Relationship of Transforming Growth Factor β1 to Angiogenesis in Gastric Carcinoma

Transforming growth factor-β (TGF-β) comprises a group of multifunctional regulatory proteins, whose effects include angiogenesis. The expression of TGF-β1 in gastric carcinomas (70 cases) has been determined and related to pathological features and microvessel count by immunohistochemical staining for TGF-β1 and Factor VIII related antigen. Prominent reactivity for TGF-β1 was associated with the depth of invasion ($r=0.2$; $p<0.05$) and increased microvessel count ($r=0.5$; $p<0.05$). Also, the microvessel count had a significant correlation with invasiveness ($r=0.34$; $p<0.05$) and lymph node metastasis ($r=0.28$; $p<0.05$). These findings indicate that TGF-β1 may have a role in tumor invasion and angiogenesis. *(JKMS 1997; 12: 427~32)*

Key Words : *Transforming growth factor beta; Angiogenesis factor; Gastrointestinal neoplasms*

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Received : June 3, 1997
Accepted : June 19, 1997

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INTRODUCTION

Transforming growth factor-β (TGF-β) comprises a group of multifunctional regulatory proteins which have many effects on physiological and pathological processes (1). TGF-β can both stimulate and inhibit cell proliferation, depending on the type of cells. It can block or effect entry into differentiation pathways (2). The effects of TGF-β on endothelial cells are complicated in that most of the in vitro effects are growth inhibitory (3), yet the peptide is angiogenic in vivo (4).

Angiogenesis is very important to the tumor because angiogenesis is required for the expansion of the primary tumor mass, and new blood vessels penetrating the tumor are frequent sites for tumor cell entry into the circulation (5). Angiogenesis is also required for expansion of the metastatic colony.

We have previously studied the immunohistochemical expression of TGF-β1 in gastric carcinoma (6). TGF-β1 expression in gastric carcinoma is related to the depth of invasion, the degree of invasiveness and the presence of metastasis. The TGF-β1 in gastric carcinoma cells may play an important role in carcinomatous invasion resulting in metastasis.

The present study has considered TGF-β1 in gastric carcinoma in relation to angiogenesis and tumor characteristics to further consider the potential role of TGF-β in invasion and metastasis.

MATERIALS AND METHODS

Patients and tissue samples

Gastric cancer tissues were obtained from 27 female and 43 male patients from the surgical pathology files of the Chun Chon 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.

Freshly removed tissue samples were fixed in 10% neutral formalin for 12 to 24 hr and paraffin-embedded for histological analysis.

According to the classification of the Korean Research Society for gastric cancer (7), there were nineteen well differentiated tubular; twenty-six moderately differentiated tubular; twelve poorly differentiated tubular; nine signet ring cell; and four mucinous carcinomas. The clinical data included age, sex, differentiation, depth of invasion, presence of lymphatic emboli and lymph node metastasis.

Immunohistochemical staining for TGF-β1

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 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 (8).

This antibody recognizes bovine, mouse and human TGF-β1. 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 a reported method (9). 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 $\times$100 magnification, and 2 for results in which the staining was clearly identified by $\times$40 magnification (Fig. 1). 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-β1.

Staining for microvessels

All blood vessels were highlighted by staining endothelial cells for factor VIII related antigen (Dako Polyclonal, Dako, Santa Barbara, Calif.) with a standard immunoperoxidase technique described previously (10).

Microvessel density was assessed without knowledge of the patient's outcome; areas of invasive tumor containing the most capillaries and small venules were examined by light microscopy. Tumors were frequently heterogenous in their microvessel density, but the areas of highest neovascularization were found by scanning the tumor sections at low power (40$\times$ and 100$\times$, so-called 'hot spots') and identifying the areas of invasive carcinoma with the highest number of discrete microvessels staining for factor VIII related antigen (brown). In each section, the three most vascular areas were chosen. A 200$\times$ field in each of these three regions was counted, and the average counts of the three fields were recorded. This analysis was also performed in a 200$\times$ field in each of these three regions. Large vessels with lumina greater than approximately eight red blood cells were excluded from the count. A vessel lumen was not required for identification of a microvessel; single cells or cell clusters were counted. Counts are expressed as total number of microvessels per 200$\times$(11).

Statistical analysis

Data are summarized as mean $\pm$ 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.

