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Original Article

Expression of apoptosis-related proteins bcl-2 and bax in tumor infiltrating dendritic cells in human endometrial cancer

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Abstract: The purpose of this study was to investigate changes in apoptosis-related proteins bcl-2 and bax on tumor infiltrating dendritic cells (TIDCs) in human endometrial cancer. Expression of bcl-2 and bax proteins in TIDCs of 52 patients with human endometrial cancer were studied by double-labeling immunehistochemistry and image analysis, respectively. 28 cases of normal endometria served as control. DCs in these specimens were labeled by S-100 monoclonal antibody. Statistically significant differences ($P<0.05$) in bcl-2 and bax proteins expression were noticed on TIDCs in human endometrial cancer when compared to normal endometrium. The levels of expression of bcl-2 on TIDCs in human endometrial cancer were significantly lower compared to those on DCs in normal endometrium ($P<0.05$). The intensity of bax on DCs was considerably higher in human endometrial cancer compared to that in normal endometrium ($P<0.05$). Taken together, the data suggest that those apoptosis-related proteins bcl-2 and bax on TIDCs in human endometrial cancer are unbalanced. Therefore, the changes may lead to the occurrence of tumor immune escape, which promotes the evolution of endometrial cancer.

Keywords: Endometrial cancer, tumor infiltrating dendritic cells (TIDCs), apoptosis, bcl-2, bax

Introduction

As one of the common malignancies of the female genital tract, endometrial cancer typically presents as endometrioid-type adenocarcinoma [1, 2]. Nevertheless, the incidence of endometrial cancer has ascended considerably throughout the world in the past few years [3, 4].

It has been known that the delivery of antigen from tumor cells to professional antigen-presenting cells (APCs) could generate strong tu mor specific T-cell immunity. Among these APCs, dendritic cells (DCs) have already been shown to play a critical role in the initiation of effective T cell-mediated immune response, which was initially extracted from rat spleen in 1973 [5]. Therefore, DCs are believed to be the most potent professional antigen-presenting cells and have the most powerful antigen-presenting capacity. In the antitumor immunity, T-mediated Cell immunity has a significant function. By this token, the quantity of DCs and their activity directly relate to the antitumor immunity reactions [6-8].

The study on DCs defect has become heated in recent years. Previous studies have shown that TIDCs with “abnormal” morphology and “defective” antigen-presenting function exist in tumor tissue but lack constructive function in preventing biological behavior changes of tumors [9]. The mechanism of TIDCs dysfunction in tumor-bearing hosts is not clear. Furthermore, our findings indicated that the quantity of TIDCs in tumor tissues is less than that in normal tissues [10].

Apoptosis is a critical process for normal development. However, apoptosis is regarded as an active and organized form of cell death triggered in response to physiologic or pathologic stimuli [11]. Cell death by apoptosis is a part of normal development and maintenance of hom-

eostasis, but it is also involved in pathological situations associated with tumor. So far, only few studies have explored the detailed mechanisms of endometrial cancer immune escape from apoptosis of DC angles. We endeavor to conduct the following studies to try to find the answer.

Under normal conditions, tissue DCs are said to be immature because they express very little class II protein on their surfaces and are unable to activate T cells, although they store large amounts of class II MHC protein in late-endosomal vesicles in the cytoplasm. These DCs are characterized by high endocytic activity and low T cell activation potential. Once immature DCs constantly sample lipopolysaccharide (LPS) or other bacterial macromolecules, they become activated into mature DCs. As a highly effective APC, these mature DCs have great ability to activate T cells. Although several kinds of antibodies may label DCs, different antibodies label only DCs of different stages of DCs. As previously reported, S-100 protein was the most powerful specificity distinguishing from other conventional markers of dendritic cells [12, 13]. In this study, DCs in these specimens were labeled by using anti-S-100 protein antibody.

