Elevated levels of proinflammatory and anti-inflammatory cytokines in patients with bladder cancer depending on a tumor stage

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Abstract

Bladder Cancer (BC) is a common disease worldwide. Chronic inflammation is one of the key mechanisms for the development of BC. This study enrolled 40 patients. Preoperative plasma levels of IL-1β, IL-4, IL-6, IL-10, IL-12β, TNF-α and IFN-γ were determined by ELISA. In our study, we observed diverse changes in the levels of cytokines in patients with BC Stage I, II, III and IV. The levels of IL-1β was increased for stage I, stage II, and stage III. The level of TNF-α was increased for stage II, stage III, stage IV. The levels of IL-4, IL-6, IL-10 and IL-12β were increased in patients with stage III and IV only. The levels of IFN-γ declined for stage II, stage III and stage IV with the lowest levels in patients with Stage IV.

In our study, we investigated alteration in levels of Th-1 and Th-2-like cytokine profile, but some deficiency in Th1- status discovered in patients with BC.

Introduction

BC is the ninth most frequently diagnosed cancer worldwide, and the seventh most common in men, with the highest incidence rate observed in developed countries. About 75% of patients with BC are men.[1]

BC is a highly immunogenic disease. An imbalance in the proliferation and differentiation of cytotoxic cells and cytokine-producing macrophages leads to dysregulation of the immune system, thus promoting the growth of BC cells.[2] During the chronic inflammatory process, cytokines are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction.[3] Cytokines regulate the inflammatory response, *i.e.* they are major determinants of the make-up of the cellular infiltrate, the state of cellular activation, and the systemic responses to inflammation. The exact mechanism by which the immune system regulates BC still unclear.

CD4 t cells are also known as T-helpers (Th) are central mediators of immunity to infections and cancers.[4] CD4 t cells differentiate to Th1, Th2, Th17, and regulatory T-cells. Th1 cells produce interferon (IFN) γ, IL-12 and tumor necrosis factor α, IL-1 and some other cytokines, which are involved in the cell-mediated pro-inflammatory response. Contrarily, Th2 cells secrete IL-4, IL-6, IL-10 and some other cytokines, which mediate anti-inflammatory humoral response and immune suppression via the inhibition of Th1 cytokine production.[5]

Nowadays, no generally accepted mechanism describes the regulation of the growth and spread of bladder cancer by the immune system. It is necessary to investigate the key regulatory molecules involved in inflammatory processes as inflammation is one of the hallmarks of cancer.[6] Also, unlike other urological cancers, BC lacks a clinically useful biomarker for predicting disease stage and clinical outcome.[7]

Therefore, this study aimed to investigate the immune status by measuring levels of TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-10 and IL-12 in plasma of patients with BC on various tumor stages.

Materials and Methods

This study enrolled 40 patients (males), aged 52-76 years, who underwent radical surgical treatment for BC. All the patients were patients of Alexandrovsky city clinical hospital of Kyiv, Ukraine All the patients and donors or their relatives had been informed about the conduct of clinical research and provided written informed consent on participation in the study. All patients underwent standard preoperative check-up, including general blood analysis, urine analysis, blood chemistry, blood immunogram, computed tomography of pelvis, abdomen and chest cavity with contrast enhancement to determine the depth of the tumorous lesion in the bladder, and the presence of regional or distant metas-

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tases. To characterize the tumor stage, we used TNM clinical classification of AJCC, 8th edition,[8] according to which all patients were classified as Stage I = 10 patients; Stage II= 10 patients; Stage III= 10 patients; Stage IV = 10 patients. The control group consisted of 10 healthy donors (males) of respective age. The current study has been approved by the ethics committee at ESC "Institute of Biology and Medicine", Kyiv, Ukraine. Preoperative blood samples were collected from hospitalized patients the morning before the operation. All patients fasted for 8 h before blood sampling. Citrated anticoagulated blood was separated from whole blood and plasma was obtained via centrifugation at 3000 rpm at 4 °C, and then freeze and stored at -20°C.

