Serum interleukin-18 and nitric oxide activity in bladder carcinoma

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ABSTRACT. Background: Both interleukin-18 and nitric oxide are multifunctional molecules that are involved in the different steps of carcinogenesis.
Methods: In the present study, we measured serum interleukin-18 and nitric oxide activity in 51 bladder cancer patients with different tumor stage and grade, and in 8 healthy controls. Serum nitrite-nitrate levels were measured as an index of nitric oxide generation.
Results: Serum interleukin-18 levels were significantly higher in bladder cancer patients when compared to the control subjects ($p > 0.05$). Serum interleukin-18 levels were found to be higher in patients with Ta stage than patients with T1 and T2, T3, T4 stages and in patients with grade 1 tumors than patients with grade 2 and grade 3 tumors, but this was not statistically significant ($p > 0.05$). There was no significant difference in serum nitrite + nitrate levels between bladder cancer patients and control subjects.
Conclusions: Elevated serum interleukin-18 levels in bladder carcinoma patients may be a result of host defence mechanism against the growth and progression of bladder cancer cells.

Keywords: interleukin-18, nitric oxide, nitrite, nitrate, bladder cancer

INTRODUCTION

Interleukin-18 (IL-18) is a 18.3 kD cytokine that has been shown to enhance the immune defence against tumor cells by inducing interferon-γ (IFN-γ) production [1]. It has been suggested that IL-18 production may be induced in response to the tumor cells or other factors related to tumor growth [2]. Moreover, IL-18 has been shown to have potent antitumor effects that are mediated by inhibition of angiogenesis [3], induction of apoptosis [4] and reduction of tumorigenesis [1]. It has been reported that serum IL-18 measurement may be a useful marker for monitoring the clinical course of some malignancies including gastric [2] and colon carcinoma [5], and non-Hodgkin's lymphoma [6]. In our previous study, we showed an association between serum IL-18 levels and metastatic activity in breast cancer patients [7]. IL-18 has been shown to have an antitumor effect together with IL-12 gene transfer in a mouse bladder cancer model [8].

Nitric oxide (NO) is a potent biological molecule that participitates in the pathogenesis of cancer [9] by induction of apoptosis [10] and promotion of angiogenesis [9]. Some functions of NO in carcinogenesis are considered to be associated with its interactions with other molecules such as IL-18, IFN-γ and TNF-α [11, 12]. IL-18 has been shown to cause a marked increase in serum NO levels in an animal model [13]. It has been reported that NO synthesis may play different roles in tumor angiogenesis and tumor-induced immunosuppression in bladder carcinoma [14].

In the present study, we evaluated the significance of serum IL-18 and NO activity in bladder cancer patients with different tumor stages and tumor grades and correlated these levels with the inflammatory status of the patients by measuring serum C-reactive protein (CRP) and lactate dehydrogenase (LDH) levels.

MATERIALS AND METHODS

In this study, we analyzed serum samples from 51 patients with histologically confirmed transitional cell bladder carcinoma before surgery. After surgery, Ta disease (Ta: non-invasive papillary carcinoma) was detected in 19 patients, T1 disease (T1: tumor invasion to subepithelial connective tissue) in 17 patients and T2, T3, T4 disease (T2: tumor invasion to muscle, T3: invasion to perivesical tissue, T4: invasion to prostate, uterus, vagina, pelvic wall, abdominal wall) in 15 patients. Twelve patients had grade 1 (well-differentiated), 24 patients had grade 2 (intermediate differentiated) and 15 patients had grade 3 (undifferentiated) tumor. To eliminate the influence of other diseases, we

excluded patients with infectious diseases, diabetes mellitus, lung disease and synchronous secondary malignancies. The control group included 8 healthy subjects. Serum samples were obtained from patients and control subjects after overnight fasting and were stored at –70°C until analysis.

STATISTICAL ANALYSIS

The results are presented as means ± SD. Student't test, Mann Whitney U test and Pearson's correlation analysis were used in the statistical analysis. P values less than 0.05 were accepted as significant.

