Inhibition of ³H-TdR uptake for Human Gastric Carcinoma Cells by Epidermal Growth Factor Specific Antibody

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ABSTRACT

This study examined whether human gastric carcinoma cells produced EGF for self-stimulation, using TMK-1 and MKN-28 cells which express mRNA for EGF and EGF receptor. EGF protein production was confirmed by ELISA and flow cytometric analysis. The lysates of these cells replaced EGF-induced biological activity to EGF receptor bearing KB cells but the culture supernatants did not. Furthermore, EGF-radioreceptor assay revealed non-secretion of EGF product. EGF specific monoclonal antibody neutralized exogenous EGF-induced ³H-TdR uptake, and inhibited spontaneous ³H-TdR uptake of TMK-1 and MKN-28 cells. These results suggest that EGF specific antibody binds to EGF protein at the cell surface, leading to the inhibition of ³H-TdR uptake, and that EGF produced by tumor cells may act as an autocrine growth factor in human gastric carcinoma cells.

Key words: EGF, TGFα, Autocrine growth factor, Anti-EGF monoclonal antibody

EGF is a mitogenic polypeptide that binds to the EGF receptor and stimulates proliferation and differentiation of a great variety of cells in the gastrointestinal tract in vitro and in vivo[1,10,11]. Stern et al[29] have reported that constitutive release of EGF can lead to uncontrolled proliferation and cell transformation in the autocrine model.

Tahara et al have reported that patients with EGF positive gastric carcinomas have a worse prognosis than those with EGF negative carcinomas[30] and that EGF has an obvious influence on the growth of human gastric carcinoma cells[23]. Moreover, Yasui et al have demonstrated that a good correlation exists between the synchronous expression of EGF and its receptor and progression of gastric carcinoma[32]. These findings have led to the question whether or not some gastric carcinomas, with a EGF receptor, show autocrine production of EGF for self-stimulation.

In order to answer this question, a study was conducted to determine whether EGF acts as an autocrine growth factor in human gastric carcinoma cell lines TMK-1 and MKN-28 cells that express mRNA for EGF receptor.

MATERIALS AND METHODS
Cell culture

Two cell lines established from human gastric carcinomas were used. As reported previously[24], TMK-1 cell line, a poorly differentiated adenocarcinoma, was established in our laboratory. MKN-28 cell line, a well differentiated adenocarcinoma, was kindly provided by Dr. T. Suzuki (Fukushima University Medical School, Japan). KATO-III cell line, established from signet ring cell carcinoma, was kindly provided by Dr. Sekiguchi, M. (Tokyo University, Japan). EGF receptor bearing KB epidermoid carcinoma cell line was obtained from the American Type Culture Collection (ATCC). Cells were routinely propagated in monolayer culture at 37°C in a 5% CO₂/95% humidified-air atmosphere and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), glutamine and penicillin-streptomycin. Conditioned medium for EGF assay were obtained by incubating subconfluent TMK-1 and MKN-28 cells with RPMI 1640 media containing 0.5% FCS for 24 hours.

EGF and MAbs

Human recombinant EGF was kindly provided by Wakunaga Pharm. Co., ltd., Hiroshima, Japan[25]. The preparation and characterization of MAbs against human EGF (KEM-10) and human TGFα (WA-3) used in this study (kindly provided by

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Wakunaga Pharm. Co., ltd.), have been described previously[19]. All MAbs used in this study were purified by DEAE negative-ion exchange chromatography. The purity of MAbs was over 95% according to gel-permeation high performance liquid chromatography.

EGF assay

EGF was assayed by a highly specific sandwich ELISA with anti-EGF (KEM-1, KEM-10), which recognize different sites of recombinant hEGF and do not react with human TGFα. Briefly, cell pellets (1 × 10⁷ cells) were homogenized in 1 ml of extraction buffer (10 mM Tris-HCl pH 7.2, 0.15 M NaCl, 0.02 % NaN₃, 0.5 % NP40). Aliquots (100 μl) of the extract were added to anti-EGF MAb (KEM-10)-coated plates and incubated at 4°C overnight. After washing, horse-radish peroxidase-labelled anti-EGF MAb (KEM-1-HRPO) was added. Standard and cell samples were assayed in triplicate. Its detection limits was 100 pg/ml[33].