To our knowledge, this is the first report on the significance of apoptosis and apoptosis-related factors in TIDCs in human endometrial cancer. Accordingly, the aims of this study were to evaluate, by double-labeling immunohistochemistry, the effects of expression of bcl-2 and bax proteins on TIDCs in human endometrial cancer, as a step towards understanding the role of these cells in the pathogenesis of the disease, in particular in tumor immune escape.

Materials and methods

Patient selection

This study was specifically approved by the First Affiliated Hospital Ethical Committee of Jinan University, China. The samples for this study were collected between 2010 and 2014 from the archives of the Department of Obstetrics and Gynecology with the permission of the current ethical committee. The written informed consent was obtained from each patient undergoing total abdominal hysterectomy, neither of whom had received chemotherapy, radiotherapy and hormone replacement therapy prior to the surgery.

Hematoxylin-eosin stained slides from all cases were reviewed and the diagnosis and typing were confirmed. Tumor histological features and grade were determined according to the criteria of the World Health Organization. Clinical staging was determined according to the Federation of International Gynecology and Obstetrics (FIGO). The study samples did not include other histological types of endometrial cancer. It comprised only 52 cases of endometrioid adenocarcinoma. The age of these cancer patients ranged from 42 to 73 years (average age 51.2±2.8 years). The control group consisted of 28 samples of normal endometrium. Control samples were collected from patients undergoing hysterectomy or endometrial biopsy for benign indications. The age of these patients from the control group ranged from 38 to 65 years (average age 45.1±2.3 years). Differences in the ages between two groups did not differed significantly.

Double-labeling immunohistochemistry (Double-labeling IHC)

The critical role of in immunohistochemistry (IHC) techniques is the fixation of the endometrium tissue to obtain the best preservation of antigen and the fixation inside the cells. The pH and temperature of buffers allow for an optimal reaction of fixatives with proteins.

All tissues were respectively fixed in 10% formalin and embedded in paraffin. Formalin-fixed, paraffin-embedded tissue blocks from 52 cases of endometrioid adenocarcinoma and 28 cases of normal endometrium were selected from our previous studies. Consecutive sections at 5 μm thickness were prepared from each block and cut and mounted on gelatin-coated glass slides. The sections were stained with hematoxylin and eosin (H&E). H&E stained slides of all samples were first reviewed to confirm the diagnosis. Sections were dewaxed, washed in distilled water for 5 min, and rinsed in PBS for 15 min to prepare for the following unmasking procedures. They were incubated first with 4% Block Ace solution (Osaka, Japan) to reduce nonspecific background staining. After antigen retrieval, sections were washed in water and phosphate-buffered saline (PBS). The sections were then incubated for 10 min in

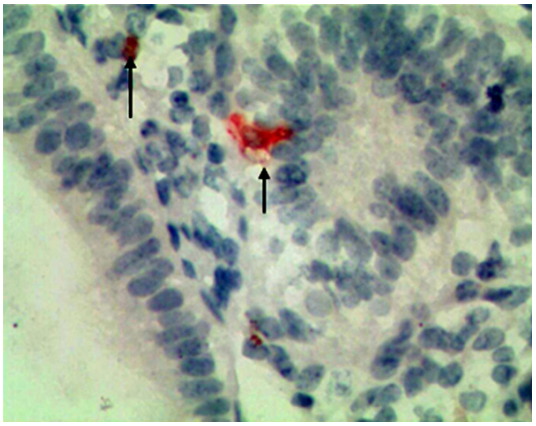

Figure 1. Bcl-2 expression in DC (↑) in normal endometrium (original magnification ×400). Bcl-2 protein of S-100 (+) DCs was stained red.

