Effect of surgical treatment on the cellular immune response of gastric cancer patients

C. Barbieri[1], M.M. Fujisawa[1], C.L. Yasuda[1], I.L. Metze[1], E.C. Oliveira[2], L.M.B. Santos[2], L.R. Lopes[1] and N.A. Andreollo[1]

[1]Departamento de Cirurgia, Faculdade de Ciências Médicas, and
[2]Unidade de Neuroimunologia, Departamento de Microbiologia e Imunologia, Universidade Estadual de Campinas, Campinas, SP, Brasil

Abstract

Correspondence

N.A. Andreollo
Rua Francisco Hummberto Zuppi, 1234
13083-350 Campinas, SP
Brasil
E-mail: nandreollo@hotmail.com

Patients with gastric cancer have a variety of immunological abnormalities. In the present study the lymphocytes and their subsets were determined in the peripheral blood of patients with gastric cancer (N = 41) both before and after surgical treatment. The percent of helper/inducer CD4 T cells (43.6 ± 8.9) was not different after tumor resection (43.6 ± 8.2). The percent of the cytotoxic CD8+ T cell population decreased significantly, whether patients were treated surgically (27.2 ± 5.8%, N = 20) or not (27.3 ± 7.3%, N = 20) compared to individuals with inflammatory disease (30.9 ± 7.5%) or to healthy individuals (33.2 ± 7.6%). The CD4/CD8 ratio consequently increased in the group of cancer patients. The peripheral blood lymphocytes of gastric cancer patients showed reduced responsiveness to mitogens. The defective blastogenic response of the lymphocytes was not associated with the production of transforming growth factor beta (TGF-ß) since the patients with cancer had reduced production of TGF-ß1 (269 ± 239 pg/ml, N = 20) in comparison to the normal individuals (884 ± 175 pg/ml, N = 20). These results indicate that the immune response of gastric cancer patients was not significantly modified by surgical treatment when evaluated four weeks after surgery and that the immunosuppression observed was not due to an increase in TGF-ß1 production by peripheral leukocytes.

Introduction

The incidence of gastric cancer has been increasing in many countries, except in Japan and a few other countries where the health care system has implemented effective strategies for the early diagnosis of the disease (1). Many gastric cancer patients have a variable degree of immunological impairment, including decreased cellular immunity (2,3). Immunity to cancer is known to be affected by the function of the lymphocyte subsets, consisting of helper/inducer CD4 T cells and cytotoxic/suppressor CD8+ T cells. Patients with malignant disease present poor *in vitro* lymphocyte transformation that is usually associated with a poorer prognosis of the disease (4). The reduction in proliferative response is associated with the increase of cytokines with immunosuppressive effects such as transforming growth factor beta (TGF-ß).

TGF-ß are multifunctional polypeptides that influence the growth and differentiation of cells, as well as their adhesion, migration, angiogenesis, extracellular matrix formation and immune functions (5). These polypeptides can have either positive or negative effects on *in vitro* cell proliferation depending on cell type, epithelial origin and immune systems. TGF-ß is an immunosuppressive cytokine which inhibits T and B cell proliferation (6), natural killer cell cytotoxic activity, and the generation of T cell cytotoxicity (7,8). Elevated levels of TGF-ß1 have been reported in breast (9), brain (10) and pancreatic (11) cancers and this overexpression due to its immunosuppressive effect has been associated with the progression of the disease.

The present study was conducted to examine the quantitative changes in the subsets of lymphocytes and the level of TGF-ß in the proliferative response of gastric cancer patients on two occasions, i.e., before and after surgical treatment.