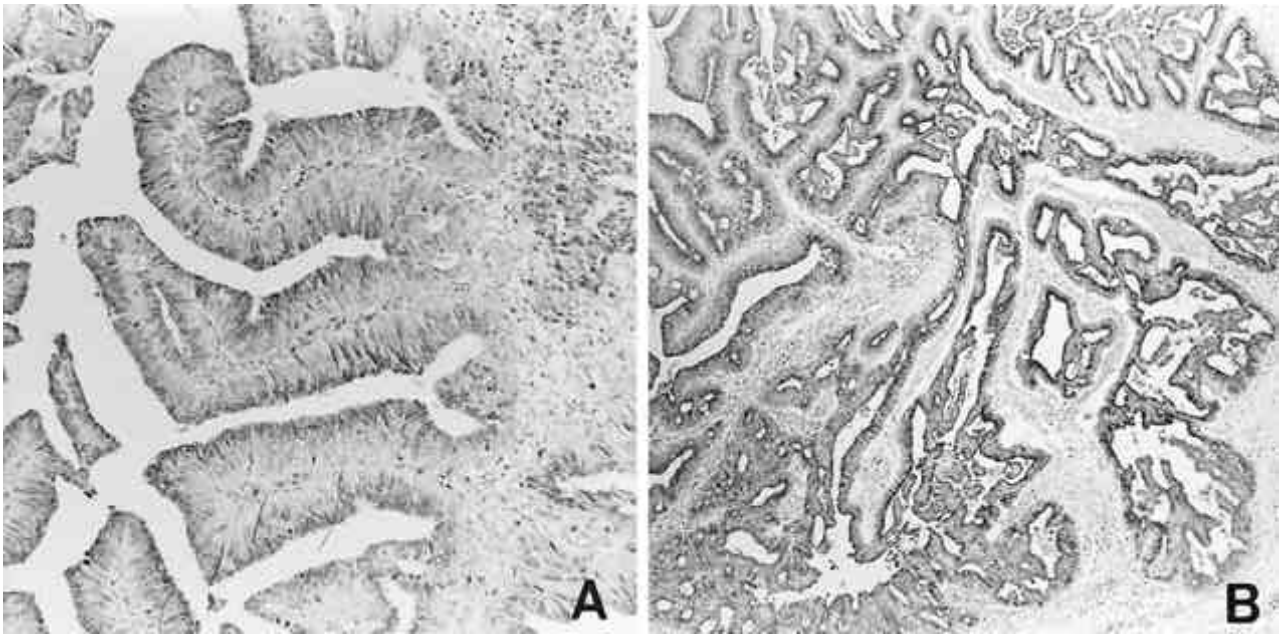

Fig. 1. Evaluation of the immunohistochemical staining for TGF-β1 according to the intensity of the staining; 1 for results in which the staining was clearly identified by $\times$100 magnification (A), and 2 for results in which the staining was clearly identified by $\times$40 magnification (B).

RESULTS

Immunoreactivity of TGF-β1

Immunoreactivity for TGF-β1 was confined to the cytoplasm of tumor cells and was not seen in the adjacent muscle layer or stroma (Fig. 2). The semiquantitative expression of TGF-β1 is presented in Table 1.

Relationship between TGF-β1 and clinicopathological parameters

Invasion depth was significantly related with TGF-β1 expression ($r=0.2$; $p<0.05$, Table 1), but other variables, such as age, sex, lymph node metastasis or differentiation were not correlated.

Relationship with microvessel count

The extent of microvessel count ranged from 2.33 to 141 per ×200 magnification (Fig. 3). There is a significant correlation between microvessel count and TGF-β1 immunoreactivity($r=0.49$; $p<0.05$; Fig. 4; Table 2). Also

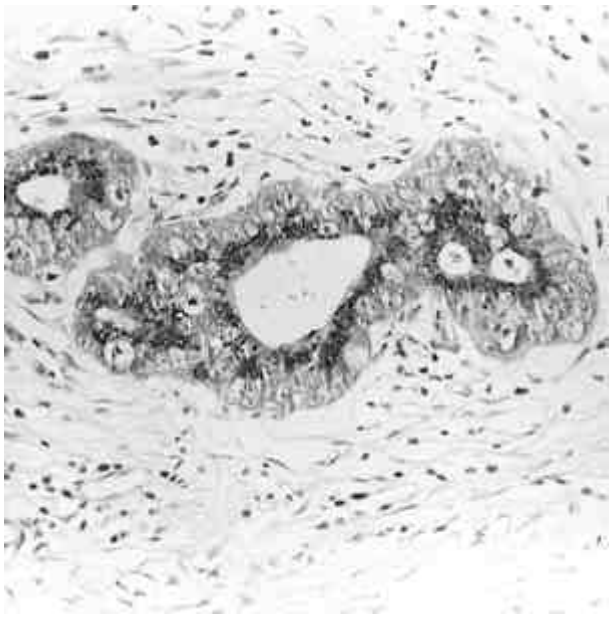

Fig. 2. Immunohistochemical staining for TGF-β1 in gastric carcinoma.