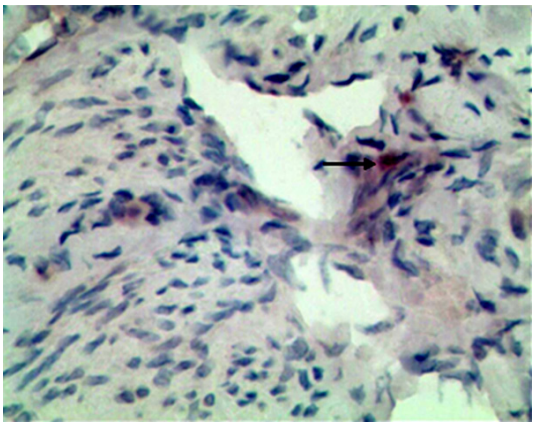

Figure 3. Bcl-2 expression in TIDC (⟶) in endometrial cancer (original magnification ×400). Bcl-2 protein of S-100 (+) DCs was stained red.

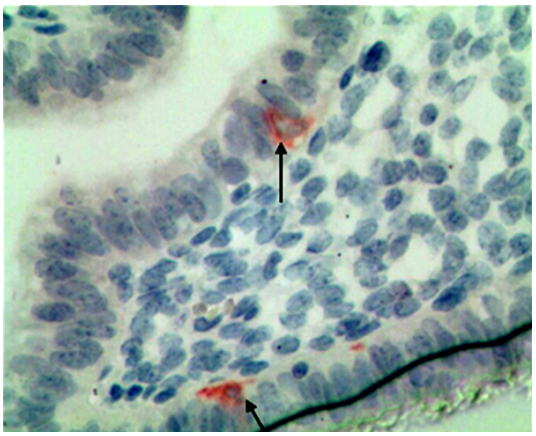

Figure 2. Bax expression in DC (↑) in normal endormetrium (original magnification ×400). Bax protein of S-100 (+) DCs was stained red.

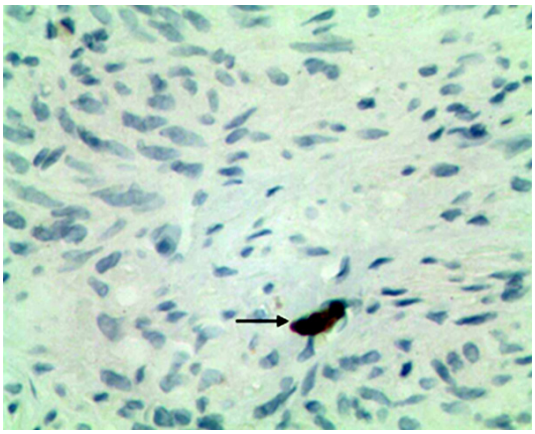

Figure 4. Bax expression in TIDC (⟶) in endometrial cancer (original magnification ×400). Bax protein of S-100 (+) DCs was stained red.

3% hydrogen peroxide to quench endogenous tissue peroxidase and for 30 min in non-immune serum. The sections were subsequently incubated with a polyclonal rabbit antibody against human S-100 protein (code no. Z0311; Dako) at 1:400 dilution for 12 hours at 4°C. After incubation with the primary antibody, the sections were washed with PBS and sequentially incubated with the biotinylated secondary antibody, avidin-biotin-alkaline phosphatase solution and BCIP/NBT (Vector Laboratories, Burlingame, CA, USA) for 10 min, respectively. After the chromogen reaction, sections were thoroughly washed with PBS for 10 min and incubated either in primary monoclonal mouse anti-bcl-2 antibody (clone 124; Dako) diluted 1:40 or monoclonal mouse anti-bax antibody (TC-100, Santa Cruz. Biotech) diluted 1:50 for 12 hours at 4°C. The antigen-antibody complex was detected with the corresponding secondary antibody using an avidin-biotin-peroxidase detection kit and a Zymed AP-red substrate kit (Zymed Laboratories Inc., South San Francisco, CA, USA).