Material and Methods

Subjects

Forty-one patients with advanced stomach cancer (27 males and 14 females, median age 60.6 years, range 37-76 years) and 20 controls (with duodenal ulcers or reflux gastritis, 12 males and 8 females, median age 50.7, range 21-70 years) were studied. All patients were hospitalized in the Department of Surgery, Campinas University Hospital, Campinas, SP, Brazil, during the period from March to November, 1998. Twenty patients underwent extended radical gastrectomy and lymphadenectomy D2, and 21 patients, whose condition prevented resection, received palliative treatment. Criteria for inclusion in the study were histologically proven adenocarcinoma of the stomach, no previous therapy, no liver metastasis, peritoneal carcinomatosis or ascites. Cancer staging was determined according to the TNM staging groups approved by the International Union Against Cancer (12). The study was approved by the Ethics Committee of Universidade Estadual de Campinas, UNICAMP, and the volunteers gave written informed consent to participate in the study.

Measurement of the quantity of lymphocytes and their subsets

Three milliliters of peripheral venous blood was collected into EDTA tubes and analyzed with a CELL DYN 1700 counter. The white blood cells in the sample were counted immediately using an automatic blood cell counter and the result is reported both as a percent and an absolute number. Lymphocytes and their subsets were identified with a FACScan (BD Biosciences, San Diego, CA, USA) using fluorescent monoclonal antibodies and flow cytometry. Monoclonal antibodies, anti-CD3, T cell, anti-CD4, helper/inducer and anti-CD8, suppressor/cytotoxic cells were used. Before counting, the lymphocytes were gated among the white blood cells, with lymphocyte size being used to distinguish them from neutrophils and monocytes.

Mitogen-induced blastogenesis

Peripheral blood lymphocytes were isolated from heparinized blood by centrifugation on a Ficoll-Hypaque density gradient. The cells were suspended (2×10^5 cells/well) in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% AB human serum, 0.125% gentamicin and 1% glutamine. Cell suspensions (200 µl) supplemented with concanavalin A (10 µg/ml) were cultured in 96-well microplates at 37ºC in a humidified atmosphere of 5% CO_2 in air. After 72 h of culture, 1 µCi [³H]-thymidine (New England Nuclear, Boston, MA, USA) was added to each well. After an additional 12-h incubation, [³H]-thymidine uptake was determined using a liquid scintillation spectrometer (Beckman Coulter Inc., San Jose, CA, USA).

Capture ELISA for cytokine quantification (13)

TGF-ß antibody (polyclonal antibody obtained from R&D Systems, Minneapolis, MN, USA; 1 µg/ml in PBS, pH 7.4) was added to 96-well microtiter plates (Immulon I, Nunc, Roskilde, Denmark). After overnight incubation at 4ºC, the plates were washed three times with ELISA washing buffer (PBS containing 0.05% Tween 20 and 0.001% Thimerosal) and blocked for l h with ELISA diluent (PPB containing 0.05% Tween 20 and l% BSA). The plates were washed three times with washing buffer and 100 µl of standard, control, or sample blood was added to duplicate wells for overnight incubation at 4ºC. The plates were again washed three times with washing buffer and incubated for l h at room temperature with 1 µg/ml TGF-ß monoclonal antibody (Genzyme Diagnostics, Cambridge, MA, USA) in ELISA buffer. The plates were then washed three times with ELISA washing buffer and incubated for an additional hour with 1:2000 biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA). The avidin-peroxidase complex and the substrate were then added. Orthophenylene diamine (Sigma) prepared at 0.5 mg/ml in 50 mM hydrogen peroxide was added and left to stand 30 min at room temperature and the plates were read at 492 nm.

Statistical analysis

The statistical significance of the results was determined by the Student *t*-test and the Friedman and Kruskal-Wallis test. A P value smaller than 0.05 was considered to be significant.

Results

Lymphocytes and their subsets

Lymphocyte subsets were quantified in normal control individuals, in patients with gastric cancer both before and after surgical treatment, and in patients with inflammatory non-neoplastic disease. Single fluorescence analysis was performed with a flow cytometer after gating the lymphocyte populations. The results for the T cell subpopulations are reported as percent of lymphocytes.

There was no significant difference in white blood cell count between the controls and the patients with advanced gastric cancer prior to surgery. However, after surgery, the number of white blood cells increased.

