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Gene Expression for Tumor Necrosis Factor α and Its Production in Gastric Cancer Patients

Masakazu Ohno,[1] Michio Kato,[2] Takeshi Nakamura[1] and Yoichi Saitoh[1]

[1]*First Department of Surgery, Kobe University School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650 and* [2]*Second Department of Surgery, Kinki University School of Medicine, 377-2 Oonohigashi, Osakasayama, Osaka 589*

To determine the gene expression for tumor necrosis factor (TNF) α and its main site of production in gastric cancer patients, serum levels in the peripheral venous blood of 50 patients and the portal blood from 15 of these 50 patients were measured by enzyme-linked immunosorbent assay (ELISA). TNF gene expression in the peripheral blood mononuclear cells (PBMC) and in the surgically resected tissues was then studied in 16 patients by reverse transcription-polymerase chain reaction (RT-PCR) assay. Whereas TNF mRNA expression was detected in the PBMC from 13 of 16 gastric cancer patients (81.3%), it was detected in only one tumor tissue (6.3%). Preoperatively, TNF was detected in the serum from 13 of 50 patients (26.0%). In the portal blood sampled immediately after laparotomy, TNF was positive in 4 of 15 patients (26.7%). TNF gene expression was much more frequently detected in PBMC than in other resected tissues, and its expression was higher than in the serum. Various clinicopathological factors for gastric cancer were not related to the preoperative detection of TNF in the serum. It appears that TNF is produced mainly in PBMC but not in the cancer regions or the regional lymph nodes of gastric cancer patients. It is suggested that TNF is not always secreted even when TNF mRNA is expressed, and its preoperative production is not related to tumor progression.

Key words: Tumor necrosis factor α — Gene expression — Gastric cancer — RT-PCR — ELISA

Tumor necrosis factor (TNF) was first detected as a substance causing the necrosis of implanted tumors[1] and it was initially thought to be a cytokine with an anti-tumor activity.[2,3] On the other hand, TNF has been considered to cause cachexia.[4] In the case of neoplasms, TNF gene expression and protein production were detected in some hematological and solid tumor (colon and breast cancer) cell lines.[5] Serum TNF protein level has been reported to be significantly higher in cancer patients than in normal individuals,[6] and TNF gene expression and protein production have been detected in tumor infiltrating leukocytes (TIL) of colorectal cancer patients,[7,8] as well as in tumor cells of renal cancer patients.[9] However, there has been no detailed report on TNF gene expression and protein production in human gastric cancers.

In order to evaluate the major site of TNF production in gastric cancer patients, we examined TNF mRNA expression in both peripheral blood mononuclear cells (PBMC) and resected tissues using reverse transcription-polymerase chain reaction (RT-PCR) assay[10] and determined the serum TNF level by enzyme-linked immunosorbent assay (ELISA).[11] We compared the serum TNF levels in gastric cancer patients with those in other types of cancer patients (colorectal cancer and pancreatic cancer) and non-cancer controls by ELISA. Furthermore, we evaluated the TNF gene expression and protein production in gastric cancer cell lines to examine the potential for TNF production. We also studied the relationship between TNF protein production and TNF gene expression, and the association between TNF production and the clinicopathological features of gastric cancer.

MATERIALS AND METHODS

Subjects This study involved 50 gastric cancer patients and other types of cancer patients (33 colorectal cancer patients and 2 pancreatic cancer patients) who had undergone surgery at Kobe University Hospital between January 1990 and December 1992. TNF gene expression was assessed by RT-PCR assay in PBMC collected preoperatively and in tissues resected during operation (tumor, regional lymph node and non-cancerous gastric mucosa) in 16 of 50 gastric cancer patients. Serum samples were collected from all cancer patients before operation and 10 healthy volunteers as controls. Portal blood from 15 gastric cancer patients was collected by inserting a catheter through the umbilical vein to the portal vein after laparotomy and the serum TNF level was determined immediately after laparotomy, as well as before and after gastrectomy. The serum TNF level was determined by ELISA.