Flow cytometric analysis

Propidium diiodide and RNase A were obtained from Sigma and fluorescein-conjugated goat anti-mouse immunoglobulin (secondary antibody) from TAGO. Before the appropriate MAb treatment, the cells were fixed in 70% (v/v) ethanol and incubated for 30 min in goat serum. Cells were suspended in 100 μg per ml of anti-hEGF MAb or anti-hIgE MAb (negative control) and following overnight incubation at 4°C, the cells were pelleted and washed 3 times with PBS(−). The cells were incubated for 30 min with secondary antibody and after the last wash the aliquots were used for fluorescence microscopy and photomicrography, and then RNase A was added to a final concentration of 1-2 mg/ml (50-75 Kunitz/mg protein). After the final wash, propidium diiodide was added. The resulting fluorescence measurements were made on a FACSCAN flow cytometer/cell sorter (Becton Dickinson, Sunnyvale, CA). The data for analysis were obtained only for $G_{1/0}$ cells.

EGF-radioreceptor assay

Conditioned media were obtained by incubating confluent TMK-1 and MKN-28 monolayers with RPMI 1640 medium containing 0.5% FCS for 24 hours. The media (2 liters) were clarified by centrifugation and immunoadsorbed with CNBr-activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden) coupled with monoclonal anti-hEGF antibody KEM-10 for the batch method through overnight shaking. The Sepharose gels were separated by centrifugation, washed with PBS, and eluted with 1 M acetic acid. The samples were dialyzed against water, and then lyophilized. The powders were reconstituted with 2 ml of RPMI 1640 medium, and analyzed for their ability to inhibit ¹²⁵I-labeled hEGF binding to TMK-1 and MKN-28 cells, respectively[21,22].

RNA extraction and northern blot analysis

RNA was isolated by the guanidium thiocyanate/cesium chloride method[9]. Poly (A)⁺ RNA was isolated by one cycle of oligodeoxythymidylic acid cellulose chromatography[4]. Human EGF cDNA insert from EGF (1.9 kilobase pair) was kindly provided by Dr. Graeme I. Bell[6]. Hybridization was carried out as described previously[33,34].

Neutralization of the activity of exogenous and endogenous EGF by EGF specific monoclonal antibody

TMK-1 and MKN-28 cells were inoculated at 1 × 10⁵ cells per well in 24 well plates (Falcon) in 1 of RPMI 1640 medium. This was supplemented wiht 10% FCS, and after 24 hours the medium was exchanged with fresh medium supplemented with 0.5% FCS. After 24 hours, the cells were cultured with EGF (0 or 100 pM) in the presence or absence of various concentrations of MAb, specific or non-specific to EGF, for 24 hours. The cells were pulsed with 0.2 μCi per well of ³H-TdR (5 μCi mmol⁻¹, New England Nuclear) for the last 4 hours of incubation.

EGF replacing biological activity of the culture supernatants and the cell lysates

TMK-1 and MKN-28 cells were cultured at 1 × 10⁶ cells per dish in tissue culture dish (Falcon) in 10 ml of RPMI 1640 media supplemented with 0.5% FCS for 2 days, and the culture supernatants or the cell lysates were measured for EGF replacing activity utilizing EGF receptor bearing KB cells. KB cells were cultured in 24 well plates at 1 × 10⁵ cells per well with various concentrations of the culture supernatants or the cell lysates of TMK-1 and MKN-28 cells for 24 hours, and pulsed with ³H-TdR for the last 4 hours of incubation. The cell lysates were prepared as described previously[24].

Statistics

The results are expressed as the means ± SD. Statistics were analyzed by t-test.

RESULTS

EGF receptor number and EGF content

The number of surface EGF receptors per cell was obtained by carrying out a saturation binding study at 4°C with increasing concentration of ¹²⁵I-EGF, as previously reported[24].
Various histological types of six human gastric carcinoma cell lines had relatively high affinity EGF receptors (dissociation constant Kd = 10^{-9} to 10^{-10} M, receptor number = $1.5 × 10^4$ to $1.4 × 10^5$)[24]. EGF protein was present in the cell lysates of TMK-1 and MKN-28 cells at concentration of 358 ± 36 and 218 ± 11 pg/10⁷ cells, respectively, but was not detected in the supernatants (Table 1).

Flow cytometric analysis of EGF production

To investigate EGF production in TMK-1 and MKN-28 cells, propidium/fluorescence staining was used. EGF specific fluorescence in these cells is shown in Fig. 1. Anti-EGF MAb treated cells showed strong immunofluorescence when compared