IL-18 MEASUREMENT

ELISA for determination of IL-18 in serum was performed according to a previously described method [15]. Immediately after blood sampling, serum was obtained by centrifugation at X 800 g at 4°C for 15 minutes and stored at –70°C until use. The IL-18 level in each sample was determined by using a commercially available ELISA kit, which was to be used for the *in vitro* quantitative determination of IL-18 in human serum, EDTA-plasma, cell culture supernatant, and buffered solution (BioSource International human IL-18 Colorimetric solid phase Sandwich ELISA, California, USA). Sensitivity was determined by assaying serially diluted hIL-18 Calibrator. The mean absorbance, plus 2 standard deviations for Calibrator diluted to 6.25 pg/ml was lower than the mean absorbance minus 2 standard deviations for Calibrator diluted to 12.5 pg/ml. The minimum detectable dose is therefore 12.5 pg/ml.

NITRITE AND NITRATE MEASUREMENT

Nitrite was measured using the Griess reaction and the results are given as micromoles per liter [16]. Nitrate was measured using the enzymatic one-step assay with nitrate reductase [17]. The method was based on the reduction of nitrate to nitrite by nitrate reductase in the presence of β-NADPH. We equilibrated tubes at 25^{0}C containing 250 µL of 100 mmol/L potassium phosphate buffer (pH 7.5) and 50 µL of 12 mmol/l β-NADPH with 100 µL of sample. To start the enzymatic reaction, we added 40 µL of 500 U/L nitrate reductase. We incubated the tubes in the dark for 45 minutes. The concomitant oxidation of β-NADPH was monitored by the decrease in absorbance at 340 nm. The method of standard addition was used to minimize the effect of interfering substances via serum. The results were expressed as micromoles per liter. We also used samples with internal standard, serum blanks and reagent blank.

CRP AND LDH MEASUREMENT

CRP levels were measured using Beckman-Coulter 360 Array Nefelometry. LDH activity was determined by Abbott-Aeroset autoanalyzer using original kit.

RESULTS

There were no significant differences among bladder cancer patients with different tumor stages and tumor grades, and control subjects in terms of age ($p > 0.05$) (Table 1). Serum IL-18 levels were significantly higher in bladder cancer patients when compared to the control subjects (IL-18: 754.4 ± 503.7 *versus* 167.6 ± 39.5 pg/ml, $p > 0.05$). Serum IL-18 levels were found to be higher in patients with Ta tumors (887.0 ± 720.2 pg/ml) when compared to the patients with T1 (706.7 ± 284.3 pg/ml) and T2, T3, T4 tumors (640.5 ± 326.2 pg/ml), but this was not statistically significant ($p > 0.05$) (Table 1). Individual serum IL-18 levels according to tumor stage are shown in figure 1. Serum IL-18 levels were found to be higher in patients with grade 1 tumors (858.7 ± 666.7 pg/ml) when compared to the patients with grade 2 (792.4 ± 540.5 pg/ml) and grade 3 tumors (610.2 ± 207.2 pg/ml), but this was not statistically significant ($p > 0.05$) (Table 1). Individual serum IL-18 levels according to tumor grade are shown in figure 2.

There was no significant difference in serum nitrite + nitrate levels between bladder cancer patients (51.5 ± 38.7 µmol/l) and control subjects (29.9 ± 22.6, µmol/l, $p > 0.05$). Serum nitrite + nitrate levels were no different in bladder cancer patients in terms of tumor stage and tumor grade ($p > 0.05$) (Table 1). No correlation was found between serum IL-18 and nitrite + nitrate levels in bladder cancer patients ($p > 0.05$).