Tablel 1. Association of TGF-β1 and vWF staining with clinicopathologic parameters

Parameters	TGF-β1[a]			vWF staining[b]				
	Grade1	Grade2		Grade1	Grade2	Grade3	Grade4	
age			p=NS					p=NS
>50	8(11.43)	7(10)		5(7.69)	4(6.51)	0	5(7.35)	
≤50	26(37.14)	29(41.43)		11(16.92)	20(30.77)	9(13.85)	14(20.59)	
sex			p=NS					p=NS
M	19(27.14)	24(34.29)		10(15.38)	10(15.38)	6(9.23)	15(22.06)	
F	15(21.43)	12(17.14)		6(9.23)	14(21.54)	3(4.62)	4(5.88)	
Depth			p<0.05					p<0.05
T1	8(11.43)	10(14.29)		5(7.69)	5(7.69)	2(3.08)	5(7.35)	
T2	11(15.17)	8(11.43)		7(10.77)	9(13.85)	2(3.08)	1(1.54)	
T3	10(14.29)	2(2.86)		3(4.62)	5(7.69)	2(3.08)	2(2.94)	
T4	5(7.14)	16(22.86)		1(1.54)	5(7.69)	3(4.62)	11(16.18)	
LN meta			p=NS					p<0.05
(−)	16(22.86)	12(17.14)		10(15.38)	8(12.31)	4(6.15)	5(7.69)	
(+)	18(25.71)	24(34.29)		6(9.23)	16(24.62)	5(7.69)	14(20.59)	
differentiation			p=NS					p=NS
Tub. well	7(10)	12(17.14)		3(4.62)	9(13.85)	3(4.62)	4(5.88)	
Tub. mod	18(25.71)	8(11.43)		8(12.31)	10(15.38)	4(6.15)	3(4.62)	
Tub. poor	4(5.71)	8(11.43)		3(4.62)	2(3.08)	1(1.54)	6(8.82)	
Signet	3(4.29)	6(8.57)		1(1.54)	2(3.08)	1(1.54)	4(6.15)	
Mucinous	2(2.86)	2(2.86)		1(1.54)	1(1.54)	0	2(3.08)	

[a] Grade I ; sum 0−3 Grade II ; sum 4,5

[b] Grade I ; 0<microvessel count≤15 Grade II ; 15<microvessel count≤30 Grade III ; 30<microvessel count≤45 Grade IV ; 45< microvessel count (count/ ×200 field)

NS ; not significant

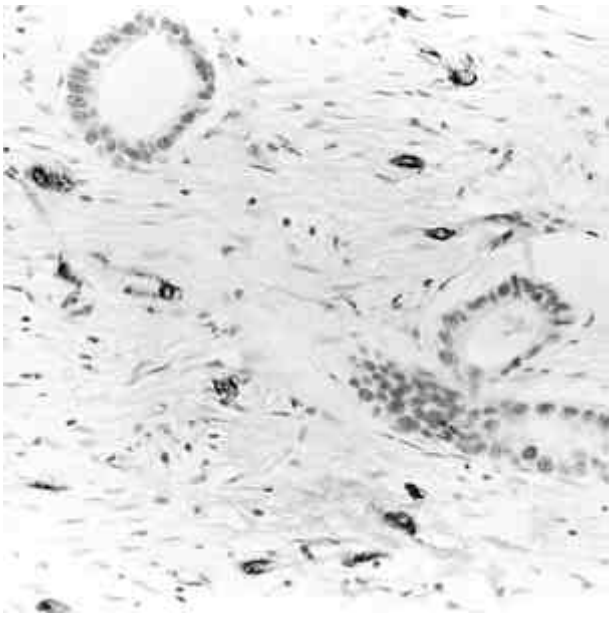

Fig. 3. Immunohistochemical staining for endothelial cells with factor VIII related antigen in gastric carcinoma.