No significant changes were observed in the number of CD4+ T lymphocytes, although CD8+ T cells (in absolute number and in percent) were decreased after surgery (P<0.05; Table 1).

Mitogen-induced blastogenesis

Changes in the proliferative response of lymphocytes were used to evaluate cellular immune responses, as shown in Figure 1. Concanavalin A-induced blastogenesis significantly decreased in gastric cancer patients before surgery and in the non-resectable group compared to normal controls (non-resectable, 5830 ± 6320 cpm; preoperative, 9176 ± 4342 cpm *vs* normal, 45,000 ± 8000 cpm; P<0.001). However, four weeks after surgical treatment, no statistically significant differences were demonstrable (preop-

Table 1. Subsets of T lymphocytes in peripheral blood cells of patients with gastric cancer before and after surgical treatment and in patients with inflammatory disease.

	Normal individuals (N = 20)	Preoperative patients (N = 20)	Postoperative patients (N = 20)	Inflammatory disease group (N = 21)
Leukocytes (10³)	6.7 ± 1.9	6.03 ± 1.7	6.5 ± 2.1	6.8 ± 3.1
%Lymphocytes	34.3 ± 8.3	29.9 ± 5.46	30.5 ± 9.23	29.0 ± 11.8
%CD3	27.8 ± 7.9	25.3 ± 8.9	24.8 ± 8.5	27.9 ± 12.0
%CD4	41.6 ± 8.2	43.6 ± 8.9	43.1 ± 8.2	39.5 ± 10.3
%CD8	33.2 ± 7.6	27.2 ± 5.8*	27.3 ± 7.3*	30.9 ± 7.5
CD4/CD8	1.28 ± 0.54	1.69 ± 0.63	1.75 ± 0.76	1.52 ± 0.91

The T cell subpopulations were quantified before surgery and 20 days after treatment using monoclonal antibodies and flow cytometry.
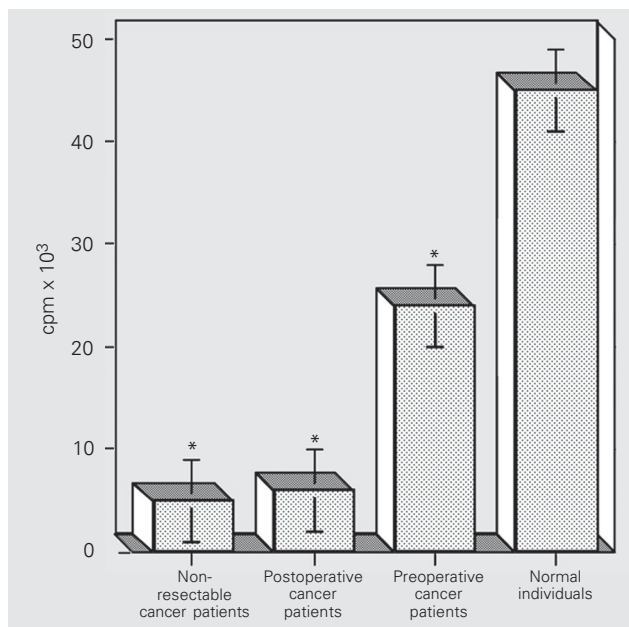*P<0.05 compared to normal individuals and the inflammatory disease group (Student *t*-test).

