Clinical immunology

Programmed lymphocytes death in pancreatic cancer patients

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Abstract

To evaluate the status of immune cell apoptosis we investigated expression of some proteins connected with programmed lymphocytes death of pancreatic cancer patients in comparison with healthy controls. The expressions of Bcl-2, Bax, Cas3, Cas9, PARP-1 and TNFR1 proteins of peripheral blood lymphocytes were assessed by western blotting. Results of the study show a significantly lower expression of Bcl-2, Bax and PARP-1 and a significantly higher expression of caspase 3, 9 and TNFR1 in the lymphocytes of patients with pancreatic cancer as compared to the healthy control. In conclusion: our studies show a down-regulation of antiapoptotic signaling system in the lymphocytes of patients with pancreatic cancer and a switch to apoptosis. These alterations may lead to lymphocytes dysfunction and immune system suppression.

Key words: pancreatic cancer, apoptosis, immune dysfunction.

Introduction

The combined burden of upper gastrointestinal cancer ranked second among the causes of death from cancer in men and third among women [1]. Survival rates for pancreatic cancer are generally low with rapid metastasis and poor prospects for cure. It is estimated that approx 90% of patients die within a year of diagnosis [2]. Ductal pancreatic adenocarcinoma, the most frequent malignancy of the pancreas, is characterized by retroperitoneal and perineural infiltration, early formation of multiple metastases, and resistance to most of the treatment regimen currently available [3-5]. Surgical resection, the patient’s only hope for cure, offers a significantly improved prognosis, with a median survival after resection of 14-20 months and up to 25% 5-year survival rates [5-7]. The mortality in early postoperative period decreased significantly in the last decade down to 1.3-7%, but the level of morbidity is still very high amounting up to 70% [8-12]. The persistent high morbidity rates have remained an important concern for patients, healthcare providers, and payers.

There are a lot of examples of strong evidences for immunosuppression in pancreatic cancer [13-15], which contribute to postoperative complications and increased morbidity. One of process especially important for the proper functioning of the immune system is apoptosis. The apoptosis or programmed cell death is a central regulator of tissue homeostasis that stimulates the elimination of redundant, damaged or infected cells. The apoptosis plays a critical role in the formation of organs, limbs and other body structures, and in maintaining the function of most of the systems in an adult body. Therefore, a dysregulation of the apoptotic signaling processes often leads to serious consequences, such as neurodegenerative diseases, cancer or autoimmunity [16-19]. It is generally accepted that 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 (DISC), and intrinsic signaling mainly through mitochondria which leads to the formation of the apoptosome (Fig. 1).
To evaluate the status of immune cell apoptosis we investigated expression of some proteins connected with programmed lymphocytes death in the peripheral blood of pancreatic cancer patients in comparison with healthy controls.

Materials and methods

Patients

In our study we included 48 patients diagnosed as having pancreatic cancer. We excluded patients over 75, patients with metastasis, the ones suffering from chronic liver or kidneys diseases and diabetes. The mean age was 62.8 years ranging from 45 to 75 years. Of the 48 patients, 25 were male and 23 were female. Patients were classified according to UICC (TNM classification of malignant tumours) – 3 patients were classified stage I, 28 patients were at stage II and 17 patients were at stage III. In 11 patients, palliative operations were performed due to irressectability of the tumor. In all patients, the diagnosis of adenocarcinoma of the pancreas was confirmed by histologival examination. In all patients, 10 ml of heparinized blood was collected after hospital admission. The control group comprised 30 healthy volunteers (14 were male and 16 were female).

Isolation of lymphocyte

10 ml of peripheral blood from patients was collected into the tubes with heparin. Then lymphocytes were iso-
lated by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) according to manufacturer’s instruction [20]. Isolated lymphocytes were suspended in PBS (phosphate-buffered saline).

