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Expression of Epidermal Growth Factor, Transforming Growth Factor-α and Their Receptor Genes in Human Gastric Carcinomas; Implication for Autocrine Growth

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The expressions of mRNA for epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and EGF receptor (EGFR) genes were examined in 7 human gastric carcinoma cell lines and 15 gastric carcinoma tissues and the corresponding normal mucosas. All of the gastric carcinoma cell lines expressed mRNA for EGFR and TGF-α genes. TMK-1 and MKN-28 cells also expressed EGF mRNA. Production of EGF, TGF-α and EGFR protein by gastric carcinoma cell lines was also confirmed by EGF and TGF-α specific monoclonal antibody binding. As for surgical specimens, EGFR and TGF-α mRNA were detected at high levels in all the tumor tissues. Interestingly, EGF mRNA was detected in 5 (33.3%) of the 15 gastric carcinomas but it was not detected in normal tissues. Moreover, anti-EGF and anti-TGF-α monoclonal antibodies inhibited the spontaneous ^{3}H-TdR uptake by gastric carcinoma cells. These results suggest that EGF and/or TGF-α produced by tumor cells act as autocrine growth factors for gastric carcinomas.

Key words: Epidermal growth factor — Transforming growth factor-α — Epidermal growth factor receptor — Autocrine growth factor — Gastric carcinoma

Recent investigations have revealed that oncogenes encode growth factors themselves, their receptors and other parts of the signal transduction mechanisms, which play an important role in controlling the growth of cancer cells and normal cells.[1)]

The v-*erb*B oncogene of erythroblastosis virus codes a product homologous to a portion of EGFR,[4] in which the binding domain is deleted.[2–4)] Binding of EGF or TGF-α to EGFR results in activation of tyrosine specific kinase activity and stimulates cell growth and DNA synthesis.[5, 6)] EGFR gene amplifications have been reported in squamous cell carcinomas,[2, 3, 7, 8)] malignant gliomas,[9–12)] breast carcinomas[13, 14)] and gastric carcinomas.[15, 16)] EGFR expressions have also been reported in a variety of tumor cells.[2, 3, 7–9, 17–22)]

We have previously demonstrated immunohistochemically that synchronous expression of EGF and EGFR was correlated with the depth of tumor invasion, metastasis and prognosis of human gastric carcinoma.[20, 21, 23)] Moreover, a good correlation was demonstrated between synchronous expression of TGF-α and c-Ha-*ras* p21 and malignancy of gastric carcinoma.[24)] These findings suggest that some gastric carcinomas show "autocrine secretion" for self-stimulation, whereby a tumor cell secretes EGF and/or TGF-α for which the tumor cell itself has a receptor.

In view of our immunohistochemical findings, it is important to search for mRNA for EGF, TGF-α and EGFR in human gastric carcinomas. In this study, we examined the expression of mRNA for EGF, TGF-α and EGFR and alterations of their genes in gastric carcinomas. Furthermore, we also confirmed the production of EGF and TGF-α protein by gastric carcinoma cell lines and also examined whether anti-EGF and anti-TGF-α MAbs inhibit the growth of gastric carcinoma cells.

MATERIALS AND METHODS

Cell cultures and tumor tissues Seven cell lines established from gastric carcinomas and A431 cells and HeLa cells were routinely grown in RPMI-1640 (Nissui Co., Tokyo) supplemented with fetal bovine serum (Whittaker M.A. Bioproducts Inc., Maryland). TMK-1 cell line (poorly differentiated adenocarcinoma) showing differentiation of parietal cells was established in our laboratory. KATO-III cell line which was established from signet ring cell carcinoma was kindly provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo). The other five human gastric carcinoma cell lines (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinoma; MKN-45,

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[4] The abbreviations used are: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; poly(A)$^{+}$, polyadenylated; cDNA, complementary DNA; kb, kilobase; T/N ratio, tumor/normal ratio.

poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima). Fifteen primary gastric carcinomas and their adjacent normal tissues were surgically removed, frozen immediately thereafter in liquid nitrogen and stored at −80°C until use. We confirmed microscopically that normal tissues did not contain tumor cells by cryostat sectioning.