Figure 1. Proliferative response of lymphocytes from gastric cancer patients and normal controls. Peripheral blood lymphocytes were cultured for 48 h after stimulation with concanavalin A (10 µg/ml). Blast transformation was evaluated by [³H]-thymidine incorporation. *P<0.05 compared to normal individuals (Kruskal-Wallis test).
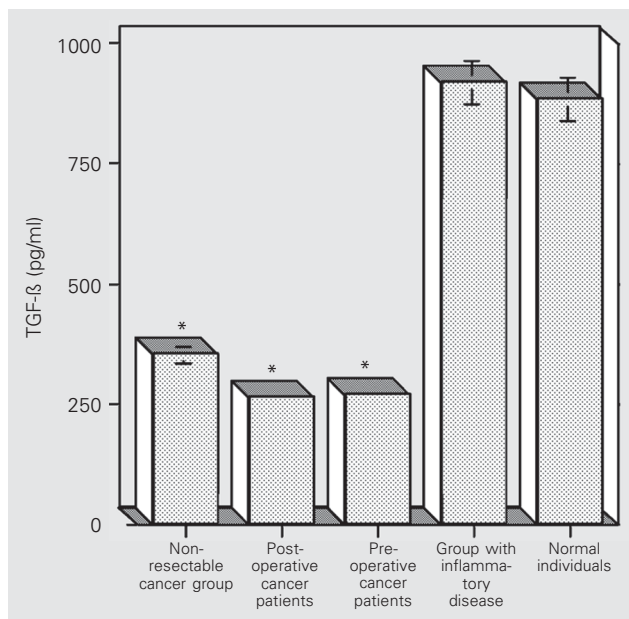

Figure 2. Transforming growth factor (TGF-ß) production by peripheral blood leukocytes from gastric cancer patients before and after surgical treatment, normal individuals, and patients with non-neoplastic disease, previously stimulated with concanavalin A (10 µg/ml) and quantified in the supernatant by ELISA. *P<0.05 compared to normal individuals and the inflammatory disease group (Student *t*-test) and P>0.05 comparing preoperative cancer patients to postoperative cancer patients (Friedman test).

erative, 9176 ± 4342 cpm *vs* postoperative, 6870 ± 2020 cpm).

Production of TGF-ß by mononuclear cells

Concanavalin A stimulation of cells for 72 h was followed by TGF-ß quantification. The mean TGF-ß production by mononuclear cells from patients was 269 ± 239 pg/ml before surgical treatment *vs* 265 ± 151 pg/ml after surgical treatment; patients with non-resectable tumors produced 354 ± 142 pg/ml. The differences were not statistically significant. Peripheral blood cells from patients with inflammatory disease produced 921 ± 229 *vs* 884 ± 175 pg/ml for normal individuals, again no significant difference was demonstrable between groups. However, there was a significant decrease in TGF-ß production by the group of patients with gastric cancer both before and after surgical treatment (P<0.01; Figure 2).

Effect of TGF-ß on the proliferative response

To determine if the suppression of the proliferative response was due to the production of TGF-ß, a neutralizing TGF-ß antibody was added to the culture (25 µg/ml), and the proliferative response was evaluated. The presence of an anti-TGF-ß monoclonal antibody in the culture did not restore the proliferative response to concanavalin A of patients with gastric cancer (Figure 3), indicating that TGF-ß was not involved in the suppression of the proliferative response.

Discussion

We studied the immune response of patients with gastric cancer both before and after surgery; the subpopulation of T lymphocytes was quantified, and the proliferative response of the lymphocytes, as well as the levels of TGF-ß were investigated.

The percent of suppressor/cytotoxic T cells (CD8) was significantly lower for both

preoperative and postoperative patients, whereas the percent of helper/inducer (CD4) cells was comparable to that of the normal control and inflammatory disease groups. Thus, the CD4/CD8 ratio increased in patients with gastric cancer. Note that the increase in the CD4/CD8 ratio contradicts data obtained in a previous study (3). Various investigators have not detected significant modifications of CD8+ lymphocyte populations in gastric cancer patients. The present data show a reduction in this cell population, which indicates an important deficiency in cell immunity since the CD8+ T cell population is directly involved in combating cancer cells. It has been reported that major histocompatibility complex class I-restricted CD8+ T cells from gastric cancer patients react specifically with autologous tumor cells (14-16). Moreover, adoptively transferred, tumor-specific CD8+ T cells induce the regression of micrometastases (17). Therefore, the reduction in the number of CD8+ T cells in the peripheral blood of cancer patients indicates a severe deficiency in the defense of the organism against the growth of tumor cells.