An intestine tissue sample served as a positive control and replacement of the primary antibody using PBS as a negative control. Negative and positive controls were examined first in each individual experiment. The sections were first subjected to S-100 staining using BCIP/NBT as chromogenic agent, followed by bcl-2 or bax proteins staining using AEC as chromogenic agent. All sections were studied under the microscope at the same magnification. The light intensity was kept constant throughout the

Table 1. Determination of bcl-2 and bax proteins expression on DCs and TIDCs ($\bar{x} \pm s$)

	Case	Bcl-2	Bax
Normal endometrium	25	13.11±2.03	7.66±1.14
Endometrial cancer	52	6.21±1.29*	12.89±2.13*

*Compared with normal endometrium, respectively, all *P*-value <0.05.

procedure. Intensity levels of immunostaining in these sections were quantified by the computer-image system (Olympus, Japan). The primary colors of the system are red, green, and blue. The computer program assigned intensity to each color on a scale of 0-255. Red color mainly represented mainly AEC binding. Different intensity of color reflected different immunoreactivity of each protein.

Statistical analysis

Scores of intensity levels were calculated for each DC. The data were expressed as $\bar{x} \pm s$. Statistical software (SPSS, version 12.0; SPSS Inc.) was used to conduct the analysis. Significant differences were calculated using the t-tests where indicated. A level of $P<0.05$ was accepted as statistically significant for all statistical comparisons. Differences between the study group and the control group in the bcl-2 and bax proteins expression were assessed by single factor analysis of variance, respectively.

Results

Immunohistochemical analysis of bcl-2 and bax proteins expression on tumor infiltrating dendritic cells in human endometrial cancer

By means of double-labeling immunohistochemistry and image analysis, the nuclei of S-100 (+) DCs and S-100 (+) TIDCs were much blacker compared to other cells. A large number of S-100 (+) DCs were found in most cases of normal endormetrium. Many S-100 (+) DCs were irregular and large in shape with thin and long cytoplasmic projections (**Figures 1** and **2**). On the other hand, few S-100 (+) TIDCs were observed in human endometrial cancer. Those were small in volume and round in shape, and had few cytoplasmic projections (**Figures 3** and **4**).

Immunoreactivities of bcl-2 and bax proteins were respectively detected in all S-100 (+) DCs of human endometrial cancer and normal endormetrium. By means of double-labeling immunohistochemistry and image analysis, the bcl-2 and bax proteins of S-100 (+) DCs were shown red (**Figures 1-4**). The levels of expression of bcl-2 protein on TIDCs in human endometrial cancer were significantly lower (6.21±1.29) compared to those on DCs in normal endometrium (13.11±2.03) ($P<0.05$). On the contrary, the expression levels of bax protein on TIDCs were considerably higher (12.89±2.13) in human endometrial cancer compared to normal endometrium (7.66±1.14) ($P<0.05$) (**Table 1**).

Discussion

Apoptosis, a type of programmed cell death, is a basic process involved in cellular development and differentiation [11]. Its functions include a normal embryogenesis, the maintenance of the cellular homeostasis, the accurate selection of the immune system and the elimination of damaged cells. In the case of tumor formation, apoptosis may be crucial and its dysregulation is involved in a wide variety of diseases such as tumors. In almost all instances, the abnormal proliferation and death of biologic cells promote neoplastic progression. Previous studies, however, have shown that disorder of apoptosis related genes promote malignant transformation of cells and tumorigenesis [11, 14-16].

Nowadays, the studies on apoptosis related genes have intensified in the field of life science. Apoptosis is a cell death process characterized by morphological and biochemical features. There are two distinct molecular signaling pathways that lead to apoptotic cell death: the intrinsic (mitochondria-mediated) and an extrinsic (extracellularly activated) pathways.

The intrinsic pathway is mainly regulated by the bcl-2 family. The bcl-2 family includes cell death suppressors and promoters, namely, anti-apoptotic proteins such as bcl-2 and pro-apoptotic proteins such as bax. Bcl-2 and bax are both important members of the bcl-2 family, which involve the regulation of cell survival or death [17]. Proliferative activity and cell death determine the growth rate of tissues. As far as apoptosis research is concerned, bcl-2 was the first of anti-apoptotic proteins to be discovered and thus lends its name to the entire protein family.