RNA preparation and Northern analysis RNA were extracted by the guanidium isothiocyanate/cesium chloride method.[25] Ten to fifteen μg of poly(A)$^+$ selected RNA was electrophoresed on 1.0% agarose/formaldehyde gels and blotted onto zeta-probe nylon filter membrane (Bio-Rad Laboratories). Filters were baked for 2 h at 80°C under vacuum. Hybridization and washing procedures were performed as we have described previously.[15] Filters were autoradiographed overnight with Kodak XAR-5 films with the use of intensifying screens at −80°C.

DNA preparation and Southern blot analysis High-molecular-weight DNAs were prepared by the phenol-chloroform method as described elsewhere.[25] DNAs were digested with *Eco*RI and electrophoresed on 0.8% agarose gel. DNAs were denatured, neutralized and then transferred to a nitrocellulose filter membrane. Hybridization and washing procedures were described above.

DNA probes The 1.9 kb human EGF cDNA insert from phEGF15 was kindly provided by Dr. Graeme I. Bell,[26] 1.4 kb human TGF-α cDNA by Dr. Rik Derynck[27] and pE7 which contains a 2.4 kb insert of human EGFR cDNA by the Japanese Cancer Research Resources Bank (JCRB). β-Actin probe was purchased from Oncor, Gaithersburg, MD.

The binding of ^{125}I-labeled anti-TGF-α (WA-3) or anti-EGF (KEM-10) specific MAb to carcinoma cells Preparation and characterization of MAbs against hEGF (KEM-10) and hTGF-α (WA-3) (kindly provided by Wakunaga Pharm. Co., Hiroshima) have been previously described.[28] All MAbs used were purified by DEAE negative-ion exchange chromatography. Purity of MAbs was over 95% by gel-permeation high-performance liquid chromatography.

Membrane-bound TGF-α[29] and EGF were examined by ^{125}I-labeled WA-3 or KEM-10 binding assay in TMK-1, and MKN-1, 7, 28, 45 and 74 cells as we have reported recently.[30] Cells were trypsinized, and plated out at 1×10^6 cells per 6-well plate (NUNC) 24 h prior to initiation of the assay. At 2 h before the assay, the medium was exchanged with RPMI 1640 containing 20 m*M* HEPES, pH 7.4. The cells were incubated for 1 h at 37°C and then cooled on ice. The medium was exchanged with ice-cold binding buffer (RPMI 1640 containing 0.5% BSA and 20 m*M* HEPES, pH 7.4). For the binding assay, wells were set up in triplicate containing 0.1 μg of ^{125}I-WA-3 or KEM-10 and incubated for 3 h on ice. The samples were washed 3 times with binding buffer, lysed with 1 *N* NaOH and then counted in a gamma counter. Non-specific binding was determined in the presence of 100 μg of unlabeled MAbs.

EGF assay EGF was assayed by a highly specific sandwich enzyme-linked immunosorbent assay (ELISA) in TMK-1, and MKN-1, 7, 28, 45 and 74 cells as we have described elsewhere.[30]

Inhibition of spontaneous ^{3}H-TdR uptake of gastric carcinoma cells by anti-EGF (KEM-10) or anti-TGF-α (WA-3) MAb To determine whether EGF and/or TGF-α act as autocrine growth factors, spontaneous inhibition of ^{3}H-TdR uptake of MKN-28, TMK-1 and MKN-7 cells by specific MAbs for EGF and TGF-α was examined. Cells were inoculated at 1×10^5 cells per well in 24-well plates (Falcon) in 1 ml of RPMI 1640 medium with 10% FCS, and after 24 h the medium was exchanged with fresh medium supplemented with 0.5% FCS. After 24 h the cells were cultured with various concentrations of MAbs specific or nonspecific to EGF and TGF-α for 24 h. The cells were pulsed with 0.2 μCi per well of ^{3}H-TdR (5 μCi/mmol, New England Nuclear) for the last 4 h of incubation.