Table 1. EGF receptor number and EGF level

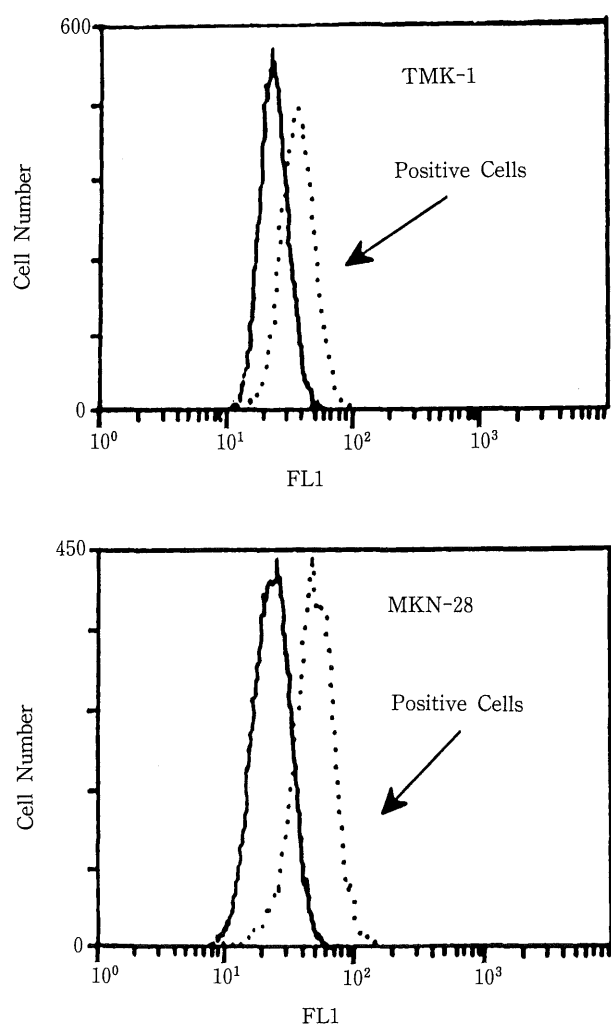| Cell line	Kd (M)	EGF receptors, no. per cell	EGF level	
			Cell lysate pg/10^7 cells	Culture supernatant
TMK-1	5.3×10^{-10}	14.4×10^4	258 ± 36	N.D.
MKN-28	1.4×10^{-10}	2.1×10^4	218 ± 11	N.D.

N.D.: not detectable

Fig. 1. Frequency distribution histograms of TMK-1 and MKN-28 cells immunofluorescently stained by anti-hEGF MAb or anti-hIgE MAb. Relative fluorescence (FL1) is expressed as a log function in the histogram (60 channels per decade, as illustrated). *Dotted line*, green fluorescence after treatment of anti-hEGF MAb; *solid line*, after treatment of anti-hIgE MAb (negative control).

with anti-hIgE MAb treated cells. These findings indicate that these cells produce EGF protein.

Neutralization of the activity of exogenous EGF by EGF specific monoclonal antibody

Recombinant hEGF stimulates the proliferation of human gastric carcinoma cells[24]. As shown in Fig. 2, exogenous EGF augmented ^{3}H-TdR uptake in TMK-1 and MKN-28 cells. Furthermore, the biological activity of hEGF was netralized by the
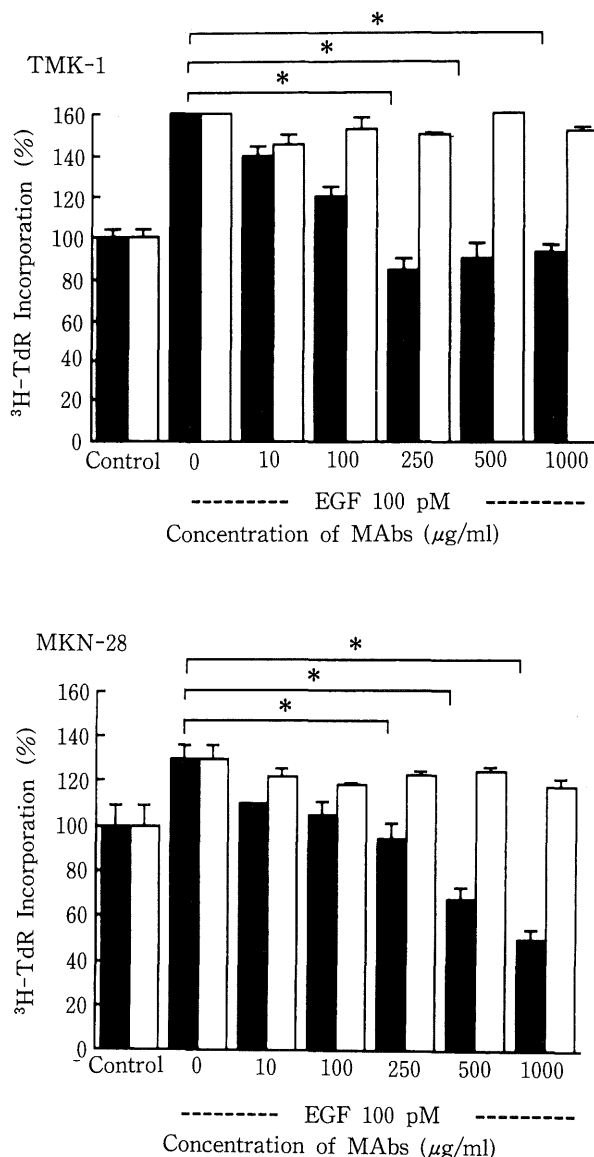
Fig. 2. Neutralization of the biological activity of exogenous EGF by the EGF specific MAb. TMK-1 and MKN-28 cells were cultured for 24 hours with recombinant human EGF in the presence of various concentrations of anti-hEGF MAb (KEM-10, ■) or anti-hIgE MAb (25B4, □). The data indicate ^{3}H-TdR incorporation in the experimental groups as a percentage of spontaneous ^{3}H-TdR incorporation and represent means ± SD of triplicate determinations. *, $P < 0.01$.