**Western blotting**

Lymphocytes suspended in PBS (phosphate-buffered saline) were mixed with equal amount of Laemmli sample buffer with 0.5% β-mercaptoethanol (Biorad, 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 TRANSBLOT SD, SEMI DRY TRANSFER CELL (Biorad, 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 RT (room temperature) and probed with specific Ab (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), Cas3 (sc-7148, rabbit polyclonal), Cas9 (sc-7885, rabbit polyclonal), TNFR1 (sc-1068, goat polyclonal) and GAPDH (sc-25778, rabbit polyclonal) (Santa Cruz Biotechnology, Santa Cruz, USA) for 1.5 h at RT (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 for 2 × 10 min at RT (room temperature). Protein-antibody binding was detected by using Alkaline Phosphatase Conjugate Substate Kit (Biorad, California, USA).

**Statistical analysis**

Statistical analysis was performed using the Statsoft Statistica v.7.0 program. To evaluate the statistical significance in apoptotic proteins expressions between group with pancreatic cancer and control group Wilcoxon signed-rank test with Bonferroni’s correction was used. Significance was set at \( p < 0.05 \).

**Results**

The expression of proteins involved in programmed lymphocytes death is altered in pancreatic cancer patients as compared to the healthy control. The expression of proteins Bcl-2, Bax and PARP-1 is significantly lower in pancreatic cancer patients as compared to the healthy control group (respectively: \( p = 0.02 \), \( p < 0.01 \), \( p < 0.01 \) (Fig. 2). On the other hand the expression of TNFR1, caspase 3 and caspase 9 in pancreatic cancer patients as compared with the control group was significantly higher (respectively: \( p = 0.01 \), \( p < 0.01 \), \( p < 0.01 \) (Fig. 3). In the control group the expression of caspase 3, caspase 9 and TNFR1 was not detected (Fig. 3). The analysis of differences in the expression of proteins inside the group of patient with pancreatic cancer shows a considerably higher expression of Bcl-2 and caspase 3 as compared with the expression of PARP-1, TNFR1.

![Fig. 2. Expression of Bcl-2, Bax and PARP-1 in peripheral blood lymphocytes in pancreatic cancer patients in comparison with healthy controls](image1)

![Fig. 3. Expression of Cas3, Cas9 and TNFR1 in peripheral blood lymphocytes in pancreatic cancer patients in comparison with healthy controls](image2)
Discussion

Our results show a significantly lower expression of Bcl-2, Bax and PARP-1 and a significantly higher expression of caspase 3, 9 and TNFR1 in the lymphocytes of patients with pancreatic cancer as compared to the healthy control, which corresponds to the underexpression of anti-apoptotic proteins and the overexpression of proapoptotic proteins. These alterations revealed the down-regulation of antiapoptotic signaling system in the lymphocytes of patients with pancreatic cancer and switch to apoptosis.

While apoptosis is an essential biological process for normal development and maintenance of tissue homeostasis, it is also involved in a number of pathological conditions including tissue injury, degenerative diseases, immune diseases and cancer [21]. Whether activated by membrane-bound death receptors [22] or by stress-induced mitochondrial perturbation with a subsequent cytochrome c release [23], 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 [24]. The several mechanisms of activation of apoptosis in different physiological or pathological conditions in cells have been proposed and studied intensively [25]. Numerous factors, such as cytosolic cyt c release, caspase 3 activation, and the expression of Bcl-2 family proteins have been suggested to play an essential role in the apoptotic process.

The Bcl-2 family proteins are important players in apoptosis and the interactions between the antiapoptotic members like Bcl-2 protein and pro-apoptotic members like Bax protein provide a mechanistic basis in modulating the apoptotic cell death [26, 27]. Bcl-2 directly or indirectly prevents the release of cytochrome c from mitochondria in a variety of tissues [28]. The Bax protein is pro-apoptotic member of the Bcl-2 family that resides in the cytosol and translocates to mitochondria upon induction of apoptosis [29].

The lymphocytes found in the peripheral blood of patients with laryngeal carcinoma showed an increased level of Bcl-2 protein expression in both subpopulations, as well as helper (CD4) and cytotoxic (CD8) of human T cells [30]. In addition, the freshly isolated and aged lymphocytes express a high amount of bcl-2 (the aged cells more than freshly isolated ones) [31]. 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 as compared with the corresponding ratios found in healthy individuals [32]. Also the Bax levels were lower in the cytosolic fraction in euthymic, depressed and manic bipolar patients [33]. However there are some data that shown the expression of Bcl-2 and Bax proteins in the peripheral blood lymphocytes in pancreatic cancer patients. We observed decreased expression of these proteins as compared with the healthy controls, which may suggest a decreased expression of Bcl-2 family proteins in the peripheral blood lymphocytes of pancreatic cancer patients.

