

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

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## Abstract

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 ( $43.6 \pm 8.9$ ) was not different after tumor resection ( $43.6 \pm 8.2$ ). The percent of the cytotoxic CD8+ T cell population decreased significantly, whether patients were treated surgically ( $27.2 \pm 5.8\%$ , N = 20) or not ( $27.3 \pm 7.3\%$ , N = 20) compared to individuals with inflammatory disease ( $30.9 \pm 7.5\%$ ) or to healthy individuals ( $33.2 \pm 7.6\%$ ). 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- $\beta$ ) since the patients with cancer had reduced production of TGF- $\beta$ 1 ( $269 \pm 239$  pg/ml, N = 20) in comparison to the normal individuals ( $884 \pm 175$  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- $\beta$ 1 production by peripheral leukocytes.

## Key words

- Gastric cancer
- Cellular immune response
- Transforming growth factor beta
- Immunosuppression

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## 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- $\beta$ ).

TGF- $\beta$  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- $\beta$  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- $\beta$ 1 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- $\beta$  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 ( $2 \times 10^5$  cells/well) in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% AB human serum, 0.125% gentamicin and 1% glutamine. Cell suspensions (200  $\mu$ l) supplemented with concanavalin A (10  $\mu$ g/ml) were cultured in 96-well microplates at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 72 h of culture, 1  $\mu$ Ci [<sup>3</sup>H]-thymidine (New England Nuclear, Boston, MA, USA) was added to each well. After an additional 12-h incubation, [<sup>3</sup>H]-thymidine uptake was determined using a liquid scintillation spectrometer (Beckman Coulter Inc., San Jose, CA, USA).

### Capture ELISA for cytokine quantification (13)

TGF- $\beta$  antibody (polyclonal antibody obtained from R&D Systems, Minneapolis, MN, USA; 1  $\mu$ g/ml in PBS, pH 7.4) was added to 96-well microtiter plates (Immulon I, Nunc, Roskilde, Denmark). After overnight incubation at 4°C, the plates were washed three times with ELISA washing buffer (PBS containing 0.05% Tween 20 and 0.001% Thimerosal) and blocked for 1 h with ELISA diluent (PPB containing 0.05% Tween 20 and 1% BSA). The plates were washed three times with washing buffer and 100  $\mu$ l of standard, control, or sample blood was added to duplicate wells for overnight incubation at 4°C. The plates were again washed three times with washing buffer and incubated for 1 h at room temperature with 1  $\mu$ g/ml TGF- $\beta$  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<sup>+</sup> T lymphocytes, although CD8<sup>+</sup> 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  $\pm$  6320 cpm; preoperative, 9176  $\pm$  4342 cpm vs normal, 45,000  $\pm$  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 (10 <sup>3</sup> )	6.7 $\pm$ 1.9	6.03 $\pm$ 1.7	6.5 $\pm$ 2.1	6.8 $\pm$ 3.1
%Lymphocytes	34.3 $\pm$ 8.3	29.9 $\pm$ 5.46	30.5 $\pm$ 9.23	29.0 $\pm$ 11.8
%CD3	27.8 $\pm$ 7.9	25.3 $\pm$ 8.9	24.8 $\pm$ 8.5	27.9 $\pm$ 12.0
%CD4	41.6 $\pm$ 8.2	43.6 $\pm$ 8.9	43.1 $\pm$ 8.2	39.5 $\pm$ 10.3
%CD8	33.2 $\pm$ 7.6	27.2 $\pm$ 5.8*	27.3 $\pm$ 7.3*	30.9 $\pm$ 7.5
CD4/CD8	1.28 $\pm$ 0.54	1.69 $\pm$ 0.63	1.75 $\pm$ 0.76	1.52 $\pm$ 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).

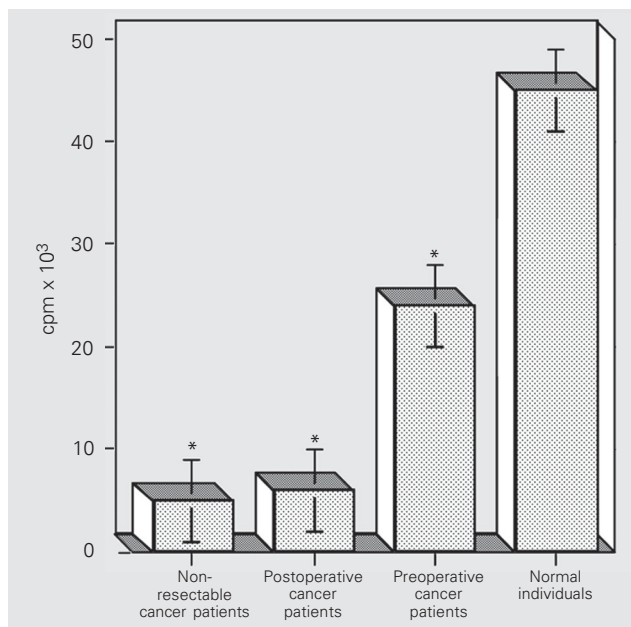


Figure 1. Proliferative response of lymphocytes from gastric cancer patients and normal controls. Peripheral blood lymphocytes were cultured for 48 h after stimulation with concanavalin A (10  $\mu$ g/ml). Blast transformation was evaluated by [<sup>3</sup>H]-thymidine incorporation. \*P<0.05 compared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