In addition to the quantitative changes in the number of T lymphocyte subsets, the functional state of these cells was investigated. As previously reported, patients with gastric cancer had a significant decrease in lymphocyte proliferative response (4), and we demonstrated that this response did not improve significantly after surgery. Immunosuppressive cytokines produced by leukocytes such as TGF-ß are associated with the inhibition of the proliferative response of lymphocytes. TGF-ß overexpression has also been associated with the progression of the various types of cancer, as well as with the decreased survival of cancer patients. This suggests that the characteristics of TGF-ß allow these polypeptides to enhance cancer cell growth. Prostatic cancer accompanied by the overproduction of TGF-ß1 exhibits faster growth and more extensive metastases

than identical tumor cells without TGF-ß1 expression.

In the present study, when the level of TGF-ß produced by peripheral blood leukocytes from cancer patients was quantified, it was possible to observe that cytokines were present at much lower levels in comparison with the control group. Although monocytes have been considered to be the principal source of TGF-ß in human peripheral blood (18), stimulated lymphocytes are also an important source of TGF-ß (6). Interestingly, TGF-ß has been reported to promote the growth of CD8+ T cells (19-22); this observation may explain the reduction of the CD8+ T cell population in gastric cancer patients. To emphasize that the decrease in the proliferative response of the lymphocytes was not exclusively due to the increase in TGF-ß production, we also demonstrated that exogenous addition of anti-TGF-ß monoclonal antibodies slightly increases the proliferative response of lymphocytes, although it does not block the observed suppression.
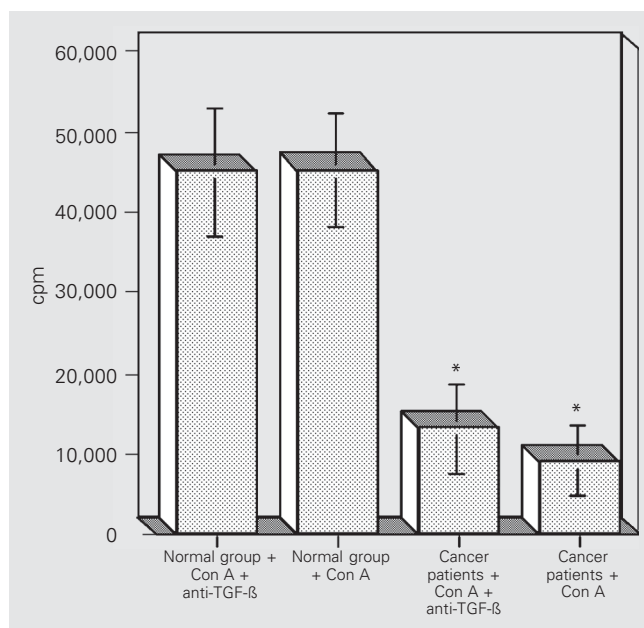

Figure 3. Proliferative response of peripheral blood lymphocytes from gastric cancer patients stimulated with concanavalin A (Con A) in the presence and absence of anti-TGF-ß antibody. *P<0.05 comparing normal group + Con A + anti-TGF-ß to cancer patients + Con A + anti-TGF-ß and normal group + Con A to cancer patients + Con A (Student *t*-test).

Although some investigators have shown elevated serum levels of TGF-ß in gastric cancer patients (22,23), our data are in agreement with others who suggested that the effect of TGF-ß in gastric cancer is concentrated at the site of tumor growth, and production of TGF-ß in the tumor does not contribute to the total amount of TGF-ß in the blood circulation (24). It is known that gastric tumor cells synthesize a large amount of TGF-ß (25-29) which may inhibit the local immune response, especially that of intratumoral lymphocytes. Inasmuch as gastric cancer cells are often resistant to TGF-ß-mediated growth inhibition (30), the local overexpression of TGF-ß may also act via these mechanisms to enhance the growth and metastasis of this cancer *in vivo*.