Cell culture KATO-III and MKN-45, humn gastric cancer cell lines, were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FBS) (Sigma Chemical Co., St. Louis, MO) in a humidified atmosphere of 5% CO_2 at 37°C.

Preparation of total RNA PBMC was separated from 10 ml of heparinized blood by Ficoll-Paque (Pharmacia, Piscataway, NJ) density-gradient centrifugation.[12] Resected tissues, PBMC and gastric cancer cell lines were homogenized in a guanidine isothiocyanate (GIT) buffer and the total RNA was extracted by the guanidine/cesium chloride method.[13] One μg of the total RNA was used as the template for RT-PCR.

RT-PCR assay Complementary DNA (cDNA) was synthesized from 1 μg of the total RNA in 10 μl of reaction solution using reverse transcriptase (GIBCO-BRL, Bethesda, MD). After heat inactivation of reverse transcriptase, 2.5 units of DNA polymerase (Takara Shuzo Co., Kyoto) and specific primers for TNFα (Table I) provided by Dr. Hirose (Kureha Chemical Ind. Co., Tokyo) were added to the above reaction solution, and cDNA was amplified by PCR using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). PCR assay for TNF and β-actin was performed in 100 μl of reaction solution using 26 cycles, each consisting of 94°C for 1 min (denaturation), 55°C for 45 s (annealing), and 72°C for 2 min (primer extension). The PCR products (10 μl) were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

ELISA TNF levels in the peripheral blood and the portal blood were determined using a kit with monoclonal antibody to TNFα (Otsuka Assay Lab., Tokushima). TNF protein in supernatants of gastric cancer cell lines (KATO-III and MKN-45) were measured after 48 h culture in serum-free medium using the same kit. This ELISA kit accurately detects TNF levels from 5.0 to 1000 pg/ml. In this study, a TNF level over 5.0 pg/ml

Table I. List of Primers Used for PCR

TNFα	5′ primer: 5′-CTTCTGCCTGCTGCACTTTGGA-3′
TNFα	3′ primer: 5′-TCCCAAAGTAGACCTGCCCAGA-3′
β-Actin	5′ primer: 5′-ATGGATGATGATATCGCCGCGCGCT-3′
β-Actin	3′ primer: 5′-CGGACTCGTCATACTCCTGCTTG-3′