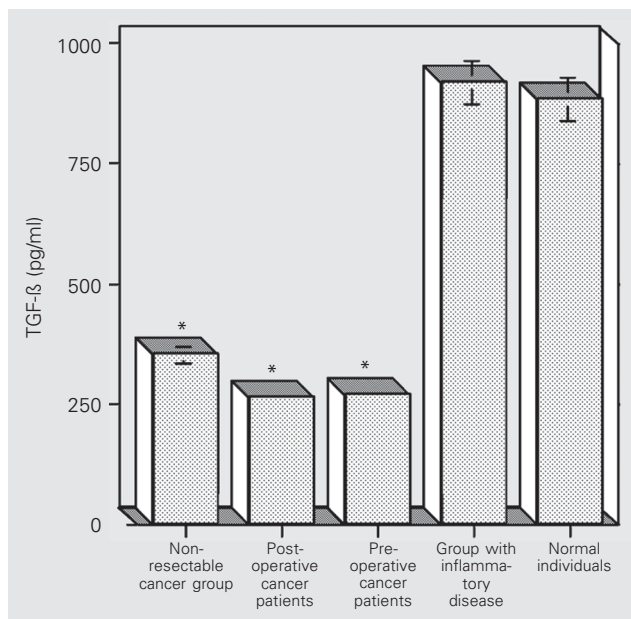


Figure 2. Transforming growth factor (TGF- $\beta$ ) production by peripheral blood leukocytes from gastric cancer patients before and after surgical treatment, normal individuals, and patients with non-neoplastic disease, previously stimulated with concanavalin A (10  $\mu$ g/ml) and quantified in the 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,  $9176 \pm 4342$  cpm vs postoperative,  $6870 \pm 2020$  cpm).

#### Production of TGF- $\beta$ by mononuclear cells

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

#### Effect of TGF- $\beta$ on the proliferative response

To determine if the suppression of the proliferative response was due to the production of TGF- $\beta$ , a neutralizing TGF- $\beta$  antibody was added to the culture (25  $\mu$ g/ml), and the proliferative response was evaluated. The presence of an anti-TGF- $\beta$  monoclonal antibody in the culture did not restore the proliferative response to concanavalin A of patients with gastric cancer (Figure 3), indicating that TGF- $\beta$  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- $\beta$  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<sup>+</sup> 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<sup>+</sup> T cell population is directly involved in combating cancer cells. It has been reported that major histocompatibility complex class I-restricted CD8<sup>+</sup> T cells from gastric cancer patients react specifically with autologous tumor cells (14-16). Moreover, adoptively transferred, tumor-specific CD8<sup>+</sup> T cells induce the regression of micrometastases (17). Therefore, the reduction in the number of CD8<sup>+</sup> 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- $\beta$  are associated with the inhibition of the proliferative response of lymphocytes. TGF- $\beta$  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- $\beta$  allow these polypeptides to enhance cancer cell growth. Prostatic cancer accompanied by the overproduction of TGF- $\beta$ 1 exhibits faster growth and more extensive metastases

than identical tumor cells without TGF- $\beta$ 1 expression.

In the present study, when the level of TGF- $\beta$  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- $\beta$  in human peripheral blood (18), stimulated lymphocytes are also an important source of TGF- $\beta$  (6). Interestingly, TGF- $\beta$  has been reported to promote the growth of CD8<sup>+</sup> T cells (19-22); this observation may explain the reduction of the CD8<sup>+</sup> 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- $\beta$  production, we also demonstrated that exogenous addition of anti-TGF- $\beta$  monoclonal antibodies slightly increases the proliferative response of lymphocytes, although it does not block the observed suppression.

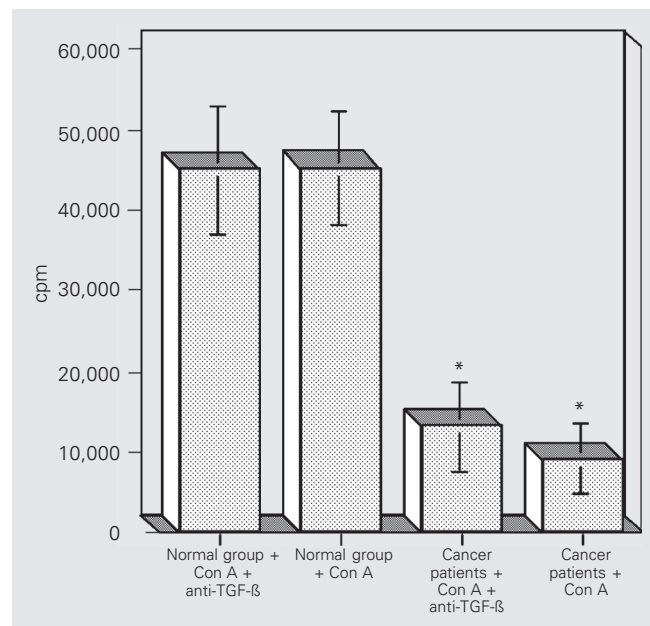


Figure 3. Proliferative response of peripheral blood lymphocytes from gastric cancer patients stimulated with concanavalin A (Con A) in the presence and absence of anti-TGF- $\beta$  antibody. \* $P < 0.05$  comparing normal group + Con A + anti-TGF- $\beta$  to cancer patients + Con A + anti-TGF- $\beta$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

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

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