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Reduced Levels of Transforming Growth Factor-beta Type I Receptor in Human Gastric Carcinomas

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The expressions of transforming growth factor beta (TGF-β) and its receptor and TGF-β inhibitory element (TIE)-binding protein were examined on human gastric carcinomas by Northern blot hybridization, immunohistochemistry, affinity labeling and gel retardation analysis. TGF-β mRNA was expressed in tumor and normal tissues at various levels. Immunohistochemically, TGF-β expression was confirmed to be present within tumor cells. Out of the 17 human gastric carcinoma tissues, 14 (82%) showed a reduction in the level of type I receptor (65 kDa) for TGF-β when compared to corresponding normal mucosas. Interestingly, in seven of the 14 tumors the level of TIE-binding protein in the tumor tissue was lower than that in normal mucosa. Human gastric carcinoma cell line TMK-1, whose growth was inhibited by TGF-β, had only type I receptor for TGF-β and showed a high level of TIE-binding protein. Conversely, MKN-1, a TGF-β-resistant cell line, exhibited an extremely low level of TGF-β receptor and had no TIE-binding protein. These results overall indicate that although human gastric carcinoma cells produced TGF-β, they showed a reduction in TGF-β type I receptor and a low level of TIE-binding protein, resulting in escape from growth inhibition by TGF-β.

Key words: TGF-β — TGF-β receptor — TGF-β inhibitory element — Gastric carcinoma

Transforming growth factor beta (TGF-β) is a multifunctional polypeptide, consisting of two 12.5-kDa monomers linked by disulfide bonds.[1] The expression of TGF-β in many types of normal cells and tumor cells was reported.[2] Although this polypeptide was first discovered as a growth factor which causes transformation of rat fibroblasts,[3] recent evidence indicates that TGF-β participates in the proliferation and differentiation of a variety of tumor cells as well as normal cells.[4, 5] Moreover, many studies have shown that TGF-β inhibited the cell growth of not only normal epithelial cells[6] but also certain carcinoma cells such as colon carcinoma[7] and breast carcinoma.[8] Therefore, it is likely that TGF-β acts as an autocrine growth inhibitor in carcinoma cells.[9]

The growth of tumor cells was regulated by both positive and negative growth factors. In fact, human gastric carcinoma cells are known to have autocrine loops of growth factors like EGF and TGF-α to maintain their autonomous growth.[10] However, we previously reported that TGF-β was preferentially expressed in scirrhous-type gastric carcinoma whose biological behavior is most malignant.[11] Therefore, it is possible that tumor cells may escape from negative growth regulation by TGF-β.[12] However, it is still unclear whether cancer cells *in vivo* express less TGF-β receptor or whether the post-receptor signal in cancer tissues is altered.

To resolve these questions, we examined the expression of TGF-β and its receptor on human gastric carcinoma tissues and cell lines, and compared them with the level of binding protein to TGF-β inhibitory element (TIE).[13]

MATERIALS AND METHODS

Chemicals Recombinant human TGF-β1 was kindly provided by Dr. Takaku (University of Tokyo, Tokyo) and ^{125}I-TGF-β by Amersham Japan Inc. (Tokyo). TGF-β and labeled TGF-β were obtained from R & D Systems Inc. (Minneapolis, MN).

Tumor tissues and cell culture A total of 17 human gastric carcinoma tissues were frozen in liquid nitrogen immediately after surgical removal and stored at −80°C. The corresponding non-neoplastic mucosa was obtained from the same patient. It was histologically confirmed that tumor tissue consisted of carcinoma tissue and that non-neoplastic mucosa did not contain any tumor cell invasion or show significant inflammatory involvement.

Human gastric carcinoma cell line, MKN-1 was kindly made available for this study by Dr. T. Suzuki (Fukushima Medical University, Fukushima) and KATO-III by Dr. M. Sekiguchi (University of Tokyo). TMK-1 was established from poorly differentiated adenocarcinoma in our laboratory.[14] These cells were routinely cultured in RPMI-1640 (Nissui, Tokyo)

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supplemented with 10% fetal bovine serum (FBS, M. A. Bioproducts, Walkersville, MD).

