Transforming Growth Factor β_1 Expression in Gastric Carcinoma

Many types of human malignant tumor have been reported to amplify transforming growth factor-β_1 (TGF-β_1) gene and overexpress its protein. However, little work has been done about the content of TGF-β_1 protein in tissue and blood of patients with malignant tumors. TGF-β_1 protein of tissue (n=29) and serum TGF-β_1 levels in patients with gastric carcinoma (n=62) were compared with those in normal subjects (n=10) using a TGF-β_1 enzyme-linked immunosorbent assay. Also, expression of TGF-β_1 mRNA (n=20) and immunohistochemical distribution of the protein (n=70) in gastric carcinoma tissues were studied. The immunohistochemial expression of TGF-β_1 protein was significanlty correlated with the tissue TGF-β_1 content ($r=0.45$: $p<0.05$). The content of TGF-β_1 was 311 ± 212 ng/g wet carcinoma tissue. TGF-β_1 mRNA was expressed in gastric carcinoma cells. However, unexpectedly serum TGF-β_1 levels in patients with gastric carcinoma were lower (97.1 ± 29.4 ng/ml) than those in normal subjects (140.3 ± 85.7 ng/ml, $P<0.05$). Our results support that the tumor cells directly produce TGF-β_1 and that semiquantitative immunohistochemical staining method for TGF-β_1 protein is a validative method for TGF-β_1 protein quantitation. *(JKMS 1997; 12 : 215~20)*

Key Words : *TGF-β_1, Gastric carcinoma*

Young-Euy Park, Young-Hee Choi, Won-Yo Lee, Kyung-Chan Choi

Department of Pathology, College of Medicine, Hallym University, Korea

Received : January 29, 1997
Accepted : April 9, 1997

Address for correspondence
Young Euy Park, M.D., Department of pathology, Chun Chon Sacred Heart Hospital, Hallym University, 153 Kyo-Dong, Chun-cheon city, Kang Won-Do 200-060, Korea
Tel : (0361) 52-9970 exc. 190, Fax : (0361) 56-4161
e-mail path@sun.hallym.ac.kr

* This work was supported by a grant from Hallym Univirsity Research Fund (1996).

INTRODUCTION

The prediction of the invasiveness and metastatic potential of a cancer at an early stage is one of the most important challenges of cancer research. A marker that can correlate with a biologic behavior such as invasiveness or metastasis will be of significant value in prognosis and targeted therapy. The development of sensitive diagnostic methods to identify patients at risk for disease progression should provide critical information to medical oncologists. Therefore, efforts are being made to identify such new biochemical markers.

Transforming growth factor-β, a 25 kDa homodimeric peptide, first discovered in an assay based on its ability to transform fibroblasts phenotypically in culture, by now has been shown to have profound effects on nearly all cell types, influencing either their proliferation, differentiation, or other aspects of their function (1, 2).

In culture, their constellation of effects is dependent on cell type, other cytokines and culture conditions (3~5). While *in vitro* studies have provided valuable information concerning the nature of TGF-β action, their relevance to *in vivo* systems is low. To understand TGF-β action *in vivo*, it is essential to be able to localize the protein in various target tissues (6).

We previously studied the immunohistochemical expression of TGF-β_1 in gastric carcinoma (7). The results suggest that the TGF-β_1 expression in the gastric carcinoma is related to the depth of invasion, the degree of invasiveness and the presence of metastasis.

Quantitative analysis of TGF-β_1 protein in gastric carcinoma tissues has not been carried out although immunohistochemical methods and Western blotting have been used to detect TGF-β_1 protein in tissues in other studies. It is also very important to know whether TGF-β_1 protein is actually overproduced in parallel with the highly expressed mRNA in the malignant tumor cells in order to understand the significance of the activated transcription of TGF-β_1 gene in malignancy (8).

The aims of this study were to investigate the correlation of immunohistochemical expression of TGF-β_1 with TGF-β_1 mRNA expression and TGF-β_1 contents in gastric carcinoma tissues.