As mentioned previously, bcl-2 protein is essential to suppress the activation of cell-death program and facilitates cell survival during the process of apoptosis. In consequence, high levels of bcl-2 expression not only prevent the bax accumulation, but inhibit the bax activation in mitochondrial membrane [18]. In response to pro-apoptotic stimuli, Bax undergoes conformational change by exposing its C-terminal hydrophobic domain and forms channels following oligomerization to facilitate mitochondrial permeabilization. It is obvious that the mitochondrial membrane permeabilizing function of bax must be tightly controlled. The subsequent death signaling, ultimately conducted by pro-apoptotic proteins, can be blocked by expression of bcl-2, which can bind and sequester the pro-apoptotic proteins.

Many studies have been done to understand the molecular mechanisms controlling cell death [11, 14, 16]. The extrinsic pathway is triggered by extracellular pro-apoptotic signals. Binding of death ligands to their cognate receptors on the cell membrane initiate a cascade of dynamic protein-protein interactions, induce the activation of caspase-8 and promote the formation of death inducing signaling complex [19, 20]. Once activated, caspase-8 either induces the activation of executioner caspases or integrates a mitochondrial amplification loop. Activation of caspase-3 is common in two major pathways, death receptors and the mitochondrial pathway that both lead to the activation of caspase-3 [21, 22]. Active caspase-9 induces the activation of the effectors caspases-3 to drive cells into programmed cell death. In addition, previous studies have shown that bcl-2 directly inhibit the members of the caspase family, including caspase-3 and caspase-9 [23, 24].

Although DCs are the most potent and professional APCs, with a unique ability to induce primary specific immune responses, only a limited number of studies investigated the functional changes in TIDCs in human endometrioid adenocarcinoma. It was very important that the morphology, functions and numbers of DCs during the progress of anti-tumor immune response. To a certain extent, the abnormal changes in morphology, functions and numbers of DCs would lead to the occur of tumor immune escape.

Jia et al. [9] also reported that TIDCs show less mature phenotype with decreased MHC molecules and co-stimulatory signals, such as CD40 and CD86, compared to normal DCs. Our management of this case was consistent with the generally held view that DC maturation and function are severely impaired under tumor conditions. Furthermore, in the previous study, we have demonstrated that the numbers of TIDCs in human endometrial cancer decreased significantly [10]. This raises the question of how the numbers of TIDC are regulated. Therefore, the purposes of this study are to explore the mechanism underlying the decrease in DCs numbers from the angle of apoptosis, which further reveals tumorigenesis. By double-labeling immunohistochemistry and image analysis, we found that the levels of expression of bcl-2 protein on TIDCs in human endometrial cancer were significantly lower compared to those on DCs in normal endometrium. In human endometrial cancer, the expression of bax protein on TIDCs was considerably higher compared to the expression of bax in normal endometrium. Obviously, the findings indicated apoptosis of TIDCs in human endometrial cancer which indirectly promotes the occurrence and progress of tumor.

The assignment of the ratio of bcl-2 to bax (bcl-2/bax) is important in deciding the prognosis of the cell under harmful insults [25-27]. Therefore, the balance between the expression levels of the bcl-2 and bax is critical for cell survival or death. The increase of bcl-2/bax rate could be considered as a poor prognostic factor of the tumor development for inhibiting apoptosis [26].

In our present study, high expression level of bax and low expression level of bcl-2 were observed on TIDCs in human endometrial cancer, suggesting an imbalance between bax and bcl-2 on TIDCs in human endometrial cancer. This would further lead to TIDCs decreasing in volumes. The rarity of TIDCs suggests that tumor antigen could not be presented validly, which would promote tumorigenesis. In conclusion, the significant differences of bax and bcl-2 expression levels further provides evidence of increased apoptosis on TIDCs in human endometrial cancer. In the future, further studies should investigate the cellular and molecular mechanisms based on the differen-

tial expression of the bax and bcl-2 proteins. Without doubt, it would be useful to perfect the apoptosis mechanism of TIDCs in human endometrial cancer.

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Disclosure of conflict of interest

None.

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