The extrinsic pathway of apoptosis can be induced through the members of the TNF/TNF receptor superfamily such as for example: receptor 1 for TNF-α (TNFR1) [34]. Our finding demonstrate a high expression of TNFR1 in the lymphocytes of patients with pancreatic cancer what suggesting an increased level of apoptosis of peripheral blood lymphocytes in patients with pancreatic cancer by the extrinsic pathway. Other studies have shown a high level of TNFRI in the lymphocytes of patients after heart transplants [35] and HIV infected patients [36]. Interestingly, no expression of TNFR1 in lymphocytes of the control group was observed. It shows that it is not the TNFR1 that starts a physiological apoptosis of lymphocytes in healthy people.

The central component of apoptosis is a family of caspases. Caspases initiate and execute cell death by inactivating anti-apoptotic proteins, shutting down DNA replication and repair [37], reorganizing the cytoskeleton [38] and disrupting the nuclear lamina [39]. There are some data that show an overexpression of caspase 3 and caspase 9 in patients with type 1 diabetes mellitus [40], HIV infected [41], the ones suffering from Alzheimer’s disease [42], patients in septic shock [43] and gastric cancer patients [44]. Similarly to these results our observations show an increased expression of caspase 3 and caspase 9 in peripheral blood lymphocytes. Similarly as TNFR1 there was not observed expression of caspases in control group.

The caspase 3 is also responsible for the proteolytic cleavage of nuclear enzyme poly-(ADP-ribose) polymerase (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. 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 [45]. However, in the lymphocytes there is a desirable high expression of PARP-1 to prevent from breaking down DNA. Our study shown a decreased expression of PARP-1 as compared with the healthy control group, which suggests damage to DNA in the lymphocytes of pancreatic cancer patients. Rajaee-Behbahari et al. [46] have obtained similar results, but in this particular case the patients suffered from laryngeal cancer. The peripheral blood lymphocytes showed significantly lower levels of poly(ADP-ribose) formation as compared with those found in healthy controls [46]. These data show that the lymphocytes of cancer patients show a lower expression of PARP-1, which may indicate a higher level of DNA damaged.

Our results show a decreased expression of antiapoptotic proteins and an overexpression of proapoptotic proteins in the lymphocytes of patients with pancreatic cancer.

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as compared with the healthy control. These alterations revealed the down-regulation of antiapoptotic signaling system in the lymphocytes of patients with pancreatic cancer and a switch to apoptosis. It suggests that patients with pancreatic cancer may show an increased number of lymphocytes dysfunction and, consequently, higher immune system suppression. Then, it can lead to a greater number of infectious complications, organ failure and a higher level of death rate, specially after pancreatic resection. One of the possible explanation of inappropriate changes in the apoptotic proteins in lymphocytes of patients with pancreatic cancer is that these patients are mostly malnourished. The frequency of malnutrition in patients with pancreatic cancer ranges from 80 to 85% [47]. This is the malnutrition that except for reduced skeletal, cardiac and respiratory muscle function, poor wound healing and loss of non-fat body mass causes an impairment of immune functions as a response to undernutrition of immune cells [48]. To improve a nutritional status of immune cells it may be required to introduce immune-enhancing diets. It may lead to some changes in the expression of apoptotic proteins and perhaps a decrease or even an elimination of the differences in the expression of these proteins between patients with pancreatic cancer and healthy people.

In conclusion, our studies show a down-regulation of antiapoptotic signaling system in the lymphocytes of patients with pancreatic cancer and a switch to apoptosis as compared with healthy people. These alterations may lead to lymphocytes dysfunction and immune system suppression. This problem can be resolved by introducing immune-enhancing diets. Further research needs to be undertaken to examine this issue.

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