MATERIALS AND METHODS

Gastric cancer tissues were obtained from 27 female and 43 male patients selected from the surgical pathology files of the Chun Cheon Sacred Heart Hospital of Hallym University. The median age of the gastric cancer patients was 58 years, with a range of 27 to 77 years. The sera also were obtained from the same above gastric cancer patients before gastrectomy (24 female, 38 male;

range, 32~77 years). The sera for control were obtained from 4 healthy female and 6 male individuals without gastric cancer. Freshly removed tissue samples were fixed in 10% neutral formalin for 12 to 24 h and paraffin-embedded for histological analysis. Tissues destinated for protein extraction were frozen in liquid nitrogen immediately upon surgical removal and maintained at -80℃.

According to the classification of the Korean Research Society for gastric cancer (9) there are nineteen, well differentiated tubular ; twenty-six, moderately differentiated tubular ; twelve, poorly differentiated tubular ; nine, signet ring cell ; four, mucinous carcinoma.. The clinical data included age, sex, differentiation, depth of invasion, presence of lymphatic emboli, presence of venous emboli and lymph node metastasis.

Purification of TGF-β_1 from gastric carcinoma tissues

Frozen carcinoma tissues were extracted as described (10) except that the homogenized tissue (0.5~1.5g) was stirred in the acid/ethanol solution at room temperature for 2 to 3 hr prior to centrifugation. The resulting supernatant was adjusted to pH 3.0 and protein was precipitated with ether and ethanol. The precipitate was collected by filtration and dissolved in 1M acetic acid (1 ml/g of tissue). Insoluble material was removed by centrifugation, the supernatant were analyzed in duplicate by ELISA.

Immunohistochemical staining for TGF-β_1 protein

Sections were treated with hyaluronidase (1 mg/ml ; Sigma) for 30 min at room temperature, blocked with 10% normal goat serum in phosphate-buffered saline (PBS) for 30 min, and incubated with biotinylated TGF-β_1 antibody (Genzyme 80-1835-03) overnight at 4℃. After incubation with peroxidase (DAKO LSAB kit), the substrate (LSAB kit, hydrogen peroxide) was applied for 20 min. Slides were counterstained with Mayer's hematoxylin (11). This antibody recognizes bovine, mouse and human TGF-β_1 protein. The positive control was a case of advanced gastric cancer. Normal mouse serum at the same protein concentrations as the primary antibodies was used in place of the primary antibodies as a negative control.

Evaluation of the immunohistochemical staining was done according to the reported method(12). This entails a three-step categorization according to the intensity of the staining : 0 for negative results, 1 for results in which the staining was clearly identified by x100 magnification, and 2 for results in which the staining was clearly identified by x40 magnification. Areas that showed positivity were further quantified into four levels : 0 when none of the cancer cells were stained, 1 when one third or fewer of the cancer cells were stained, 2 when two thirds or less of the cancer cells were stained, and 3 when two thirds or more of the cancer cells were stained. When total score (sum of the intensity and quantification measurements) was 4 or greater, the tumor was considered positive for TGF-β_1 protein.

In situ hybridization for TGF-β_1 mRNA

A digoxigenin-labelled single-stranded oligonucleotide probe for TGF-β_1 (R&D, Mineapolis, MN) was prepared using In Situ Workstation kit (R&D, Mineapolis, MN, BBS2) according to the instruction manual. In brief, tissues fixed in 10% neutral buffered formalin were cut into 4 μm thick sections and mounted on coated slides. After proteinase K (0.5 mg/ml) digestion, prehybridization was performed at 37℃. A hybridization solution containing 500 ng/ml of probe was applied to the section, which was then covered with cover slide and hybridized at 37℃ overnight in a moist chamber. The next day, the slides were washed briefly with 4x SSC/30% formamide, followed by 2xSSC/30% formamide. Hybridized digoxigenin-labelled probe was identified using a In Situ Hybridization Detection kit-Digoxigenin kit (R&D, Mineapolis, MN, BBS8) according to the instruction manual. Sections were mounted in glycerin and coverslips were applied. Controls included : (i) no probe (ii) RNase pretreated tissue (iii) Positive control probe (iv) negative control tissue

Twenty cases examined by in situ hybridization involved the use of the same blocks.