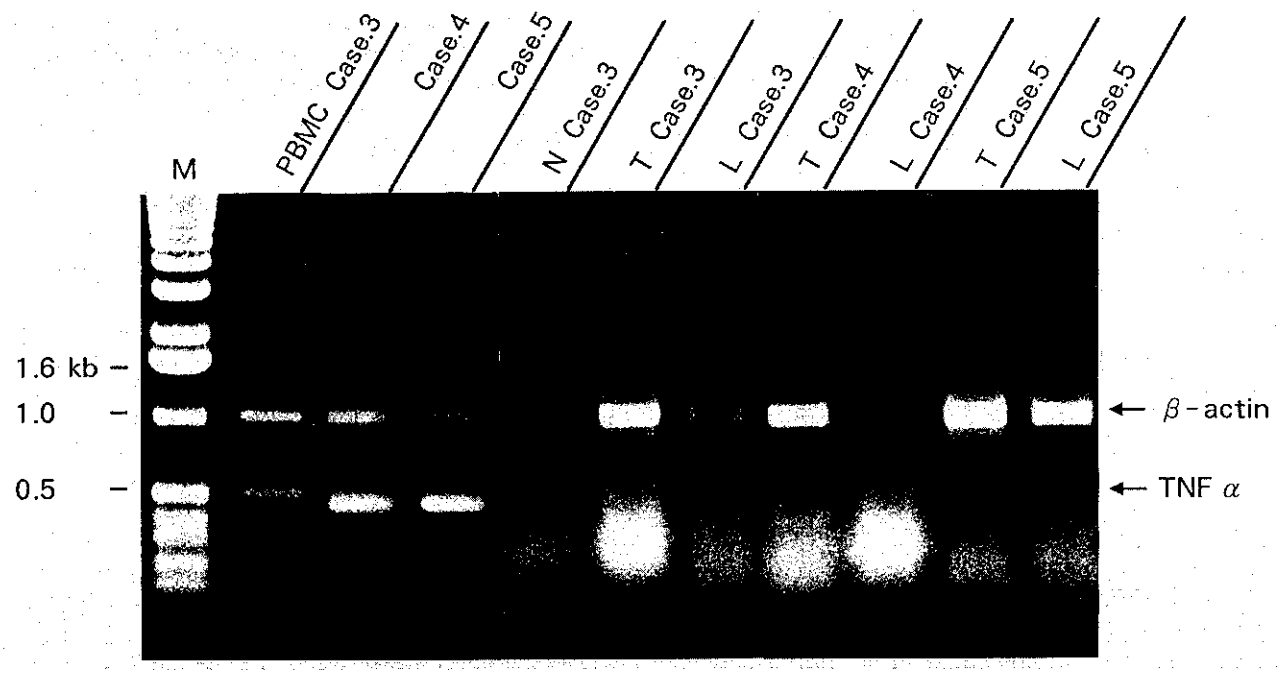

Fig. 1. TNFα mRNA expression detected by RT-PCR assay. M: size marker. N: normal mucosa. T: tumor tissue. L: lymph node.

was defined as positive and one under 5.0 pg/ml as negative.

Relationship between the serum TNF protein level and other clinicopathological factors The preoperative levels of serum tumor markers (CEA and CA19-9) and various clinicopathological factors including percentage of monocytes in PBMC, tumor size, hepatic metastasis, peritoneal dissemination, lymph node metastasis and serosal involvement were compared among patients with or without preoperative serum TNF protein.

Statistical analysis Quantitative differences between groups were statistically analyzed by means of Student's *t* test and the chi-squared test. A *P* value below 0.05 was taken as the criterion of significance.

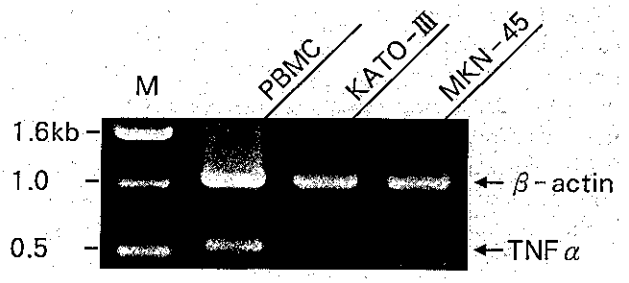

Fig. 2. TNFα mRNA expression in human gastric cancer cell lines. M: size marker. PBMC: positive control; stimulated with 1 μg/ml lipopolysaccharide for 3 h.

RESULTS

TNF mRNA expression PBMC and resected tissues (tumor, regional lymph node and non-cancerous mucosa) were collected from 16 gastric cancer patients. Following extraction of the total RNA, the expression of TNF mRNA was analyzed by RT-PCR assay using TNFα-specific primers, as shown in Table I. TNF mRNA was found preoperatively in the PBMC from 13 of 16 patients (81.3%). However, TNF mRNA expression was found in the tumor tissue from only 1 patient (6.3%) and was undetectable in the lymph nodes and non-cancerous gastric mucosa samples (Fig. 1). KATO-III and MKN-45 cell lines expressed no TNF mRNA, as assessed by RT-PCR (Fig. 2).

TNF protein production The serum TNF levels in the peripheral venous blood and portal blood were assessed by ELISA with a detection limit of 5.0 pg/ml. TNF was detected preoperatively in the serum from 13 of 50 gastric cancer patients (26.0%). We also detected serum TNF protein in 6 of 33 colorectal cancer patients (18.1%) and both of 2 pancreatic cancer patients (100%). In addition, serum TNF protein was detected in 2 of 10 healthy volunteers (20.0%) as controls (Fig. 3).