Surgical resection is the treatment of choice in gastric cancer, although the majority of patients with advanced gastric cancer have a poor prognosis even after curative resection. These data show that the surgical removal of a tumor is insufficient to overcome the profound immunological depression found in patients with gastric cancer. Therefore, other treatment modalities mainly involving the modulation of the immune response must be associated with the surgical treatment against the growth of neoplastic cells.

References

1. Breaux JR, Bringaze W, Chappuis C & Cohn I (1990). Adenocarcinoma of the stomach: A review of 35 years and 1710 cases. *World Journal of Surgery*, 14: 580-586.

2. Maeta M, Shimizu N, Oka A, Kondo A, Yamashiro H, Tsujitani S, Ikeguchi M & Kaibara N (1994). Perioperative allogeneic blood transfusion exacerbates surgical stress-induced postoperative immunosuppression and has a negative effect on prognosis in patients with gastric cancer. *Journal of Surgical Oncology*, 55: 149-153.

3. Lee W-J, Chang K-J, Lee C-S & Chen KM (1994). Selective depression of T-lymphocyte subsets in gastric cancer patients - An implication of immunotherapy. *Journal of Surgical Oncology*, 55: 165-169.

4. Santos LMB, Yamada FT & Scheinberg MA (1985). Monocyte and lymphocyte interaction in patients with advanced cancer. Evidence for deficient IL-1 production. *Cancer*, 56: 1553-1558.

5. Sporn MB & Roberts AB (1992). Transforming growth factor beta: recent progress and new challenges. *Journal of Cell Biology*, 119: 1017-1021.

6. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarezmon M, Derynck R, Sporn MB & Fauci AS (1986). Production of transforming growth-factor-ß by human T lymphocytes and its potential role in the regulation of T cell growth. *Journal of Experimental Medicine*, 163: 1037-1050.

7. Rook AH, Kehrl JH, Wakefield LM, Roberts AB, Sporn MB, Burlington DB, Lane HC & Fauci AS (1986). Effects of transforming growth factor ß on the functions of natural killer cells - depressed cytolytic activity and blunting of interferon responsiveness. *Journal of Immunology*, 136: 3916-3920.

8. Ranges GE, Figari IS, Espevik T & Palladino Jr MA (1987). Inhibition of cytotoxic T cell development by transforming growth factor ß and reversal by recombinant tumor necrosis factor α. *Journal of Experimental Medicine*, 166: 991-998.

9. Gorsch SM, Memoli VA & Stukel TA (1992). Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in breast cancer. *Cancer Research*, 52: 6949-6952.

10. Johnson MD, Federspiel CF, Gold LI & Moses HL (1992). Transforming growth factor beta and transforming growth factor beta-receptor expression in human meningioma cells. *American Journal of Pathology*, 141: 633-642.

11. Friess H, Yamanaka Y, Buchler M, Ebert M, Beger HG, Gold LI & Korc M (1993). Enhanced expression of transforming growth factor ß isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology*, 105: 1846-1856.

12. Holtz J, Meyer HJ & Schmoll HJ (1989). *Gastric Carcinoma: Classification, Diagnosis, and Therapy*. Springer-Verlag, New York, NY, USA.

13. Santos LMB, Al-Sabbagh A, Londono A & Weiner HL (1994). Oral tolerance to myelin basic protein induces regulatory TGF-ß-secreting T cells in Peyer's patches of SJL mice. *Cellular Immunology*, 157: 439-447.

14. Shimizu Y, Weidmann E, Iwatsuki S, Herberman RB & Whiteside TL (1991). Characterization of human autotumor-reactive T-cell clones obtained from tumor-infiltrating lymphocytes in liver metastasis of gastric carcinoma. *Cancer Research*, 51: 6153-6162.

15. Ikeda H, Sato N, Matsuura A & Kikuchi K (1993). Analysis of T-cell receptor V region gene usage of cytotoxic T-lymphocytes and tumor-infiltrating lymphocytes derived from human autologous gastric signet-ring cell carcinomas. *Cancer Research*, 53: 3078-3084.