RESULTS

EGFR mRNA levels in gastric carcinoma cell lines and surgical specimens We initially examined human gastric carcinoma cell lines and surgical specimens for the presence of EGFR mRNA. As shown in Fig. 1, EGFR transcripts of 10.8 kb and 5.6 kb were clearly detectable in most of the cell lines examined. RNAs from A431 and HeLa cells were used as positive controls. In MKN-74 cells, the levels of 10.8 kb and 5.6 kb transcripts were very low, but about 2.9 kb transcript, which codes the sequence for the extracellular domain of EGFR in A431 cells, was also detected. As for surgical specimens, all the tumor specimens and the corresponding normal tissues exhibited 10.8 and 5.6 kb transcripts (Fig. 1). The ratio of EGFR expression in the gastric carcinomas relative to that of the corresponding normal mucosas was calculated as the T/N ratio. It was calculated by summing the densitometric signals of the two transcripts of EGFR mRNA and the results are summarized in Table I. The T/N ratio ranged from 0.6 to 20 and most tumor specimens showed higher mRNA levels than the corresponding normal tissues. In case 198, the tumor sample overexpressed EGFR mRNA with an additional band of about 4.4 kb, while its normal mucosa exhibited a low level of EGFR mRNA. β-Actin probe was used as an internal control (Fig. 1) and the T/N ratio was calculated as shown in Table I.

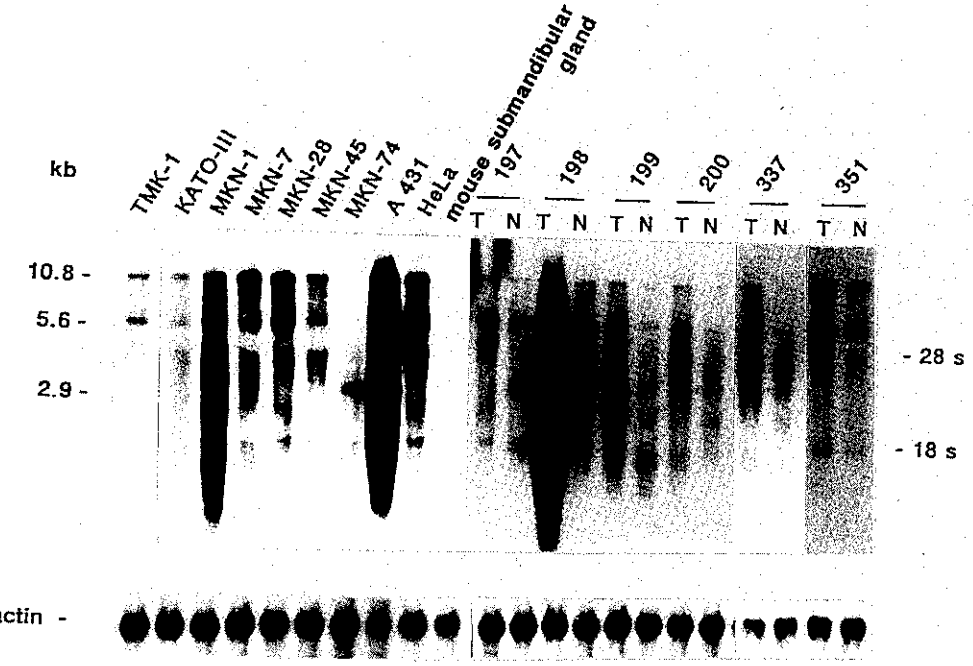

Fig. 1. EGFR mRNA levels determined by Northern hybridization in gastric carcinoma cell lines and surgically removed tumor specimens (T) and corresponding normal tissues (N). Numbers above the lanes are sample numbers. RNAs from A431 cells and HeLa cells were used as positive controls. β-Actin probe was applied as an internal control. The intensities of autoradiographic signals were determined by densitometry.