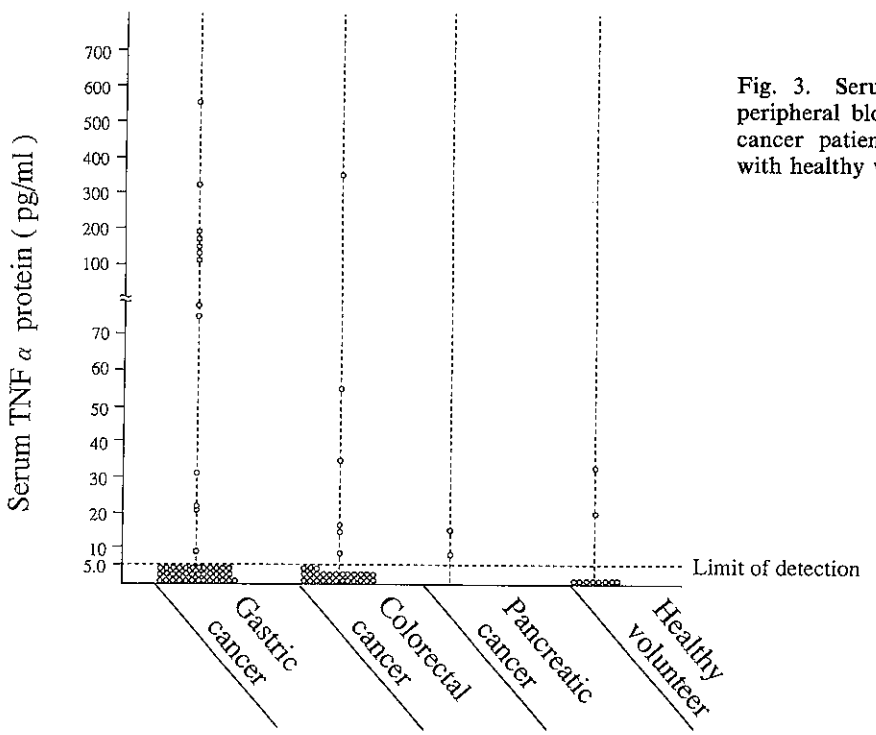

Fig. 3. Serum TNFα protein level in the peripheral blood of gastric cancer and other cancer patients before operation, compared with healthy volunteers.

In addition, TNF was positive in the portal blood from 4 of 15 gastric cancer patients (26.7%) immediately after laparotomy and no remarkable changes were found in serum levels after gastrectomy compared to the levels before (Fig. 4). TNF protein was undetectable in culture supernatant from KATO-III and MKN-45 cell lines (data not shown).

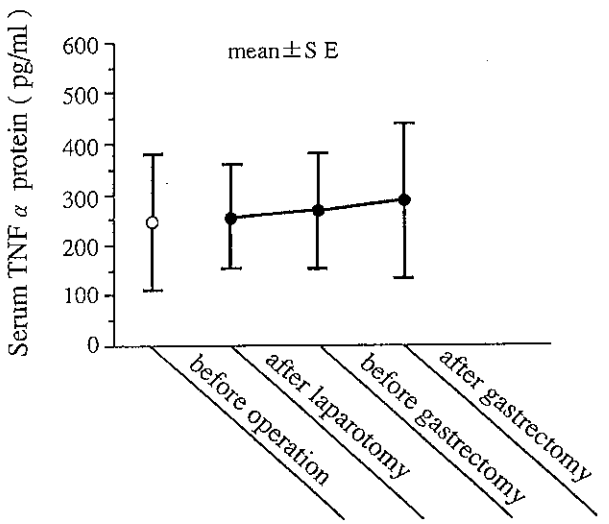

Fig. 4. Serum TNFα protein level in the portal blood of gastric cancer patients during operation. (○): peripheral venous blood. (●): portal blood.

Relationship between TNF mRNA and TNF protein The relationship between TNF mRNA expression and TNF protein production was examined in 16 gastric cancer patients. TNF mRNA expression was the highest in PBMC and was positive preoperatively in 13 of 16 patients (81.3%). Serum TNF was detected in 4 of the 13 patients (30.8%), in whom mRNA expression was positive, but was not observed in the other 3 patients who were negative for TNF mRNA expression (Table II). Thus, there was a difference between TNF mRNA expression and TNF protein production, with the positivity rate being obviously lower for protein production.