DNA synthesis analysis Approximately 5×10^4 cells were inoculated in a 12-well plate and cultured for 24 h. The medium was replaced with serum-free medium in the presence or absence of TGF-β. After incubation for 24 h, ^{3}H-thymidine (0.2 μCi/ml) was added to the medium and cultured for a further 3 h. The samples were washed 4 times and solubilized by 1 *N* NaOH and 1 *N* HCl. Radioactivity was estimated by using a liquid scintillation counter.

Northern blot analysis Total RNA was extracted from 13 pairs of samples by the guanidine isothiocyanate method.[15] Northern blot analysis was carried out as described previously.[11] Human TGF-β probe (1.0 kb cDNA insert of plasmid pβC$_1$) was kindly provided by Dr. Derynck.[16] β-Actin probe was purchased from Oncol (Gaithersburg, MD).

Immunohistochemistry Immunohistochemical analysis was performed by using a modified immunoglobulin enzyme bridge technique as described previously.[17] Frozen sections were subjected to methanol fixation, followed by primary reaction using affinity purified anti-TGF-β polyclonal antibody, which was kindly supplied by King Brewing Co. (Kakogawa). Peroxidase-labeled secondary antibody was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Specificity of the reaction was confirmed by using nonspecific IgY in the primary reaction as well as the primary antibody preabsorbed with a 3-fold excess of TGF-β for 24 h at 4°C.

Affinity labeling of TGF-β receptors Crude membrane fraction was prepared as described previously.[18] Membrane proteins were incubated with 150 p*M* ^{125}I-TGF-β in binding buffer containing 0.5% bovine serum albumin (BSA) for 4 h at 4°C.[19] After intensive washing, the samples were incubated with 0.25 m*M* disuccinimidyl suberate (DSS) for 15 min at 4°C. Samples were washed with washing buffer (10 m*M* Tris, pH 7.0, 250 m*M* sucrose, 1 m*M* EDTA, 0.1 m*M* PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin), followed by solubilization with Laemmli's sample buffer.[20] When intact cells were used, samples were treated with ^{125}I-TGF-β and DSS as described above. After scraping off in washing buffer, samples were extracted with extraction buffer (10 m*M* Tris, pH 7.0, 1% Triton X-100, 1 m*M* EDTA, 0.1 m*M* PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin) for 40 min at 4°C. Twenty μg of soluble fraction was subjected to SDS polyacrylamide gel electrophoresis. The experiment was repeated twice.

Gel retardation analysis Nuclear protein was extracted as described previously.[18] TIE oligonucleotide (GAGTTGGTGA)[13] was kindly provided by Tonen Inc. (Saitama). Gel retardation analysis was carried out by the method of Johnson *et al.* with some modification.[21] Twenty μg of nuclear extract was incubated with 25 m*M* Tris, pH 7.5, 12.5 m*M* spermidine and 2 μg of poly dI-dC (deoxyinosinate-deoxycytidylate) at room temperature. After 5 min, 2 μg of labeled DNA was added and incubation was continued for 45 min with shaking. Twenty μl aliquots of samples were subjected to 4% polyacrylamide gel electrophoresis.

RESULTS

Expression of TGF-β in human gastric carcinoma tissues TGF-β mRNA was expressed at various levels in all samples. Particularly high expression was observed in scirrhous gastric carcinoma, as in Case 5 (Fig. 1). Immunohistochemically, TGF-β immunoreactivity was detected within tumor cells as shown in Fig. 2a. The immunoreactivity was decreased by using primary antibody preincubated with an excess of TGF-β (Fig. 2b) and control IgY (data not shown). Gastric carcinoma cell lines, TMK-1 and MKN-1, also showed high expression of TGF-β mRNA (Fig, 1). Human gastric carcinoma cell line KATO-III was used as a positive control, because KATO-III was reported to express TGF-β mRNA and secrete TGF-β protein in an active form into the conditioned medium.[11, 22] TGF-β mRNA and TGF-β protein were expressed as shown in Fig. 1 and 2c.