Table 1
Patient characteristics

	n	Age (median) (years)	IL-18 (mean) (pg/ml)	Nitrite + nitrate (mean) (µmol/l)	CRP (mean) (mg/L)	LDH (mean) (U/L)
All patients	51	64.5 (27-85)	754.4 ± 503.7	51.5 ± 38.7	8.5 ± 15.7	97.7 ± 47.1
Control subjects	8	61.0 (55-65)	167.6 ± 39.5*	29.9 ± 22.6	4.2 ± 1.6	74.7 ± 31.1
Tumor stage						
Ta	19	63.0 (27-75)	887.0 ± 720.2	49.0 ± 35.1	4.4 ± 5.3	92.0 ± 34.9
T1	17	64.0 (46-85)	706.7 ± 284.3	65.0 ± 46.9	13.4 ± 20.7	106.3 ± 67.7
T2, T3, T4	15	65.0 (48-78)	640.5 ± 326.2	39.4 ± 29.6	8.1 ± 15.1	95.2 ± 31.1
Grade						
1	12	59.0 (27-75)	858.7 ± 666.7	46.4 ± 130.5	3.8 ± 5.4	99.0 ± 41.6
2	24	63.5 (46-85)	792.4 ± 540.5	59.8 ± 44.0	11.2 ± 17.6	97.2 ± 58.4
3	15	68.0 (48-77)	610.2 ± 207.2	42.39 ± 34.8	8.1 ± 15.6	97.5 ± 30.8

*$p < 0.05$

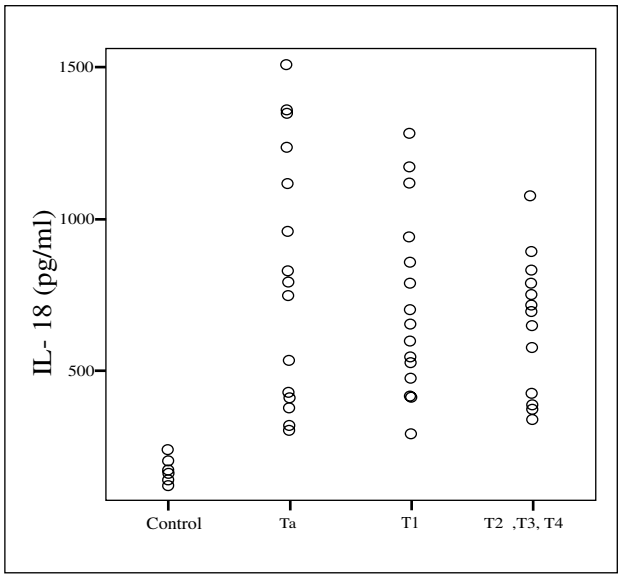

Figure 1
Individual serum IL-18 levels in patients with different tumor stages and controls.

No significant difference was observed in serum CRP and LDH levels between bladder cancer pateints and healthy controls ($p > 0.05$, Table 1). These levels were not correlated with serum levels of IL-18 and nitrate + nitrite ($P < 0.05$).

DISCUSSION

IL-18 is a multifunctional cytokine that was originally described as IFN-γ - inducing factor based on its ability to induce IFN-γ secretion by natural killer (NK) and Tcells [1, 18]. IL-18 is produced by activated macrophages, Kupffer cells, keratinocytes, intestinal epithelial cells and osteoblasts as a biologically inactive form that is cleaved by interleukin-1β - converting enzyme to generate the active form [19]. IL-18 also has other important functions including enhancing the cytotoxic activity of NK cells, proliferation of T cells and inducing secretion of granulocyte-macrophage colony stimulating factor from NK and T cells [19, 20]. IL-18 has been reported to have antitumor effects, which are mediated by inhibition of angiogenesis [3], reduction of tumorigenesis [1] and induction of apoptosis [4].

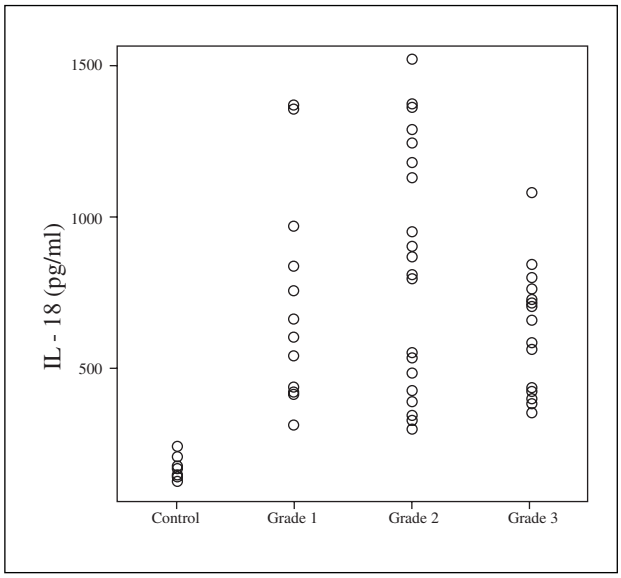

Figure 2
Individual serum IL-18 levels in patients with different tumor grades and controls.