Enzyme linked immunosorbent assay for TGF-β_1 protein in serum and tissue extracts

Double determinant ELISA method was used (13). The microtiter plates were incubated with 50 μl of mouse monoclonal anti TGF-β_1 antibody (Genzyme 80-1835-03), as the capturer antibody, at a concentration of 1.2 μg/ml in PBS at 4℃ overnight. The wells were washed three times with PBS-0.05% Tween-20, as well as subsequent washings between incubations with different reagents. The wells were blocked with PBS-0.5% gelatin solution for 2 h at 37℃ incubator and then washed again. Dilutions of sera and tissue extracts were acidified by 6 M hydrochloric acid and neutralized with 6 N sodium hydroxide. The 50 μl of each sample or standard TGF-beta proteins were added to the wells of the microtiter plates. The plates were incubated at 37℃ for 2 h, and then washed.

For the detection of TGF-β_1 protein, 50 μl of chicken anti-TGF-β_1 antibody (R&D, Minneapolis, MN, AB-100-

NA), as the detector antibody, were added to the wells at a concentration of 5.0 μg/ml, per well for 2 h. The wells were washed and peroxidase labeled rabbit anti-chicken antibody (1 : 1000) was added to the wells and incubated for 2 h at 37℃. Following the incubations, the plates were washed and the substrate 2, 2' azenobis-3-ethylbenthiazolie sulfonic acid (ABTS ; Sigma) was added to wells for 20 min. The reaction was measured at 405 nm with an ELISA reader (Microplate Autoreader, Bio-Rad Instruments). The concentration of TGF-β_1 protein was determined from a standard curve prepared using serial dilutions of standards obtained from the manufacturer.

TGF-β_1 protein in the sample was converted to the activated form using an acidification/neutralizing process.

Statistical analysis

Data are summarized as mean±SD. The data were analysed using the Kruskal-Wallis test, Spearman correlation coefficients, Wilcoxon rank sum test and regression analysis. $P<0.05$ was considered statistically significant.

RESULTS

ELISA system for measuring TGF-β_1 protein

The standard curve showed satisfactory linearity within the range of the TGF-β_1 protein concentration from 0.1 to 20 ng/ml. The curve of serially diluted samples, obtained from serum and tissue extraction, satisfactorily parallelled the standard curve. These data indicated that the ELISA system was reliable for determining TGF-β_1 protein concentrations.

Immunoreactivity, In situ hybridization and the content of TGF-β_1 protein

Immunoreactivity was confined to the cytoplasm of tumor cells and was not seen in the adjacent muscle layer or stroma (Fig. 1). The TGF-β_1 mRNA expression was detected at the same tumor cells (Fig. 2).

There was labelling of tumor cells for TGF-β_1 mRNA in 9 of 20 carcinomas. There was no labelling in tissue in which RNA preservation must be poor. The content of TGF-β_1 protein in gastric adenocarinoma tissue was significantly higher (311±212 ng/g wet tissue ; mean± standard deviation) than that in normal gastric tissue (65 ±36 ng/g) (Fig. 3 : $p< 0.05$). Those content showed correlation with the immunoreactivity of TGF-β_1 protein in the tissue ($r=0.45$; $p<0.05$).

Relationship between tissue TGF-β_1 protein overexpression and clinicopathological parameters

There was no significant correlation between TGF-β_1 protein overexpression and clinicopathologic variables, such as age, sex, lymph node metastasis or differentiation except invasion depth ($r=0.2$; $p< 0.05$, Table 1).