Plasma levels of IL-1β, IL-4, IL-6, IL-10, IL-12, TNF-α and IFN-γ were done by enzyme-linked immunosorbent assay (ELISA) according to the standard instructions. ELISA plates were coated overnight at 4°C with samples (100 µl each) of plasma previously 10 times diluted with Tris-HCl buffer pH 7.4. After being washed, plates were blocked with 5% nonfat dry milk for 1 h at 37°C with specific polyclonal antibodies («Santa Cruz», USA) against cytokines such as TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-10, and IL-12. Plates were washed and incubated for 1 h at 37°C with corresponding secondary antibodies conjugated to horseradish peroxidase («Bio-Rad», USA). After washing, the substrate (OPD and hydrogen peroxide) was added. The reaction was stopped by the addition of 2.5 N H_2SO_4. Plates were read at 492 nm by a microplate reader (µQuantTM, BioTek Instruments, Inc). In our study, standard units represent optical density.

Data entry and analysis were performed using StatSoft Statistica ver.7.0 for Windows. After testing for normality (by Shapiro-Wilk), a one-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were statistically significant when $p<0.05$. Data were reported as means ± Standard Deviation (SD).

Results

The results of the measuring levels Th1 cell cytokines in plasma from BC patients are presented in Figure 1. IL-1 plays a central role in acute and chronic inflammation, both locally and systemically. IL-1 is produced primarily by monocytes and macrophages, but it is also produced by astrocytes, endothelial cells, T-cells, and fibroblasts. The most extensively studied function of IL-1 is an initiation of inflammation. In our study, patients with BC showed significantly higher plasma levels of IL-1β than controls patients. The results of the ELISA test have shown rise in levels of IL-1β in patients with all stages of BC, in particular by 1.42 (0.186 ± 0.017), 1.73 (0.227 ± 0.009), 1.83 (0.24 ± 0.03) and 1.54 (0.202 ± 0.025) times compared with control values (0.131 ± 0.005) in BC patients of Stage I, II, III and IV, respectively (Figure 1), results obtained herein agree with previously obtained by other authors.[9]