Table I. Clinical Features and mRNA Levels of EGFR, TGF-α, and EGF in 15 Primary Human Gastric Carcinomas

Case No.	Sex	Age	Histology[a]	Stage[a]	Relative expression T/N[b]			
					EGFR	TGF-α	EGF	β-actin
196	F	73	sci	III	1.4	1.2	−[c]	1.0
197	M	74	sci	IV	1.7	1.5	−	1.0
198	M	80	por	III	10–20	1.8	+[d]	1.1
199	F	63	well	I	7.5	1.4	−	1.1
200	F	55	sci	III	5.0	0.8	−	0.9
201	F	46	sci	IV	1.2	2.3	−	1.0
202	M	68	well	III	1.7	1.3	+	1.0
203	M	54	por	III	4.6	1.4	+	1.0
204	F	39	sci	III	0.6	1.1	−	0.8
67	M	63	por	IV	1.9	0.8	−	1.0
332	F	65	well	II	2.0	1.2	−	0.9
333	F	73	por	I	1.5	1.8	−	1.0
337	F	63	por	III	1.2	1.2	+	0.8
338	M	58	por	III	1.4	1.2	−	1.1
351	M	70	well	III	1.3	1.1	+	1.0

a) According to the criteria of the Japanese Research Society for Gastric Cancer (1985). Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; por, poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; sci, scirrhous gastric carcinoma.
b) The ratio of direct densitometric measurement of autoradiographic signals from Northern hybridization in gastric carcinoma tissues (T) and corresponding normal tissues (N).
c) −, Not detectable in tumor tissues or normal tissues.
d) +, Detected in tumor tissues but not detected in normal tissues.

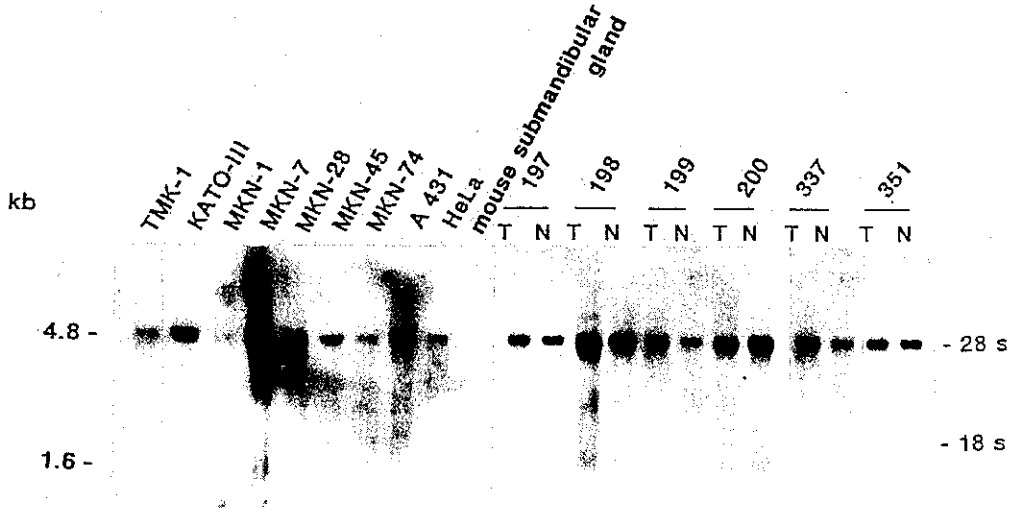

Fig. 2. TGF-α mRNA levels determined by Northern hybridization in gastric carcinoma cell lines and surgically removed tumor specimens (T) and corresponding normal tissues (N).

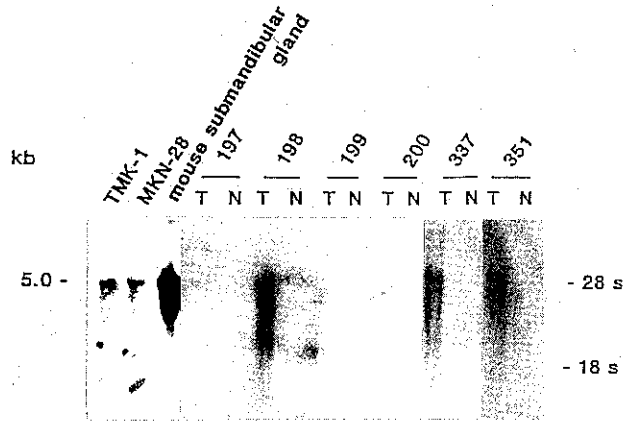

Fig. 3. EGF mRNA levels determined by Northern hybridization in TMK-1, MKN-28 cells and surgical specimens. Positive expression was observed in tumor tissues of case 198, 337 and 351. RNA from male mouse submandibular glands was used as a positive control for EGF.