TGF-β receptor expression in human gastric carcinoma tissues As shown in Fig 3a, both tumor and normal tissues showed preferential expression of type I receptor (65 kDa) and the levels were clearly reduced in tumor tissues when compared to normal mucosas. Type II or

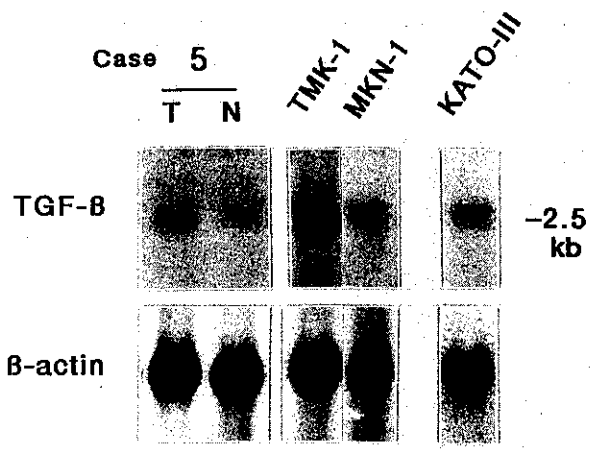

Fig. 1. TGF-β mRNA expression in human gastric carcinomas and cell lines. Ten μg of total RNA was used in each lane. β-Actin was used as an internal control. The number above the lane is the case number. T, tumor tissue; N, normal mucosa.

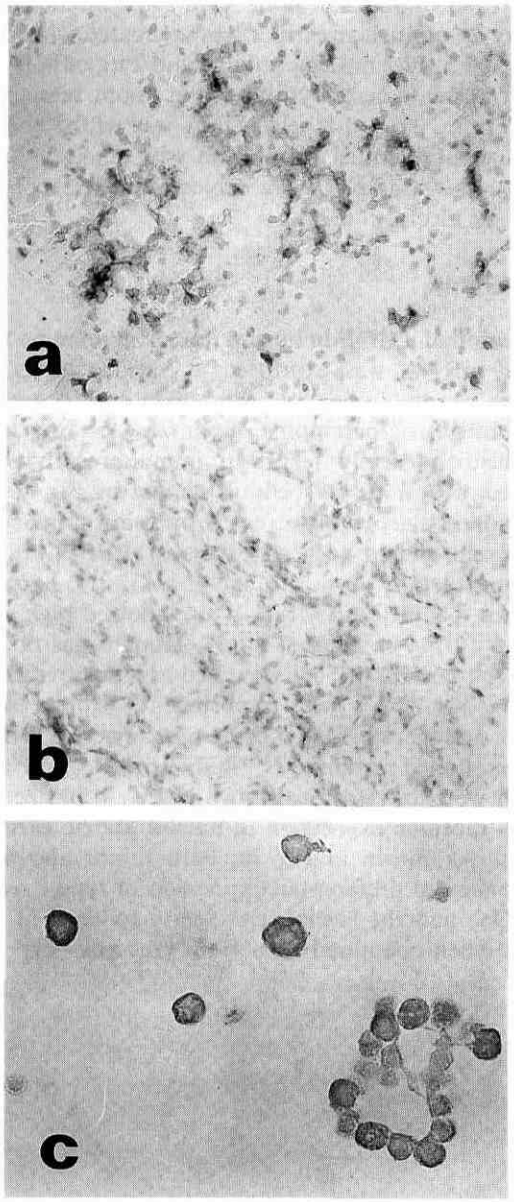

Fig. 2. Immunostaining of TGF-β on human gastric carcinoma tissue (Case 5). Tumor tissue was stained with anti-TGF-β antibody (a) and primary antibody preabsorbed with an excess of TGF-β (b). ($\times$120) KATO-III cells were fixed with ethanol and subjected to immunostaining with anti-TGF-β antibody (c). ($\times$800)