Recently, we reported that serum IL-18 levels were significantly higher in metastatic breast cancer patients when compared to nonmetastatic patients [7]. It has been suggested that the preoperative serum IL-18 levels may have a prognostic importance in gastric cancer, and the patients in each stage, with high serum IL-18 levels, experienced poorer survival than patients with lower levels [2]. Pages *et al.* [5] reported that a decrease in IL-18 production may be an early event in tumor progression of colon cancer. Yamanaka *et al.* [8] reported that IL-18 has an antitumor together with IL-12 gene transfer in a mouse bladder tumor. It has been suggested that expression of IL-18 in the urine after Bacillus Calmette-Guerin (BCG) therapy in superficial bladder cancer patients is a useful marker for predicting freedom from disease [21].

In this study, we found significantly higher serum IL-18 levels in bladder carcinoma patients when compared to the healthy controls. These elevated serum IL-18 levels in bladder carcinoma patients may be a result of host defence mechanisms to prevent tumor growth and progression. At the present time, we do not know the source of increased serum IL-18 levels in bladder carcinoma. This issue can be clarified in future studies, which should comprise measurements of IL-18 in urine and the determination of expression of IL-18 in tumor tissue and normal uroepithelium by immunohistochemistiry in bladder carcinoma patients. However, it is generally believed that IL-18 production is induced in response to the tumor cells or other factors related to tumor development possible from the tumor associated macrophages. In our study, serum IL-18 levels were not found to be correlated with other inflammatory markers (CRP and LDH).

It has been reported that immune responses decrease with the progression of tumor growth in bladder carcinoma [22, 23] and profoundly depressed, cell-mediated immunity responses were observed in advanced stage bladder carcinoma patients [22, 24]. Shapiro *et al.* [25] reported that IFN-γ production was lower in patients with Stage C or D bladder cancer than in those with Stage A or B. Ikemoto *et al.* [26] reported that the IFN-γ producing capacity was normal in early stage bladder cancer patients but depressed in late stage cancer patients. In our study, despite statistical non-significance, patients with Ta tumors showed higher serum IL-18 levels when compared to the patients with T1 or T2, T3, T4 tumors. Moreover, higher serum IL-18 levels were observed in patients with grade 1 tumors when compared to the patients with higher grades, but this was also not statistically significant. These relatively lower serum IL-18 levels in patients with more invasive and higher grade tumors may be explained by an impairment of immunological responses in these patients, in accord with previous reports mentioned above. The decreased levels of IFN-γ in patients with advanced stage bladder cancer in Shapiro's and Ikemoto's studies [25, 26], may be related to the lower production of IFN-γ - inducing factor, «IL-18».

The lack of statistical significance of our results may be due to the small number of patients within each group.

In the literature, there were some conflicting results in regard to IL-18 serum levels. One of the study on this issue [27] revealed that some specific circulating inhibitory factors, such as soluble reseptors and binding proteins that were present in serum samples may interfere with the IL-18 assay. However, in the current study, our main purpose was to evaluate IL-18 levels in bladder cancer patients as regards to well-known pathological prognostic factors.

NO is an important bioactive agent that is involved in a variety of biological functions such as vasodilatation, host defence and carcinogenesis [9, 28, 29]. NO has both promoting and inhibitory effects on carcinogenesis, such as induction of apoptosis [10] and promotion of angiogenesis [9]. Some specific functions of NO in tumor biology are thought to be related to its interactions with other molecules including IFN-γ and IL-18 [11, 12]. Chikano *et al.* [11] reported that changes in serum IFN-γ levels were positively correlated with NO after induction of IL-18. It has been reported that increased production of NO in malignant bladder epithelium may play different roles in carcinogenesis [14]. It was suggested that the generation of NO may cause cytotoxicity as a result of an immune defence mechanism and it may also promote tumor growth by increasing tumor blood flow and angiogenesis in bladder cancer [14].