In order to ascertain that the mechanism of EGFR mRNA expression was due to its gene alteration, Southern blotting was performed. Neither amplification nor rearrangement was observed in gastric carcinoma cell lines and tissues in the present study.

TGF-α and EGF mRNA levels in gastric carcinoma cell lines and surgical specimens To examine whether EGFR-expressing tumors express its ligands for autocrine growth promotion, an attempt was next made to detect the mRNA levels of TGF-α and EGF. We used 15 μg of poly(A)$^+$-selected RNA from cell cultures and 10 μg of poly(A)$^+$-selected RNA from surgically removed samples. The same filters were used to examine the mRNA levels in these studies. TGF-α mRNA of about 4.8 kb was clearly expressed at various levels by all gastric carcinoma cell lines tested, but it was not detected in mouse submandibular gland (Fig. 2). MKN-7, MKN-28 and KATO-III expressed rather high levels of TGF-α and the expression levels in A431 and HeLa cells were consistent with the data previously reported.[31] In the examination of surgical specimens, we could detect TGF-α mRNA not only in all the tumor tissues but also in the corresponding normal tissues. Some cases which expressed a high level of 4.8 kb transcript also faintly expressed 1.6 kb transcript as reported previously.[27,28] The T/N ratio was calculated (Table I) and ranged from 0.8 to 2.3. TGF-α mRNA levels in tumor tissues of most cases were slightly higher than those in normal mucosas.

Expression of mRNA for EGF of about 5.0 kb was weakly but evidently detected in TMK-1 cells and MKN-28 cells. However, it was not detected in other gastric carcinoma cell lines after long exposures (Fig. 3). RNA extracted from the male mouse submandibular gland was used as a positive control. In surgical specimens, out of 15 gastric carcinoma tissues, five tumor tissues (33.3%) exhibited EGF mRNA of 5.0 kb (Fig. 3), but EGF mRNA was not detectable in normal tissues or other tumor tissues (Table I). There was no amplification or rearrangement of TGF-α and EGF genes in the present study. The clinico-pathological features and the T/N

Table II. Expression of EGFR, TGF-α and EGF in Gastric Carcinoma Cell Lines

	EGFR			TGF-α		EGF	
	mRNA	Binding capacity[a] (sites/cell)	Kd (M)	mRNA	pg/10^6 cells[b]	mRNA	pg/10^7 cells[c]
TMK-1	++	144.0×10^3	529×10^{-12}	+	1.7	+	358
KATO-III	+	NE	NE	++	NE	−	NE
MKN-1	+++	34.5×10^3	218×10^{-12}	+	1.4	−	85
MKN-7	++	14.5×10^3	245×10^{-12} 6500×10^{-12}	+++	22.8	−	ND
MKN-28	++	21.4×10^3	144×10^{-12}	++	14.7	+	218
MKN-45	+	22.3×10^3	244×10^{-12}	+	6.4	−	163
MKN-74	+	ND	ND	+	1.6	−	98

a) Specific binding determined by ^{125}I-hEGF.
b) Binding of ^{125}I-WA-3 to membrane-bound precursor of carcinoma cells (cpm). ^{125}I-WA-3 1000 cpm=10 pg TGF-α precursor.
c) EGF content was determined by ELISA.
NE, not examined; ND, not detected.

ratio was also examined, but we found no correlation between these parameters and the T/N ratio.

The binding of ^{125}I-labeled anti-TGF-α (WA-3) or anti-EGF (KEM-10) MAb to gastric carcinoma cells and EGF assay We next determined the specific binding of ^{125}I-labeled anti-TGF-α (WA-3) and anti-EGF (KEM-10) MAb to six gastric carcinoma cell lines to detect membrane-bound TGF-α[29] and EGF, respectively. Specific binding of ^{125}I-anti-TGF-α (WA-3) MAb was detected at various levels in gastric carcinoma cells as shown in Table II. The binding ranged from 2282 cpm to 142 cpm and MKN-7 and MKN-28 cells revealed relatively high binding activity (Table II). Specific binding of ^{125}I-anti-EGF (KEM-10) MAb was not detectable in these cell lines.