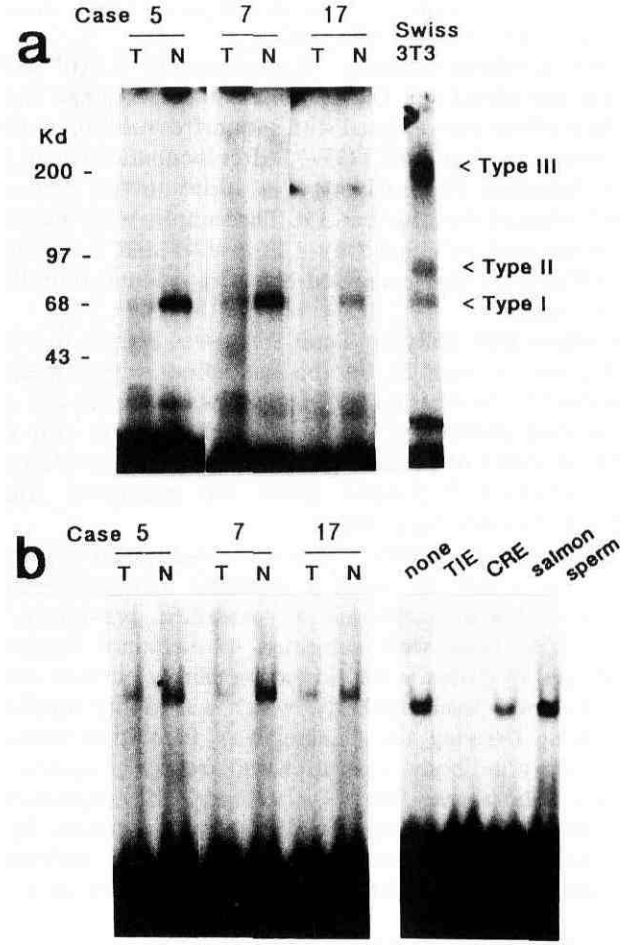

Fig. 3. Affinity labeling of TGF-β receptor (a) and gel retardation analysis of TIE-binding protein (b) in human gastric carcinoma tissues. Affinity labeling and gel retardation assay were performed as described in "Materials and Methods." The number above the lane is the case number (T, tumor; N, normal). Swiss 3T3 cells were used as a positive control (a). Nuclear extract was incubated with ^{32}P-labeled TIE oligonucleotide (b). The sample extracted from normal mucosa of Case 5 was preincubated with unlabeled DNAs and subjected to gel retardation analysis in the same manner (b). CRE oligonucleotide was kindly provided by Tonen Inc.[32]

type III receptor was undetectable. The band of 65 kDa was confirmed to disappear upon addition of an excess of unlabeled TGF-β to the reaction mixture. Moreover, it was confirmed by gel staining that the lanes contained equal amounts of proteins without degradation (data not shown). As a positive control, membrane fraction was prepared from Swiss 3T3 cells in the same manner, and three types of receptors were detected.[23] The results of affinity labeling on 17 tissue samples are summarized in Table I. Out of the 17 tumor tissues, 14 (82%) revealed a lower level of type I receptor expression when compared to normal mucosa. No correlation was found between the reduction in the level of type I receptor and sex, age, location or histological type. However, the reduction in the level of type I receptor was obvious as the invasion of tumors became deeper. Out of four

Tabre I. TGF-β Receptor and TIE-binding Activity in Human Gastric Carcinoma Tissues

No.	Sex	Age	Location[a]	Depth[b]	Histology[c]	TGFβ-receptor (type I) expression[d]	TIE-binding protein[d]
1	M	75	A	sm	pap	T<N	T=N
2	M	69	A	sm	tub	T<N	T<N
3	M	79	M	sm	por	T=N	T=N
4	F	60	M	pm	por	T=N	T=N
5	F	70	A	ss	por (sci)	T<N	T<N
6	M	65	A	ss	tub	T=N	T=N
7	F	84	A	ss	por	T<N	T<N
8	M	76	M	ss	tub	T<N	T=N
9	M	71	MA	ss	pap	T<N	T<N
10	M	75	C	ss	pap	T<N	T<N
11	M	58	M	ss	tub	T<N	T=N
12	M	51	M	ss	tub	T<N	T=N
13	M	44	M	ss	tub	T<N	T=N
14	M	73	M	ss	pap	T<N	T=N
15	F	53	M	s	por (sci)	T<N	T<N
16	F	84	A	s	por	T<N	T<N
17	F	84	A	s	tub	T<N	T=N
					T<N:	14/17 (82%)	7/17 (41%)

a) C, cardia; M, body; A, antrum.
b) sm, submucosa; pm, propria muscularis; ss, subserosa; s, serosa.
c) tub, tubular; pap, papillary; por, poorly differentiated; sci, scirrhous carcinoma.
d) Estimated by one-dimensional densitometry and classified by tumor/normal ratio: T=N; from 2.0 to 0.5, T<N; less than 0.5.

tumors which were limited to the propria muscularis (Cases 1–4), two showed reduced levels of type I receptor. Conversely, out of 10 tumors which extended into the subserosa (Cases 5–14), nine (90%) showed this reduction. Moreover, all tumors extending beyond the serosa (Cases 15–17) showed reduced type I receptor.