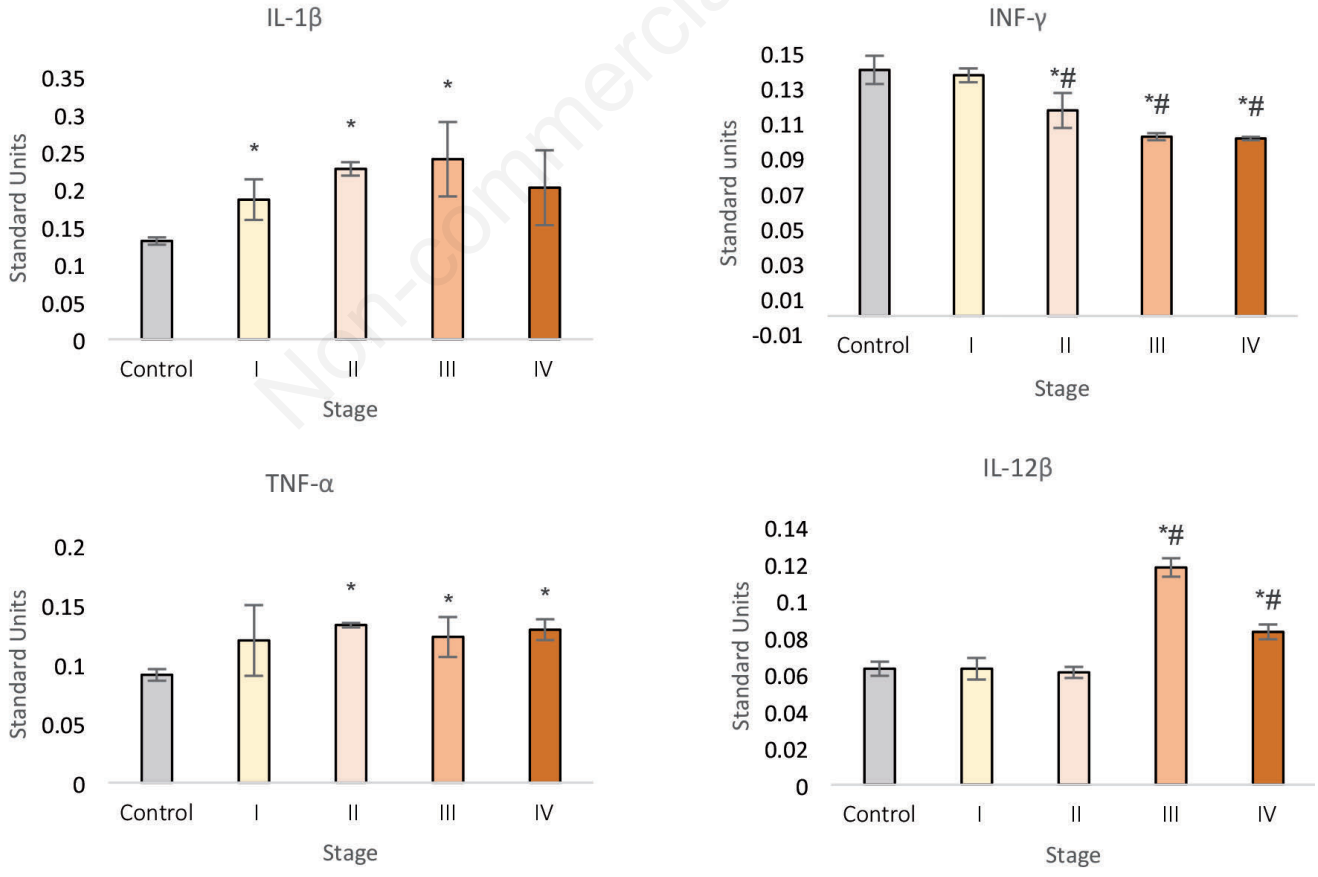

Figure 1. The levels Th1-like cytokine profile in patients with BC in different stages (standard units) (mean SD). *<0,05 compared with the control group; #p<0.05 compared to stage 1 BC.

IFN-γ, or type II interferon, is a cytokine that is important for innate and adaptive immunity against intracellular bacterial infections and tumor control.[10] Current knowledge suggests that the induction of the Th1-like cytokine profile, represented by, IL-1, IL-12, IFN-γ, and TNF-α is essential in the development of the cell-mediated antitumor activity. IFN-γ has been shown to have an important role in protecting against tumor development and cancer immune editing.[11] In our study, plasma levels of INF-γ in patients with BC tended to drop with a growing stage of BC. While in Stage I patients the level of INF-γ was equal to control (0.14 ± 0.008), with a higher stage of cancer the gap in values increased, reaching the biggest decline of 1.39 (0.101 ± 0.005) times in patients with Stage IV BC.

TNF-α mediated inflammation has been linked to cancer.[12] TNF-α is one of the major mediators of inflammation and has been linked to all steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis.[13] TNF-α induces MMP-9 expression in a variety of cell types. Also, recent results have demonstrated that TNF-α-induced MMP-9 expression is mediated by increased activities of NF-κB.[14] Previous studies indicate that increased production of the TNF-α in the serum is related to bad prognosis especially in patients with BC and prostate cancer.[15] In our study, the level of TNF-α was elevated in BC patients in all stages, in particular, it was increased by 1.32 times in Stage I (0.12 ± 0.01), 1.45 times in Stage II (0.132 ± 0.002), 1.35 times in Stage III (0.123 ± 0.015) and 1.42 times in Stage IV (0.129 ± 0.009) compared with control values (0.091 ± 0.005). Our study has revealed a correlation between the level TNF-α and the BC, as it was equally increased in patients of all four stages. During the tumor growth the level of TNF-α in the blood increases, firstly, due to its production by tumor cells, and secondly, as a result of enhanced secretion by macrophages.

IL-12 is an immune-regulatory cytokine that triggers the development of a specific T-cell-mediated immune response. IL-12 is mainly produced by monocytes, macrophages, and dendritic cells in response to bacterial products such as lipopolysaccharides or tumor cells. In different cancer types, a relationship between serum levels of IL-12 and tumor progression or prognosis has been reported as significant. Although, reports concerning IL-12 levels in cancer patients have been contradictory.[16] The serum levels of IL-12 were found to be decreased in colon cancer,[17] gastrointestinal tumors.[18] In patients with ovarian cancer elevated levels of IL-12 were discovered in ascitic fluid samples and were found to be correlated with progression.[19] In another study, Lissoni *et al*. found abnormally high levels of IL-12 in metastatic cancer patients.[20] In our study, a statistically significant change in the plasma level of IL-12 was determined in patients with Stage III BC only, where it was higher by 1.87 (0.118 ± 0.005) times compared to control values (0.063 ± 0.004). In stages I and II its level was equal to control, whereas in Stage IV it was higher than control, though statistically insignificant.