On the other hand, EGF assay was performed by an ELISA that does not react with human TGF-α. EGF protein was present in the lysates of gastric carcinoma cells (Table II). TMK-1 and MKN-28 cells had EGF protein at concentrations of 358 and 218 pg/10^7 cells, respectively, but it was not detected in MKN-7 cells. These results strongly indicate that gastric carcinoma cells produce EGF and TGF-α protein.

Inhibition of spontaneous ^{3}H-TdR uptake of gastric carcinoma cells by anti-EGF (KEM-10) or anti-TGF-α (WA-3) MAb The ^{3}H-TdR uptake by MKN-28 cells when treated with MAbs was compared to that of a non-treated group (Table III). Anti-EGF MAb (KEM-10) reduced the ^{3}H-TdR incorporation by 67% at the concentration of 1000 μg/ml. Interestingly, anti-TGF-α MAb (WA-3) also reduced the ^{3}H-TdR uptake by 65%. In the case of TMK-1 cells, anti-EGF (KEM-10) MAb inhibited the ^{3}H-TdR uptake by 82%, while anti-TGF-α (WA-3) MAb inhibited the uptake by 32%. On the other hand, the KEM-10 and WA-3 reduced the ^{3}H-TdR uptake of MKN-7 cells by 44% and 74%, respectively, at the concentration of 1000 μg/ml but the control MAb had little effect on the cells. These results indicate that anti-EGF (KEM-10) and anti-TGF-α (WA-3) MAbs inhibit the biological activity of endogenous EGF and/or TGF-α by blocking their binding to the receptors.

Table III. Percent Inhibition of Spontaneous ^{3}H-TdR Uptake by MAbs in MKN-28

	Dose of MAbs (μg/ml)		
	0	100	1000
Anti-EGF MAb	0	7	67
Anti-TGF-α MAb	0	21	65
Anti-IgE MAb	0	−5	30

DISCUSSION

We have previously demonstrated that gastric carcinoma cell lines, including TMK-1, MKN-1, MKN-7, MKN-28, MKN-45, have high-affinity EGFR.[6] In the present study, we initially examined the expression of mRNA for EGFR by gastric carcinoma cell lines and compared the expression levels to the total binding sites/cell as shown in Table II. The expression level of mRNA and the amounts of binding site/cell showed slight discrepancies among the cell lines, but most of the gastric carcinoma cell lines synthesized EGFR mRNA and protein. In MKN-74, only 2.9 kb transcript was detected, but EGFR binding activity was not detected. Moreover,

MKN-74 cells showed no response to hEGF in cell growth and DNA synthesis.[6]

On the other hand, EGFR mRNA was detected in all the surgical specimens tested. We have recently reported the amplification and overexpression of the EGFR gene in a gastric carcinoma.[15] In the present study, no amplification of EGFR gene was detected but EGFR mRNA was detected not only in tumor tissues but also in normal mucosas. However, tumor tissues exhibited higher mRNA expression levels than those in the adjacent normal tissues. Higher levels of EGFR mRNA might provide for uncontrolled growth of carcinoma cells. The incidence of EGFR mRNA expression in tumor tissues was not consistent with our previous immunohistochemical finding that EGFR immunoreactivity was present in about 30–40% of gastric carcinomas.[20] Further study is necessary to elucidate whether mRNA expression mirrors the protein synthesis or kinase activities and also which cells synthesize mRNA.