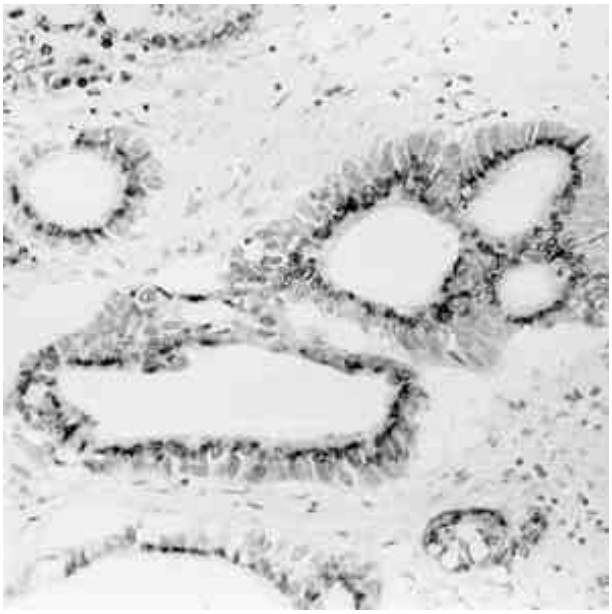

Fig. 1. Immunohistochemical staining for TGF-β_1 protein shows positive reaction in the cytoplasm of tumor cells.

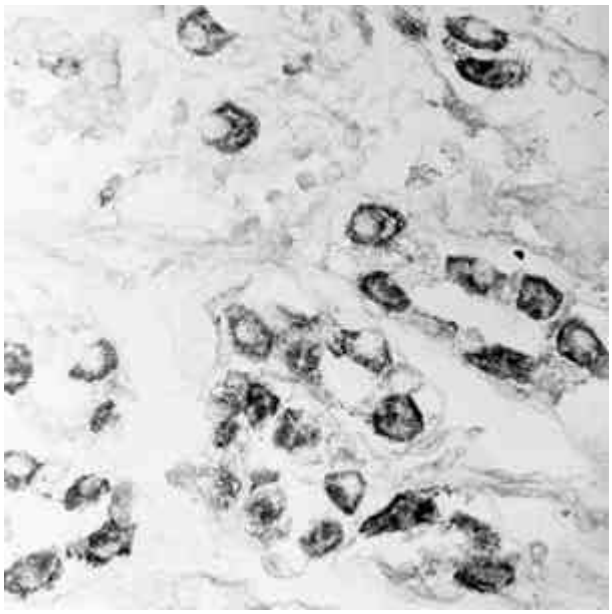

Fig. 2. Labelling of gastric carcinoma cells for TGF-β_1 mRNA shows intranuclear positive reaction.

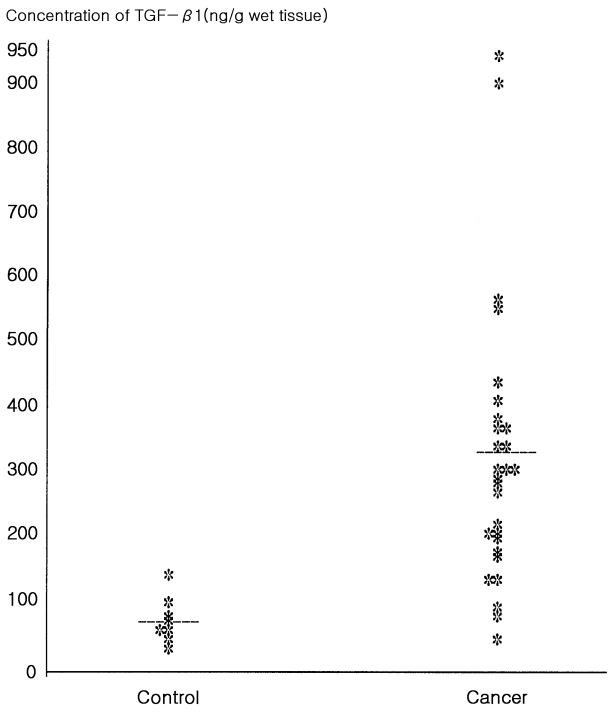

Fig. 3. Content of TGF-β_1 protein in gastric carcinoma tissue. Specimens were derived from gastric carcinoma and 8 normal controls. Tissue TGF-β_1 content was measured using a TGF-β_1 enzyme-linked immunosorbent assay. Bars represent mean values.