In this study, serum nitrite + nitrate levels were measured as an index of NO generation [30]. We found no significant difference in serum nitrite + nitrate levels between bladder cancer patients and healthy controls. Serum nitrite + nitrate levels were similar in bladder cancer patients with different tumor stages and grades. Moreover, there was no correlation between serum nitrite + nitrate and IL-18 levels in these patients.

In conclusion, we have shown that bladder cancer patients had significantly higher serum IL-18 levels than healthy controls. Despite statistical non-significance, patients with more invasive and higher grade tumors showed lower serum levels of IL-18 than other patients. This may be related to decreased immunological responses of these patients. Serum NO activity was not found to be different between bladder cancer patients and healthy controls. Serum IL-18 levels may reflect the status of the host defence mechanisms against tumor growth in bladder cancer. However, larger studies are needed to clarify this suggestion.

REFERENCES

1. Fukumoto H, Nishio M, Nishio K, Heike Y, Arioka H, Kurokawa H, *et al.* 1997. Interferon-gamma inducing factor gene transection into Lewis lung carcinoma cells reduces tumorigenicity *in vivo*. *Jpn J. Cancer Res.* 88: 501.

2. Kawabata T, Ichikura T, Majima T, Seki S, Chochi K, Takayama E, Hiraide H, Mochizuki H. 2001. Preoperative serum interleukin-18 level as a postoperative prognostic marker in patients with gastric carcinoma. *Cancer* 92: 2050.

3. Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE. 2000. Evidence of IL-18 as a novel angiogenic mediator. *J. Immunol.* 167: 1644.

4. Ohtsuki T, Micallef MJ, Kohno K, Tanimoto T, Ikeda M, Kurimoto M. 1997. Interleukin-18 enhances Fas ligand expression and induces apoptosis in Fas-expressing human myelomonocytic KG-1 cells. *Anticancer Res*. 17: 3253.

5. Pages F, Berger A, Henglein B, Piqueras B, Danel C, Zinzindohoue F, *et al.* 1999. Modulation of interleukin-18 expression in human colon carcinoma: consequences for tumor immune surveillance. *Int. J. Cancer* 84: 326.

6. Takubo T, Kumura T, Nakao T, Nakamae H, Aoyama Y, Nishiki S, *et al.* 2000. Comperative study on complete remission rate and overall survival in three groups classified based on the seum interleukin-18 level in non-Hodgkin's lymphoma patients. *Acta Haematol*. 104: 220.

7. Günel N, Coşkun U, Sancak B, Günel U, Hasdemir O, Bozkurt S. 2002. Clinical importance of serum interleukin-18 and nitric oxide activities in breast carcinoma patients. *Cancer*. 95: 663.

8. Yamanaka K, Hara I, Nagai H, Miyake H, Gohji K, Micallef MJ, *et al.* 1999. Synergistic antitumor effects of interleukin-12 gene transfer and systemic administration of interleukin-18 in a mouse bladder cancer model. *Cancer Immunol. Immunother.* 48: 297.

9. Ohshima H, Barthch H. 1994. Chronic infections and inflamatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. (Review) *Mutat. Res.* 305: 253.

10. Cui S, Reichner JS, Mateo RB, Albino JE. 1994. (Review) Activated murine macrophage induce apoptosis in tumour cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res*. 54: 2462.

11. Chikano S, Sawada K, Shimoyama T, Kashiwamura SI, Sugihara A, Sekikawa K, *et al.* 2000. IL-18 and IL-12 induce intestinal inflamation and fatty liver in mice in an IFN-gamma dependent manner. *Gut* 47: 779.

12. Brennan PA, Palacios-Callender M, Zaki GA, Spedding AV, Langdon JD. 2001. Type II nitric oxide synthase (NOS2) expression corralates with lymph node status in oral squamous cell carcinoma. *J. Oral. Pathol. Med.* 30: 129.