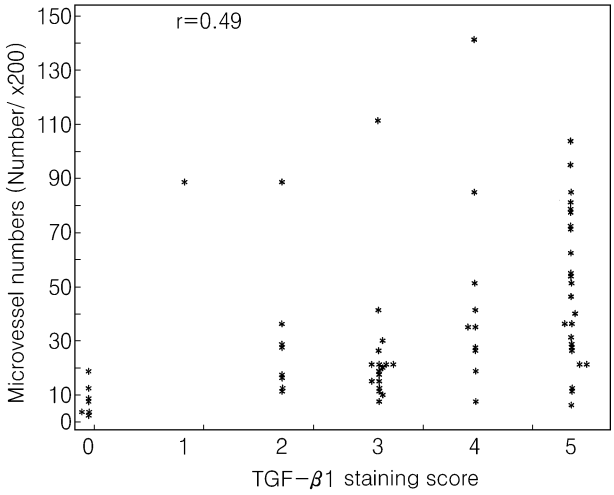

Fig. 4. Scatter diagram shows TGF-β1 staining score versus vessel number.

invasiveness (r=0.34; p<0.05) and lymph node metastasis (r=0.28; p<0.05) are significantly correlated with the microvessel count (Table 1). Gastric carcinoma with nodal metastasis had a mean microvessel count of 42 per ×200 magnification (SD=31.7; range, 6 to 141). For those carcinomas without nodal metastasis the corresponding value was 27 per ×200 magnification (SD=27.3; range, 2 to 95).

However, microvessel count showed no correlation with other clinicopathological factors, i.e., age, sex and differentiation.

Table 2. Angiogenesis in relation to the extent of staining for TGF-β (number(%))

TGFβ1 staining score \ Grade of vessel count[a] [number(%)]	I	II	III	IV
0	6(8.82)	1(1.47)	0	0
1	0	0	0	1(1.47)
2	2(2.94)	4(5.88)	1(1.47)	1(1.47)
3	4(5.88)	11(16.18)	1(1.47)	1(1.47)
4	1(1.47)	3(4.41)	3(4.41)	3(4.41)
5	3(4.41)	5(7.35)	4(5.88)	13(19.12)

[a] Grade I ; 0<microvessel count≤15
Grade II ; 15<microvessel count≤30
Grade III ; 30<microvessel count≤45
Grade IV ; 45<microvessel count (count/ ×200 field)

DISCUSSION

We have shown that the immunoreactivity of TGF-β1 significantly correlated with mcirovessel count per ×200 field. TGF-β1 inhibits endothelial cell proliferation and migration in vitro but stimulates vessel formation in vivo and thus is an "indirect" angiogenic factor because TGF-β1 is a potent chemotactic agent for monocytes (12), fibroblasts (13), macrophages (14) and neutrophils (15), which are then capable of releasing direct angiogenic factors. TGF-β1 also stimulates the expression of a variety of extracellular matrix molecules including proteoglycans, fibronectin and collagen, and increases their incorporation into the extracellular matrix. These matrix proteins play crucial roles in the angiogenic process (16). TGF-β1 also stimulates tenascin expression, which correlates with angiogenesis in astrocytomas. Endothelial cells can attach and spread on tenascin in vitro. The attachment is mediated by integrins such as $\alpha_v\beta_3$, which is required for angiogenesis (17).

In this study, microvessel count was significantly correlated with invasion depth and nodal metastasis. Obviously, the new vessels allow exchange of nutrients, oxygen and waste products by a crowded cell population through perfusion. Also endothelial cells may release important paracrine growth factors for tumor cells [eg. basic fibroblast growth factor (bFGF), insulin growth factor-2, platelet-derived growth factor, and colony stimulating factors] (18). Furthermore, the invasive chemotactic behavior of endothelial cells at the tips of growing

capillaries is facilitated by their secretion of collagenases, urokinases, and plasminogen activator (19). These degradative enzymes probably facilitate spread of tumor cells into and through the adjacent fibrin-gel matrix and connective tissue stroma. Thus, the additive impact of the perfusion and paracrine tumor effects plus the endothelial cell-derived invasion-associated enzymes all probably contribute to a phase of rapid tumor growth and signal a switch to a potentially lethal angiogenic phenotype. These same effects probably contribute to a much higher metastatic potential by facilitating entry of tumor cells into the lymphatic vascular system. Intratumor microvessel count has independent prognostic significance when compared with traditional prognostic markers by multivariate analysis. This has been shown in studies of patients with carcinomas of the breast (21), lung (22, 23), prostate (24, 25, 26), head and neck (squamous) (27, 28), rectum (29), testicles (30) and bladder (31), as well as in malignant melanoma (32), soft tissue tumors (33), central nervous system tumors (34) and multiple myeloma (35).

Those findings suggest that TGF-β1 may have a role in the invasion and metastasis of gastric carcinoma by stimualtion of angiogenesis.

Acknowledgments

We express our gratitude to Jae N. Seo and Seong C. Ju for their expert immunohistochemical staining.

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