TIE-binding protein in human gastric carcinomas We next tried to elucidate the post-receptor signaling by measuring the level of TGF-β inhibitory element (TIE)-binding protein.[13] A summary of TIE-binding protein data and a representative autoradiograph are shown in Table I and Fig. 3b, respectively. In seven of the 17 cases, tumor tissues showed TIE-binding protein at lower levels than the corresponding normal mucosas. Interestingly, all the seven tumors also had type I receptor at lower levels than the normal mucosas (Table I). No tumor tissues showed increased TIE-binding activity. Specificity of the reaction was examined by adding an excess of unlabeled DNAs, and the band specifically disappeared upon addition of TIE oligo-DNA (Fig. 3b).

TGF-β receptor and TIE-binding activity in human gastric carcinoma cell lines As regards the expression of TGF-β receptor in human gastric carcinoma cell lines,

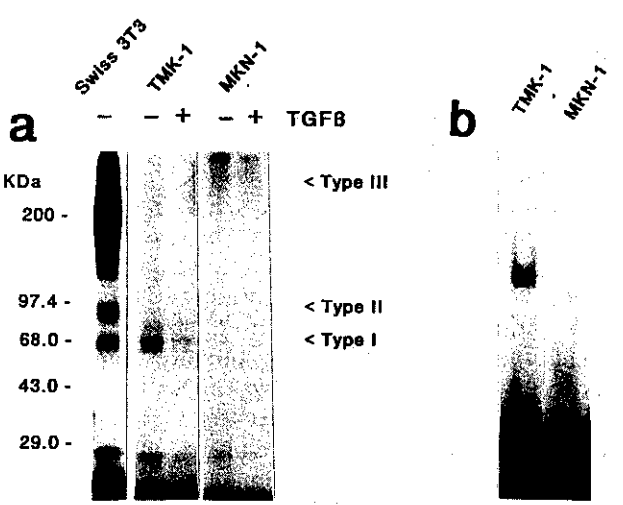

Fig. 4. Affinity labeling of TGF-β receptor and gel retardation analysis of TIE-binding protein in human gastric carcinoma cell lines. Cell lines were subjected to TGF-β affinity labeling in the presence (+) or absence (−) of an excess of unlabeled TGF-β (a). Gel retardation assay was carried out using TMK-1 and MKN-1 cells which had been preincubated with 100 p*M* TGF-β for 24 h before extraction (b).

Table II. Effect of TGF-β on DNA Synthesis of Human Gastric Carcinoma Cell Lines

Cells	^{3}H-Thymidine incorporation (% control)	
	TGF-β 100 p*M*	TGF-β 200 p*M*
TMK-1	65.6±12.9[a)]	47.3±6.3
MKN-1	96.6±12.0	116.0±12.7

a) Data represent the average of triplicate experiments (±SD).

TMK-1 expressed type I receptor for TGF-β. On the other hand, MKN-1 had a very much lower level of each type of receptor (Fig. 4a). In addition, TMK-1 cells preincubated with 100 p*M* TGF-β for 24 h showed a high level of TIE-binding protein (Fig. 4b). On the other hand, MKN-1 preincubated with TGF-β showed an extremely low level of TIE-binding protein. We confirmed that TGF-β inhibited DNA synthesis by TMK-1 but not by MKN-1, as shown in Table II.