13. Golab J. 2000. Interleukin 18- interferon gamma inducing factor- a novel player in tumour immunotherapy ? (Review) *Cytokine* 12: 332.

14. Klotz T, Bloch W, Jacobs G, Niggemann S, Engelmann U, Addicks K. 1999. Immunolocalization of inducible and constitutive nitric oxide synthases in human bladder cancer. *Urology* 54: 416.

15. Taniguchi M, Nagaoka K, Kunikata T, Kayano T, Yamauchi H, Nakamura S, *et al.* 1997. Characterization of antihuman interleukin-18 (IL-18) interferon-gamma-inducing factor (IGIF) mononclonal antibodies and their application in the measurement of human IL-18 by ELISA. *J. Immunol. Methods* 206: 107.

16. Green LC, Wagner DA, Glogowski J, Skipper PL, Whisnok JS, Tannenbaum SR. 1982. Analysis of nitrate, nitrite and (15N) nitrate in biological fluids. *Anal. Biochem*. 121: 131.

17. Bories PN, Bories C. 1995. Nitrate determination in biological fluids by an enzymatic one-step assay with nitrate reductase. *Clin. Chem*. 41: 904.

18. Okamura H, Nagata K, Komatsu T, Tanimoto T, Nukata Y, Tanabe F, *et al.* 1995. A novel costimulatory factor for g interferon induction found in livers of mice causes endotoxic shock. *Infect. Immun*. 63: 3966.

19. Okamura H, Tsutsui H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, *et al.* 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378: 88.

20. Micallef MJ, Ohtsuki T, Kohno K, Tanabe F, Ushio S, Namba M, *et al.* 1996. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur. J. Immunol*. 26: 1647.

21. Thalmann GN, Sermier A, Rentsch C, Möhrle K, Cecchini MG, Studer UE. 2000. Urinary interleukin 8 and 18 predict the response of superficial bladder cancer to intravesical therapy with bacillus Calmette-Guerin. *J. Urol.* 164: 2129.

22. Catalona WJ, Chretien PB. 1973. Correlation among host immunocompetence and tumor stage, tumor grade and vascular permeation in transitional carcinoma. *J. Urol.* 110: 526.

23. O'Toole C, Perlmann P, Unsgaard B, Moberger G, Edsmyer F. 1972. Cellular immunity to human urinary bladder carcinoma. I. Correlation to clinical stage and radiotherapy. *Int. J. Cancer* 10: 77.

24. Olsson CA, Rao CN, Menzoian JO, Byrd WE. 1972. Immunologic unreactivity in bladder cancer patients. *J. Urol.* 107: 607.

25. Shapiro A, Ratliff TL, Kelley DR, Catolona WJ. 1984. Heightened interferon-γ production by mononuclear cells from bladder cancer patients. *Cancer Res.* 44: 3140.

26. Ikemoto S, Kishimoto T, Wada S, Nishio S, Maekawa M. Clinical studies on cell-mediated immunity in patients with urinary bladder carcinoma: blastogenic response, interleukin-2 production and interferon-γ production of lymphocytes. 1990. *Br. J. Urol.* 65: 333.

27. Bresnihan B, Roux-Lombard P, Murphy E, Kane D, FitzGerald O, Dayer JM. 2002. Serum interleukin 18 and interleukin 18 binding protein in rheumatoid arthiritis. *Ann. Rheum. Dis.* 61: 726.

28. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. 1995. Nitric oxide synthase activity in human breast cancer. *Br. J. Cancer* 72: 41.

29. Coşkun U, Ozenirler S, Sancak B, Bukan N. 2001. Serum and ascitic fluid nitrate levels in patients with cirrhosis. *Clin. Chim. Acta* 306: 127.

30. Langrehr JM, Murase N, Markus PM, Cai X, Neuhaus P, Schraut W, *et al.* 1992. Nitric oxide production in host-*versus*-graft and graft-*versus*-host reactions in the rat. *J. Clin. Invest.* 90: 679.