The results of the measuring levels Th2 cell cytokines in plasma from BC patients are presented in Figure 2. IL-4 is a pleiotropic cytokine produced largely by activated Th2-polarized T-cells, mast cells and basophils. It acts upon a broad range of targets, including hematopoietic cells, endothelial cells, and tumor cells.[21] Agarwal A. reported on relatively higher IL-4 expression in BC patients and notably increased in those with recurrence.[22] Also, Goldstein R. reported that serum IL-4 increases proportionately to the cancer stage in patients with prostate cancer.[21] Tumor cells can synthesize IL-4 and its receptors to promote growth. In concordance with that fact, we observed an increase of IL-4 in patients with Stage III and IV of BC. We did not see significant changes in IL-4 levels in plasma from patients with stages I and II of BC. However, the results of the ELISA test showed increased plasma levels of mentioned IL

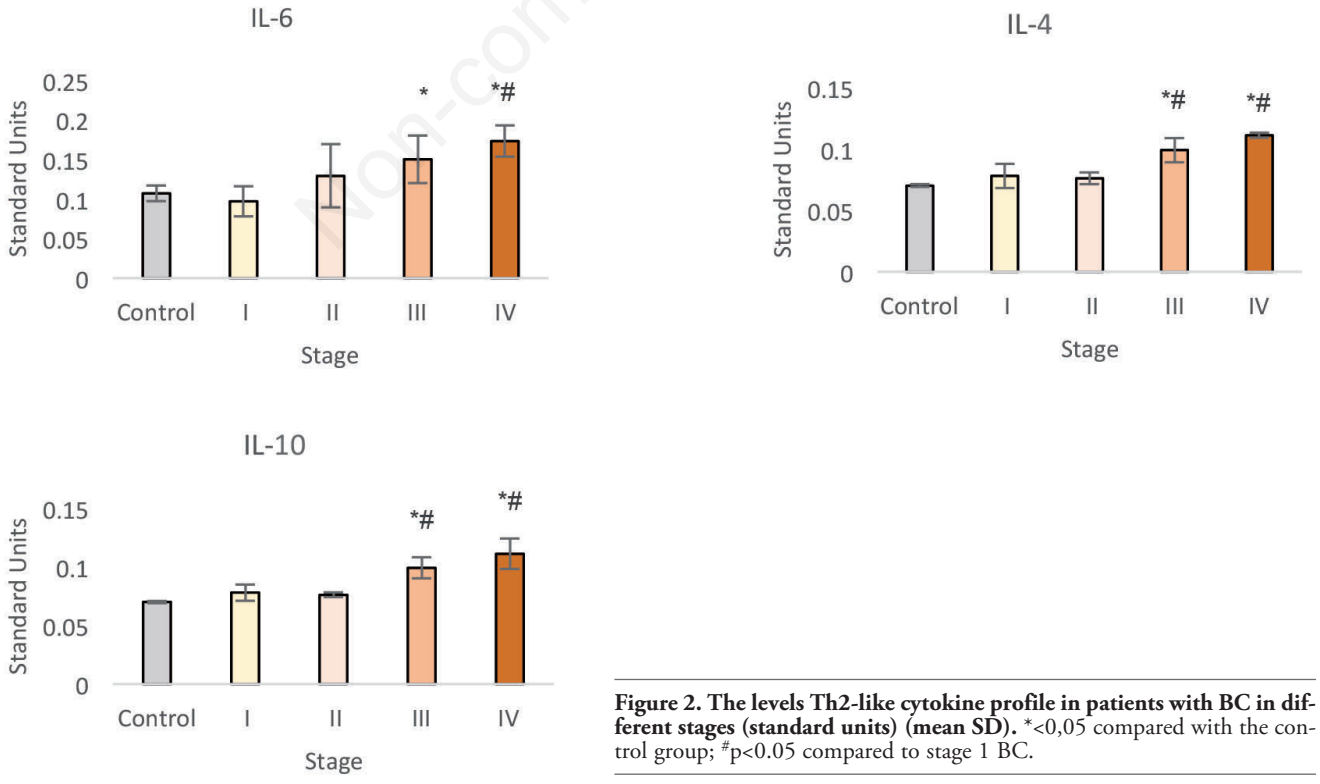

Figure 2. The levels Th2-like cytokine profile in patients with BC in different stages (standard units) (mean SD). *<0,05 compared with the control group; #p<0.05 compared to stage 1 BC.