Table 1. Relationship between immunohistochemical TGF-β_1 expression and clinicopathlogic parameters in 70 gastric carcinomas

Parameters	No(%)	TGF-β_1 expression	
		Negative	Positive
Age			p=NS
≤ 50	15 (21)	8 (11.43)	7 (10)
> 50	55 (79)	26 (37.14)	29 (41.43)
Sex			p=NS
M	43 (60.6)	19 (27.14)	24 (34.29)
F	27 (39.4)	15 (21.43)	12 (17.14)
Depth			p<0.05
T1	18 (25.7)	8 (11.43)	10 (14.29)
T2	19 (27.1)	11 (15.17)	8 (11.43)
T3	12 (17.1)	10 (14.29)	2 (2.86)
T4	21 (30.0)	5 (7.14)	16 (22.86)
LN meta			p=NS
(−)	28 (39.4)	16 (22.86)	12 (17.14)
(+)	42 (60.0)	18 (25.71)	24 (34.29)
Lym emboli			p=NS
(−)	23 (34.8)	8 (12.31)	15 (23.08)
(+)	42 (60.0)	23 (35.38)	19 (29.23)
Diff			p=NS
Tub. well	19 (27.1)	7 (10)	12 (17.14)
Tub mod	26 (36.6)	18 (25.71)	8 (11.43
Tub poor	12 (16.9)	4 (5.71)	8 (11.43
Signet	9 (12.7)	3 (4.29)	6 (8.57)
Mucinous	4 (5.6)	2 (2.86)	2 (2.86)

[a] Negative ; sum 0-3, Positive ; sum 4, 5
NS not significant

ELISA for serum TGF-β_1 levels in gastric carcinoma patients

Control and patient groups

Serum TGF-β_1 concentration ranges from 38.5 to 283.6 ng/ml on the control group and from 33.3 to 265.7 ng/ml on the gastric caricinoma group (Fig. 4). The mean TGF-β_1 level was 44% higher in the control group (140.3±85.7 ng/ml) than in the gastric carcinoma patients (97.1±29.4 ng/ml, $p<0.05$).

Clinical studies

Serum TGF-β_1 concentration was determined on 62 patients. There was no significant correlation between the TGF-β_1 level and the clinicopathological factors.

DISCUSSION

TGF-β is a polypeptide (molecular weight, 25,000) formed by separation of 112 amino acids on C end from a precursor consisting of 390 amino acids (14). It is widely distributed in the human body, including the placenta, kidney, platelet (15), and bone (16).

Current methods for detecting TGF-β_1 protein include bioassays such as colony formation of NRK cells, the inhibition study of [^{3}H]-thymidine incorporation using mink lung epithelial cells or enzyme linked immunosorbent assays. The ELISA used in this study is a sensitive method affording reproducible results. We measured the total TGF-β_1 protein, *i.e.*, both active and latent forms of TGF-β_1, with acid-ethanol extraction from tissues and serum of patients with gastric carcinoma.

Shirai et al. (17) and Tsushima et al. (18) measured plasma TGF-β_1 levels in normal control and colorectal and hepatocellular carcinoma patients. According to their data plasma TGF-β_1 levels in normal control group were ranged from 1.9 to 20 ng/ml. But in our study, serum TGF-β_1 levels in normal control were ranged from 33.3 to 283.6 ng/ml. So further studies are needed to find why these kinds of discrepancies occurred. Also, unexpectedly our results showed that serum TGF-β_1 levels in patients with gastric carcinoma were lower than those in normal subjects.

A part of the TGF-β_1 protein extracted from the gastric carcinoma tissues is probably derived from platelets in the tumor vessels. However, an immunohistochemical study demonstrated TGF-β_1 protein staining