DISCUSSION

In the present study, the level of TGF-β type I receptor in tumor tissues was almost invariably lower than that in normal mucosa. TGF-β receptors are subclassified into three types, type I (65 kDa), type II (85–95 kDa) and type III (250–350 kDa).[24] Out of the three types of receptors, type I is thought to mediate the growth-inhibitory signal.[25] In fact, human gastric carcinoma cell line TMK-1, whose growth was suppressed by TGF-β, had only type I receptor for TGF-β, whereas MKN-1, a TGF-β-resistant cell line had lost type I receptor. These *in vitro* and *in vivo* findings indicate that reduction or loss of TGF-β type I receptor in human gastric carcinomas may contribute to tumor growth by allowing escape from negative growth regulation by TGF-β. Total binding of TGF-β type I receptor is affected both by total protein amount and by the binding activity of the receptor (K_d value). We performed affinity labeling of TGF-β receptor with various concentrations of ^{125}I-TGF-β, and confirmed the concentration did not affect the result of affinity labeling (data not shown). This result suggests that the difference of K_d value between tumor and normal mucosa does not affect the result of affinity labeling. Furthermore, we should examine the K_d value by Scatchard's method and the total protein amount of TGF-β receptor by Western blotting of human gastric carcinoma tissues. On the other hand, submucosal fibroblasts of the stomach, which retains three types of receptors for TGF-β, might receive the effect of TGF-β produced by tumor cells, resulting in growth promotion and collagen synthesis[26, 27] especially in scirrhous carcinomas, which express high levels of TGF-β.[11] Besides the effect on cell proliferation, TGF-β is also known to suppress the function of T and B lymphocytes and natural killer cells *in vitro*.[1] High expression of TGF-β may produce favorable conditions for the proliferation of carcinoma cells *in vivo*. It seems to be contradictory that TMK-1 expressed TGF-β, even if the growth of TMK-1 was inhibted by exogenous TGF-β. Thus, we examined the effect of anti-TGF-β antibody on the cell growth of TMK-1 in order to assess the significance of endogenous TGF-β. Though the antibody overcame the growth-inhibitory effect of exogenous TGF-β, antibody alone had no effect on the growth of TMK-1 (data not shown). The concentration of endogenous TGF-β may be too low to induce growth inhibition, or TGF-β produced by TMK-1 may be in a latent form.

Interestingly, the reduction in TGF-β receptor type I in the tumor tissue was correlated with depth of tumor invasion. Almost all the tumors which had invaded the deep layer (ss or s) had lower levels of type I receptor than normal mucosa. We previously reported that epidermal growth factor (EGF) and TGF-α were highly expressed in advanced gastric carcinomas compared with early carcinomas.[28] Thus, not only overexpression of positive growth factor such as EGF or TGF-α, but also reduction in receptor for a negative growth factor such as TGF-β might contribute to tumor progression. Moreover, the alteration in type I receptor was observed not only in scirrhous carcinoma but also in well differentiated adenocarcinoma. Therefore, contamination by fibroblasts in the tumor tissue might not exert a marked influence on the level of type I receptor. Some *in vitro* studies have shown that responsiveness to TGF-β is not always correlated with alteration in TGF-β receptor on squamous cell carcinoma and hepatocellular carcinoma cells.[29, 30] These reports seem to conflict with our results on gastric carcinomas. It is likely that post-receptor signaling from TGF-β may be different among various types of carcinomas.

The TGF-β inhibitory element (TIE) is conserved in the promoter region of TGF-β-inhibited genes such as c-*myc* and stromelysin genes.[13] The TIE-binding protein has not been fully elucidated yet, but one element of the binding protein complex is FOS protein. The binding of the protein to the TIE element is thought to be essential for signal transduction from TGF-β. In the present study, 41% of the tumor tissues clearly showed reduced levels of TIE-binding protein, compared to corresponding normal mucosa. We further confirmed that the level of TIE-binding protein was not simply correlated with FOS expression (data not shown). Excitingly, all of the seven tumors had TGF-β type I receptor at lower levels than normal mucosas. This result suggests that the level of TGF-β type I receptor may reflect a reduction in the

level of TIE-binding protein, leading to enhanced expression of several genes including stromelysin gene, which participates in tumor invasion.[31] For example, as shown in Case 5, in which the tumor tissue expressed a low level of type I receptor for TGF-β, the level of TIE-binding protein was reduced in tumor tissue in spite of increased TGF-β expression (see Figs. 1 and 3). In addition, in MKN-1 cells, which expressed an extremely low level of TGF-β receptor, the level of TIE-binding protein was also very low.

Overall, the results of this study suggest that although human gastric carcinoma cells produce TGF-β, they exhibit a reduction in type I receptor and a low level of TIE-binding protein, resulting in escape from the inhibitory effect of TGF-β.

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