16. Stulle K, Vollmers HP, Marquardt P & Muller-Hermelink HK (1994). Human stomach carcinoma-specific T cells derived from the tumor-draining lymph nodes. *British Journal of Cancer*, 70: 1053-1059.

17. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB & Lotze MJ (1995). Bone marrow derived dentritic cells pulsed with synthetic tumor peptides elicit protective and therapeutic antitumor immunity. *Nature Medicine*, 1: 1297-1302.

18. Assoian RK, Fleurdelys BE, Stevenson HC, Miler PJ, Madtes DK, Raines EW, Ross R & Sporn MB (1987). Expression and secretion of type-beta transforming growth factor by activated human macrophages. *Proceedings of the National Academy of Sciences, USA,* 84: 6020-6024.

19. Lee G, Ellingsworth LR, Gillis S, Wall R & Kincade PW (1987). Beta-transforming growth factors are potential regulators of B lymphopoiesis. *Journal of Experimental Medicine*, 166: 1290-1299.

20. Gray JD, Hirokawa M, Ohtsuka K & Horwitz DA (1994). Generation of an inhibitory circuit involving CD8+ T cells, IL2 and NK cell-derived TGFß. *Journal of Immunology*, 160: 2248-2254.

21. Lee HM & Rich S (1993). Differential activation of CD8+ T cells by transforming growth factor ß1. *Journal of Immunology*, 151: 668-677.

22. Saito H, Tsujitani S, Oka S, Kondo A, Ikeguchi M, Maeta M & Kaibara N (2000). An elevated serum level of transforming growth factor-beta 1 (TGF-beta 1) significantly correlated with lymph node metastasis and poor prognosis in patients with gastric carcinoma. *Anticancer Research*, 20: 4489-4493.

23. Choi JH, Kim HC, Lim HY, Nam DK, Kim HS, Yi SY, Shim KS & Han WS (1999). Detection of transforming growth factor-alpha in the serum of gastric carcinoma patients. *Oncology*, 57: 236-241.

24. Maehara Y, Kakeji Y, Kabashima A, Emi Y, Watanabe A, Akazawa K, Baba H, Kohnoe S & Sugimachi K (1999). Role of transforming growth factor-beta 1 in invasion and metastasis in gastric carcinoma. *Journal of Clinical Oncology*, 17: 607-614.

25. Lee HM & Rich S (1991). Co-stimulation of T cell proliferation by transforming growth factor ß1. *Journal of Immunology*, 147: 1127-1133.

26. Hirayama D, Fujimori T, Satonaka K, Nakamura T, Kitazawa S, Horio M & Nagasako K (1992). Immunohistochemical study of epidermal growth factor and transforming growth factor-beta in the penetrating type of early gastric cancer. *Human Pathology*, 23: 681-685.

27. Mizoi T, Ohtani H, Miyazono K, Miyasawa M, Mastsuno S & Nagura H (1993). Immunoelectron microscopic localization of transforming growth factor beta 1 and latent transforming growth factor beta 1 binding protein in human gastrointestinal carcinomas: qualitative differences between cancer and stromal cells. *Cancer Research*, 53: 183-190.

28. Yanagihara K & Tsumuraya M (1992). Transforming growth factor beta 1 induces apoptotic cell death in cultured human gastric carcinoma cells. *Cancer Research*, 52: 4042-4045.

29. Naef M, Ishiwata Y, Friess H, Buchler MW, Gold LI & Korc M (1997). Differential localization of transforming growth factor ß isoforms in human gastric mucosa and overexpression in gastric carcinoma. *International Journal of Cancer*, 71: 131-137.

30. Park K, Kim SJ, Bang YJ, Park JG, Kim NK, Roberts AB & Sporn MB (1994). Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-beta. *Proceedings of the National Academy of Sciences, USA*, 9: 8772-8776.