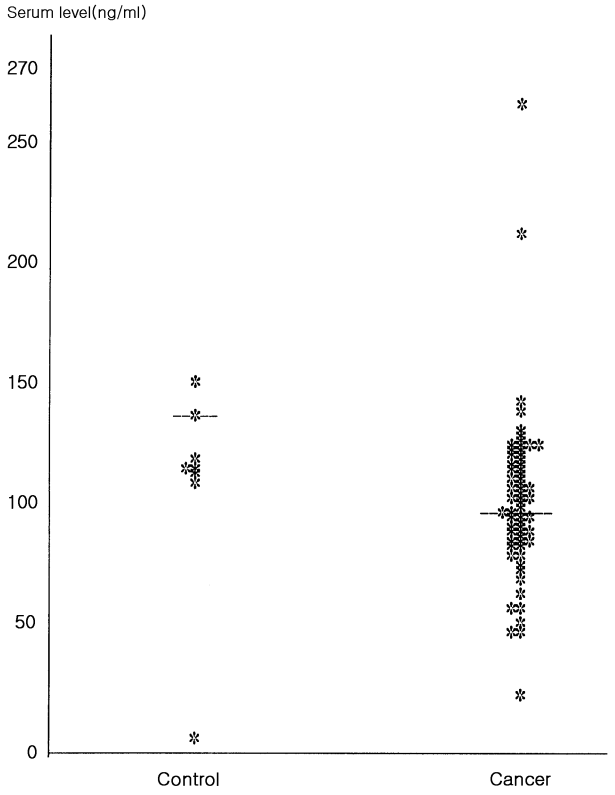

Fig. 4. Serum TGF-β_1 levels in patients with gastric carcinoma. Specimens were derived from 62 patients with gastric carcinoma and 10 normal controls. Serum TGF-β_1 level was measured using a TGF-β_1 enzyme-linked immunosorbent assay. Bars represent mean values.

in the cytoplasm of gastric carcinoma cells, indicating the presence of TGF-β_1 protein in gastric carcinoma cells. According to Assoian et al., 1 ml of peripheral blood platelets contains only about 2ng of TGF-β_1 protein (25 units of platelet concentrate contain 10μg of TGF-β_1), while the content of TGF-β_1 in the gastric carcinoma tissues was 311 ± 212 ng/g wet tissue (4). Thus, the amount of TGF-β_1 derived from platelets in the gastric carcinoma tissues seems to be negligible.

TGF-β_1 is a multifunctional polypeptide promoting collagenesis (19), inhibiting the immune system (20), suppressing epithelial cell growth (21) and enhacing the invasion (22) in gastric cancer. This present study shows TGF-β_1 protein expression was correlated with invasion depth.

The TGF-β_1 protein produced by gastric adenocarcinoma cells may be secreted as an active or latent form and then elicit its biological actions in an autocrine or paracrine fashion.

In conclusion, semiquantitavie immunohistochemical TGF-β_1 analysis is a validative method for TGF-β_1 quantitation. The tumor cells directly produce TGF-β_1. But, it is questionable to use serum TGF-β_1 levels as a predictor for gastric carcinoma.

Acknowlegements

We thank Dr. Pyeung H. Kim at Kang-Won University for excellent ELISA technical consults. We express our gratitude to Jae N. Seo and Seong C. Ju for expert immunohistochemial staining.

REFERENCES

1. Sporn MB, Roberts Ab, Wakefield LM, Assoian RK. *Transforming growth factor-beta : biological function and chemical structure. Science 1986 ; 233 : 532-4.*
2. Sporn MB, Roberts AB, Wakefield LM, de Crombrughe B. *Some recent advances in the chemistry and biology of transforming growth factor-beta. J Cell Biol 1987 ; 105 : 1039-45.*
3. Massague J. *The transforming growth factor-beta family. Ann Rev Cell Biol 1990 ; 6 : 597-641.*
4. Roberts AB, Sporn MB. *The Transforming growth factor-beta. In : Sporn MB, Roberts AB, eds. Handbook of Experimental Pharmacology, vol 95/1. Heidelberg : Springer-Verlag, 1990 ; 419-72.*
5. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB. *Type beta transforming growth factor ; a bifunctional regulator of cellular growth. Proc Natl Acad Sci USA 1985 ; 82 : 119-23.*
6. Flanders KC, THompson NL, Cissel DC, Obberghen-Schilling EV, Baker CC, Kass ME, Ellingsworth LR, Roberts AB, Sporn MB. *Transforming growth factor-beta 1 : histochemical localization with antibodies to different epitopes. J Cell Biol 1989 ; 108 : 653-60.*
7. Choi YH, Chae SW, Lee MC, Shim JW, Ahn HK, Park HR, Kang G, Shin HS, Park YE. *Immunohistochemical analysis of transforming growth factor-beta expression in gastric adenocarcinoma. Korean J Pathol 1994 ; 28 : 272-81.*
8. Ito N, Kawata S, Tamura S, Takaishi K, Shirai Y, Kiso S, Yabuuchi I, Matsuda Y, Nishioka M, Tarui S. *Elevated levels of transforming growth factor-β messenger RNA and its polypeptide in human hepatocellular carcinoma. Cancer Res 1991 ; 51 : 4080-3.*
9. Kim YI. *Guidelines for Pathologic study of gastric cancer. Korean J Pathol 1992 ; 26 : 154-63.*
10. Roberts AB, Lamb LC, Newton DL, Sporn MB, De Larco JE, Todaro GJ. *Transforming growth factors : Isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc Natl Acad Sci USA 1980 ; 77 : 3494-8.*
11. Barral-Netto M, Barral A, Brownell CE, Skeiky YAW, Ellingsworth LR, Twardzik DR, Reed SG. *Transforming growth factor-beta in Leishmanial infection : A parasite escape*