EGF and TGF-α act through a common receptor and share many biological activities. EGF plays an important role in mammary tumorigenesis.[32] Some breast carcinoma cells express EGF mRNA, and its expression is increased by progestins.[33] In gastric carcinoma patients, EGF plays an important role in progression and high malignancy.[23] But so far, little is known about the EGF mRNA levels in gastric carcinomas. In the present study, EGF mRNA was detected by TMK-1 and MKN-28 cells and these cells contained relatively high levels of EGF protein in their lysates compared to those of other cells (Table II). Furthermore, we have recently reported that exogenous EGF increased the cell growth and stimulated the synthesis of EGFR mRNA.[34] Such a phenomenon is also observed in breast carcinoma cells[35] and A431 cells.[36] Moreover, we have also confirmed as an autocrine model that anti-EGF (KEM-10) and anti-TGF-α (WA-3) inhibited the spontaneous ^{3}H-TdR uptake by MKN-28 cells.

In surgical specimens, EGF mRNA was detected in five gastric carcinoma tissues (33.3%) among 15 gastric carcinomas, but interestingly it was not detected in normal gastric mucosas. The incidence of EGF mRNA expression in tumor tissues did not differ from immunohistochemical data that EGF was found in about 20–30% of advanced gastric carcinomas.[23] In case 198, the tumor tissue expressed a high level of EGFR mRNA and also expressed mRNA for EGF. Stern *et al.* demonstrated as an autocrine model that FR3T3 fibroblasts transfected with synthetic sequences encoding EGF in expression vector revealed uncontrolled proliferation and transformation.[37] Furthermore, Velu *et al.* reported that NIH 3T3 cells transfected with EGFR gene by retrovirus vector overexpressed a full-length EGFR and that they developed a fully transformed phenotype in the presence of EGF.[38] These *in vivo* and *in vitro* findings strongly suggest that EGF acts as an autocrine growth factor through its surface receptors in some gastric carcinomas.

On the other hand, TGF-α mRNA was detected in all the gastric carcinoma cell lines tested, as reported previously in other tumor cells.[39, 40] Moreover, membrane-bound TGF-α was also detected by binding of ^{125}I-anti-TGF-α MAb (WA-3) and the levels of TGF-α mRNA were consistent with the protein levels as shown in Table II. The amount of 25 kDa TGF-α precursor level was calculated as pg/10^6 cells, on the supposition that a single antibody binds to a single antigen. Di Marco *et al.*[41] demonstrated an autocrine interaction between TGF-α and EGFR which supports our present data. In their investigation, expression of either TGF-α or EGFR alone was not sufficient to induce the transformed phenotype in NIH3T3 cells and both the TGF-α and EGFR overexpression is essential to transform the cells. Recent investigations have revealed that membrane-bound pro-TGF-α binds to EGFR on the surface of contiguous cells and induces receptor autophosphorylation[42] leading to signal transduction.[43] As for surgical specimens, TGF-α mRNA was detected not only in tumor tissues but also in all the corresponding normal tissues, but the level of mRNA in tumor tissues was generally higher than that in normal tissues. Recently, Malden *et al.*[44] revealed the expression of TGF-α mRNA in normal mucosas of esophagus, stomach, small intestine and colon. Our data are similar to their data. Moreover, we found that TGF-α in normal gastric mucosa was immunohistochemically detected not only in epithelial cells but also in macrophages. Therefore, macrophages might be one of the sources of mRNA in surgical specimens.[45] This should be further examined by *in situ* hybridization to elucidate the localization of TGF-α expression in normal tissues and we should elucidate whether the TGF-α mRNA is translated into a precursor or mature protein in the normal gut.

As this paper was being prepared for submission, Bennett *et al.*[46] reported the expression of growth factors and EGFR in gastric carcinoma tissues. But in their analysis, TGF-α and EGFR were poorly expressed compared to our data and EGF mRNA was not examined. The difference might be due to how fresh the samples were and the amount of RNA applied in each case. Furthermore, we have analyzed DNA, RNA and protein levels in gastric carcinoma cell lines. On the other hand, the expression levels of TGF-β and PDGF mRNA were similar to their data as we have reported recently.[47, 48]

The direct inhibition of MAb for EGF and TGF-α was demonstrated by MKN-28, TMK-1 and MKN-7 cells. In conclusion, these results overall strongly suggested that EGF and/or TGF-α produced by tumor cells might act

as autocrine growth factors in the progression of gastric carcinomas.

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