Relationship between serum TNF protein level and clinicopathological factors The relationship between the preoperative serum TNF level and clinicopathological factors was studied. The clinicopathological factors investigated preoperatively were serum CEA and CA19-9 levels, percentage of monocytes in PBMC, tumor size, hepatic metastasis, peritoneal dissemination, lymph node metastasis and serosal involvement. None of these factors was found to show any statistically significant relationship to TNF production (Table III). Thus, various clinicopathological factors relating to stage classification were not related to the serum TNF level.

Table II. Correlation between TNFα mRNA Expression and Protein Production of Gastric Cancer Patients before Operation

TNFα mRNA expression in PBMC	Number of cases	Monocytes in PBMC (%)	TNFα protein production in the peripheral venous blood	
			Positive	Negative
Positive	13	21.0±3.5	4 (30.8%)	9 (69.2%)
Negative	3	25.5±6.8	0 (0%)	3 (100%)
Total	16	22.5±3.1	4 (25.0%)	12 (75.0%)

Table III. Clinicopathological Factors in Positive and Negative Cases of TNFα Protein in the Peripheral Venous Blood of Gastric Cancer Patients before Operation

Factors	TNFα protein production in the peripheral venous blood	
	Positive cases (n=13)	Negative cases (n=37)
CEA (ng/ml)	9.0±2.7	15.9±7.6
CA19-9 (U/ml)	31.2±9.7	47.0±97.7
Monocytes in PBMC (%)	18.9±5.8	23.9±6.9
Tumor size (cm)	4.1±2.3	5.0±3.0
Hepatic metastasis	1 (7.7%)	8 (21.6%)
Peritoneal dissemination	0 (0%)	9 (24.3%)
Lymph node metastasis	6 (46.2%)	21 (56.8%)
Serosal involvement	4 (30.8%)	16 (43.2%)

DISCUSSION

With regard to TNF gene expression and protein production, it has not been established where the gene is expressed and the protein is produced or to what extent production occurs in gastric cancer patients. Balkwill *et al.* examined serum TNF protein levels in patients with seven representative tumors (ovarian cancer, breast cancer, pulmonary oat cell cancer, gastric cancer, non-Hodgkin's lymphoma and multiple myeloma), and found that TNF was positive in approximately 50% of all cancer patients, but in none of the 6 gastric cancer patients (0%).[6] However, we were able to detect serum TNF in 13 of 50 gastric cancer patients (26.0%), probably due to the higher sensitivity of our ELISA system (detection limit of 5.0 pg/ml, compared to 0.8 ng/ml in their system). The present report is the first describing TNF production in gastric cancer patients, in addition to colorectal and pancreatic cancer patients, using a highly sensitive ELISA kit. Positivity for serum TNF was as high in gastric cancer patients as in colorectal cancer patients.

TNF mRNA was expressed in the PBMC of many gastric cancer patients, but was rarely found in tumor tissues, and none was detected in regional lymph nodes or non-tumorous gastric mucosa. The TNF level in portal blood did not significantly change after gastrectomy, suggesting that the tumor tissues did not produce TNF protein in gastric cancer patients. Gastric cancer tissues seemed to be poor TNF producers, since gastric cancer cell lines (KATO-III and MKN-45) did not express TNF mRNA. Taken together, these results demonstrate that the main source of TNF production is neither the tumor tissue nor the regional lymph node, but is PBMC in gastric cancer patients.

The positivity of preoperative serum TNF was lower than TNF mRNA expression in PBMC, presumably due to a lack of correlation between TNF gene expression and protein production. It has been reported that most human tumor cells in which TNF mRNA is expressed do not release TNF into the culture medium.[5] Two different stages in the synthesis of TNF have been noted when endotoxin is administered *in vitro*.[14, 15] *In vivo* TNF expression may also be modulated similarly.

In our study, no statistically significant relationship was found between preoperative serum TNF levels and various clinicopathological factors. That is, clinicopathological factors, which are related to stage classification, have no relationship with TNF release into the peripheral venous blood. Therefore, preoperative production of TNF in gastric cancer patients was not related to parameters of tumor progression, such as tumor size or the existence of metastasis, but instead, might reflect differences in the host antitumor response.

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