mechanism. Science 1992 ; 257 : 545-8.

12. Hirayama D, Fujimori T, Satonaka K, Nakamura T, Kitazawa S, Horio M, Maeda S, Nagasako K. *Immunohistochemical study of epidermal growth factor and transforming growth factor-β in the penetrating type of early gastric cancer. Hum Pathol 1992 ; 23 : 681-5.*
13. Kim YJ, Lee MC, Park YE, Kim HT, Choi EY, Byeon WH, Kim PH. *Immunodetection of transforming growth factor-β_1 by an improved enzyme-linked immunosorbent assay. Korean J Immunol 1994 ; 16 : 171-6.*
14. Derynck R, Jarrett JA, Chen EY et al. *Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature 1985 ; 316 : 701-5.*
15. Assoian RK, Komoriyama A, Meyers CA, Miller DK, Sporn MB. *Transforming growth factor-β in human platelets. J Biol Chem 1983 ; 258 : 7155-60.*
16. Seyedin SM, Thomas TC, Thompson AY, et al. *Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc Natl Acad Sci USA 1985 ; 82 : 2267-71.*
17. Shirai Y, Kawata S, Tamura S, Ito N, Tsushima H, Takaishi K, Kiso S, Matsuzawa Y. *Plasma transforming growth factor-β_1 in patients with hepatocellular carcinoma. Cancer 1994 ; 73 : 2275-9.*
18. Tsushima H, Kawata S, Tamura S, Ito N, Shirai Y, Kiso S, Imai Y, Shimomukai H, Nomura Y, Matsuda Y, Matsuzawa Y. *High levels of transforming growth factor β_1 in patients with colorectal cancer : Association with disease progression. Gastroenterology 1996 ; 110 : 375-82.*
19. Kato KMJ, Takimoto TR, Horimoto M, Murakami T, Mogi Y, Watanabe N, Kohgo Y, Niitsu Y. *Transforming growth factor β_1 secreted from scirrhous gastric cancer cells is associated with excess collagen deposition in the tissue. Br J Cancer 1994 ; 69 : 777-83.*
20. Kehrl JH, Roberts AB, Assoian PK, Jalowlew S, Sporn MB, Fauci AS. *Transforming growth factor-β is an important immunomodulatory protein for human B lymphocytes. J Immunol 1986 ; 137 : 4167-71.*
21. Tucker RF, Shipley GD, Moses HL, Holley RW. *Growth inhibition form BSC-1 cells closely related to platelet type β transforming growth factor. Science 1984 ; 226 : 705-7.*
22. Hirayama D, Fujimori T, Satonaka K, Nakamura T, Kitazawa S, Horio M, Maeda S, Nagasako K. *Immunohistohcemical study of epidermal growth factor and transforming growth factor-β in the penetrating type of early gastric cancer. Hum Pathol 1992 ; 23 : 681-5.*