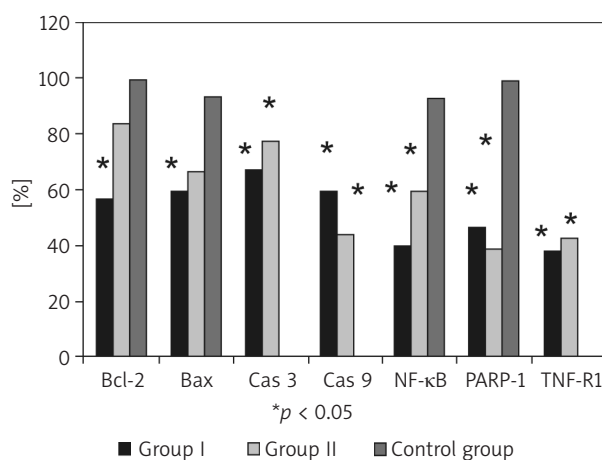


Fig. 3. The percentage of malnourished pancreatic cancer patients with pro- and anti-apoptotic protein expression after preoperative enteral standard (group I) and immune-enhancing nutrition (group II) as compared with the control group (day 1 after surgery). Caspase and TNF-R1 expression in the control group was not detected

Ryc. 3. Odsetek niedożywionych chorych z rakiem trzustki, z ekspresją pro- i antyapoptotycznych białek po przedoperacyjnym dojelitowym standardowym żywieniu (grupa I) i wspomagającym odporność (grupa II) w porównaniu z grupą kontrolną (1. doba po operacji). W grupie kontrolnej nie wykryto ekspresji kaspaz i TNF-R1

protein expression (preoperative enteral immunonutrition probably maintained Bcl-2 expression) and the lack of modulatory effect on caspase and other apoptotic protein expression was probably connected with insufficient intake of immunonutrients in the short (only 5 days) preoperative period. In the majority of patients subjected to immunonutrition a glutamine-enriched diet was used. The small group of patients receiving unsaturated fatty acids does not allow us to draw any separate conclusions, but the modulatory effect of unsaturated fatty acids on the apoptotic signalling pathways may influence glutamine activity. Why the peripheral blood lymphocytes switch to activation of the cell-intrinsic suicide programme still remains unclear. The possible explanation of inappropriate changes in the apoptotic signalling pathway proteins is related to immune system cells' malnutrition in pancreatic cancer patients. In our study disease-related malnutrition (loss of body mass by more than 5% within 2 months) detected in 70% of patients was the indication for preoperative enteral nutrition treatment.

In the presented study we also measured the expression of apoptotic proteins in patients showing normal nutritional status (group III). There were no significant differences between pancreatic cancer and healthy individuals in the frequency of Bcl-2, Bax and PARP-1 expression. 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- κ B expression may reflect pancreatic cancer progression.

Additionally, considering pancreatic cancer patients and lymphoid tissue malnutrition suggested that another explanation of pathological changes presented in the apoptotic proteins of lymphocytes (especially a significant decrease in the frequency of Bcl-2 expression in patients receiving preoperative enteral standard nutrition) may be related to the overwhelming activity of cancer tissue. The cancer cells need a lot of nutrients to multiply and survive and they show a high rate of glutamine utilization, probably also necessary to maintain Bcl-2 production and avoid apoptosis of their own cells. As a nitrogen donor for the synthesis of purine and pyrimidines, glutamine is an important component for RNA and DNA building in cancer cells that show high rates of division and/or protein secretion (e.g. Bcl-2). Current pharmacological approaches are focused on the use of peptides to neutralize anti-apoptotic Bcl-2 proteins and facilitating the apoptosis of cancer cells [29]. Researchers at the Johns Hopkins University School of

Medicine have discovered how the Myc cancer-promoting gene uses microRNAs to control the use of glutamine, which is a major energy source [30]. The authors also discovered that Myc can increase the use of glutamine by cancer cells. We suggest that the preferential utilization of glutamine by cancer cells may cause a reduction of protein supply and lowering of Bcl-2 production in peripheral blood lymphocytes. This situation may switch the peripheral blood lymphocytes of pancreatic cancer patients to apoptosis. In the experimental study a deficiency in glutamine induces apoptosis in human cells [31]. Another study revealed that glutamine deprivation initiated an intrinsic apoptotic pathway and involved the activation of both caspase-9 and -3 (in Sp2/O-Ag14 cells) [32]. It is possible that extremely high expression of caspase-3 in the lymphocytes of our patients was associated with glutamine deficiency. Our hypothesis requires further investigations including simultaneous measurements of expression of selected apoptotic signalling proteins, and serum and/or cancer tissue glutamine concentrations in malnourished patients with pancreatic cancer. As indicated earlier, Bcl-2 regulates pancreatic morphogenesis and tissue homeostasis ranging from early fetal to adult life and can be considered a phenotypic marker of normal exocrine pancreas [33]. On the other hand, the lack of expression in pre-neoplastic lesions and the low positivity found in primary tumours and lymph node metastases suggest that Bcl-2 does not play a central role in pancreatic tumourigenesis and cancer progression. In normal pancreatic and chronic pancreatitis tissues, Bcl-2 and Bax were predominantly expressed in ductal epithelial cells while p53 was not detected. In pancreatic ductal adenocarcinoma and ampullary cancer, Bcl-2 was not detected as compared with the expression seen in normal acini [34]. An immunohistochemical study of pancreatic cancer showed p53 expression in 100% of cases and Bcl-2 expression in 27.7% [35]. Overexpression of Bcl-2 has been reported for a variety of human epithelial malignant tumours, including colorectal and gastric carcinoma [36, 37]. Bcl-2 expression in cancer tissue and serum may have possible prognostic value when combined with p53 expression in gastric cancer [38, 39]. Other findings suggested that, despite the fact that Bcl-2 inhibits apoptosis, cellular proliferative activity is also suppressed [40].

In conclusion, our studies suggested the down-regulation of anti-apoptotic signalling systems in lymphocytes of malnourished patients with pancreatic cancer and a switch to apoptosis. The extremely increased frequency of caspase-3 expression not detected in healthy volunteers seemed to be the most characteristic feature of pathological alterations in the lymphocytes of

pancreatic cancer patients. These pathological alterations in apoptotic signalling pathway proteins may increase lymphocyte dysfunction and immune system suppression and may influence pancreatic cancer patients' susceptibility to infectious complications as well as to tumour metastasis. Preoperative enteral immunonutrition has no significant effect on the apoptotic signalling pathways and the anti-apoptotic impact of such nutrition in pancreatic cancer patients is still questionable.

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