in patients with Stage III and IV by 1.41 (0.099 ± 0.01) and 1.59 (0.111 ± 0.002) times compared with the control group (0.07 ± 0.005). The same trend we observe with levels of IL-6 and IL-10 in plasma of patients with BC. The level of IL-6 in patients with Stage III and IV of BC was elevated by 1.4 (0.15 ± 0.01) and 1.62 (0.173 ± 0.02) times compared with control values (0.107 ± 0.01). IL-6 is one of the cytokines that plays an important role in cell physiology.[22] The effects of IL-6 on different cells are numerous and varied. Its effects on B-cells are stimulation of differentiation and antibody secretion. IL-6 regulates the immune system via the proliferation and activation of cytotoxic T-cells and the production of acute-phase proteins. IL-6 is mainly produced by macrophages and monocytes whereas a minor amount is also formed by fibroblast, endothelial cells, amnion cells, T- and B-lymphocytes and chondrocytes.[23] It has been shown that IL-6 might provide a selective growth advantage to N-methyl-N-nitrosourea-initiated bladder epithelial cells *in vitro*, and thus accounting for the enhancement of inflammation-associated rat bladder carcinogenesis.[24] Our study shows elevation in circulating levels of IL-6 proportionately to the BC stage with the highest levels of IL-6 observed in Stage IV patients. Thus, its level could be used to differentiate the stages of BC. Previous studies indicated that elevated IL-6 levels are a significant prognostic factor in the prostate, breast, ovarian and renal cancer. Furthermore, elevated circulating levels of IL-6 have been associated with metastasis.[25]

IL-10 is secreted by a wide variety of cell types including macrophages and Th2 cells. IL-10 functions as a Th1 inhibitory cytokine in BC, it is a pleiotropic cytokine that functions as a positive or negative mediator of innate and adaptive immunity under different circumstances.[26] The level of IL-10 in patients with Stage III and IV of BC was elevated by 1.27 (0.089 ± 0.009) and 1.6 (0.112 ± 0.013) times compared with control values (0.07 ± 0.009). In plasma from patients with Stage I and II BC the levels of IL-6 and IL-10 were close to control values. In our study, we have demonstrated proportionately to the BC stage increase in IL-10 level, with statistically significant values of high levels of IL-10 only in patients with Stage III and IV of BC. Previous studies indicated that a high level of IL-10 correlated with the clinical stage of cancer.[27,28]

Discussion

Inflammation is increasingly recognized as a major factor in cancer development and progression. The microenvironment in tumor tissue resembles a status of chronic inflammation. It has long been accepted that the interaction between tumor cells and their microenvironment may affect tumor growth and metastasis formation. The tumor microenvironment may differ between tumor types, grades and disease stages, it is complex, and consists of many cell types and factors.[27] Inflammatory cells and cytokines were recently suggested to play a key role in BC. Cytokines were first discovered as secreted proteins that control different immune functions.[29] It is now clear that cytokine functions extend to many other aspects of biology, including cancer. Cytokines may be secreted not only by inflammatory cells but also by the tumor and stromal cells, together with establishing a network of factors that significantly affect BC. The progress of BC causes a misbalance between local and general immunity, acting as an external inhibitory factor for BC immunosurveillance. T-cell immunity, more precisely T-helpers (Th), is an important part of the implementation of local defense in tumors. Th cells are divided into two subsets (Th1 and Th2): Th1 cells secrete cytokines such as IFN-γ, TNF-α and support cytotoxic T lymphocytes (CTL) to exert their function; Th2 cells secrete cytokines such as IL-4, IL-6, and IL-10 and limit CTL proliferation.[30] In our study, we investigate some increase in levels of Th2-like cytokine profile, and some levels of Th2-like cytokine profile (IL-1β, IL-12β).

In our study, we observed diverse changes in the levels of cytokines in patients with BC stage I, II, III and IV. We admit an increase in levels of proinflammatory and anti-inflammatory changes, but the level of IFN-γ declined proportionately to the stage of the BC with the lowest level in patients with Stage IV. The levels of IL-4, IL-6, IL-10 and IL-12 were elevated in patients with stage III and IV only.

Conclusions

We found that studied cytokines (IL-1β, TNF-α) can differentiate the patients with BC and healthy controls. Other cytokines (IL-6, IFN-γ) can help in differentiating the stage of BC. Anti-inflammatory cytokines (IL-4, IL-10) and proinflammatory IL-12β were elevated in BC patients Stage III and IV. As for immune status – we have shown alteration in levels of Th-1 and Th-2-like cytokine profiles, but some deficiency in Th1- status discovered in patients with BC. Our study can supplement existing data on the effects of the immune system on the development of BC.

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