hEGF specific MAb but not neutralized by the hEGF nonspecific MAb. The data indicate that hEGF is actually a growth factor for human gastric carcinoma cells.
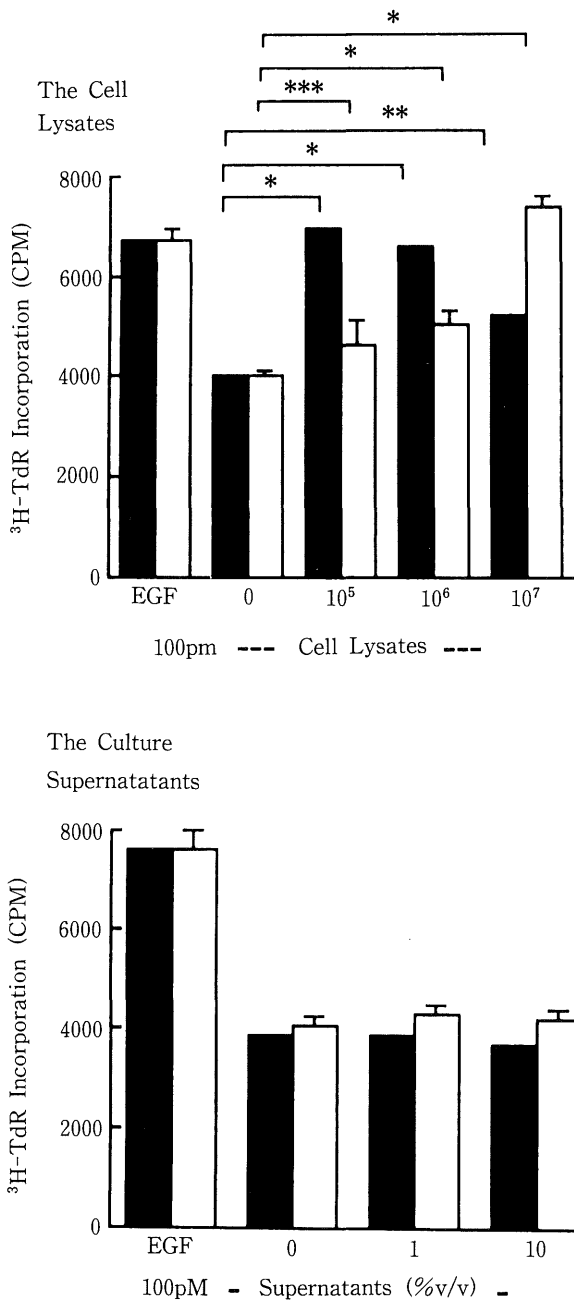
The Cell
Lysates

8000

6000

4000

2000

0

^{3}H-TdR Incorporation (CPM)

EGF   0 10^5 10^6 10^7

100pm --- Cell Lysates ---

MKN-28 cells increased ^{3}H-TdR uptake of KB epidermoid carcinoma cells. However, the culture supernatants of these cells did not increase.

EGF immunoreactivity in TMK-1 and MKN-28 conditioned medium

To investigate the nature of the translation product of the EGF gene in TMK-1 and MKN-28 cells, its ability to inhibit the binding of ^{125}I-labeled EGF to its receptor was examined (Fig. 4). Radioreceptor active material was not present in the conditioned medium of these cells. These results correspond with non-detection of EGF protein in the supernatants by ELISA.

Expression of EGF mRNA

Northern blot hybridization analysis of electrophoretically fractionated poly (A) $^+$NRNA revealed a single band corresponding to EGF mRNA 5 Kb TMK-1 and MKN-28 cells (Fig. 5). RNA extracted from the male mouse salivary gland was used as a positive control. The single mRNA species of approximately 5 Kb is also identified in some human breast cancer cell lines, and its expression is in
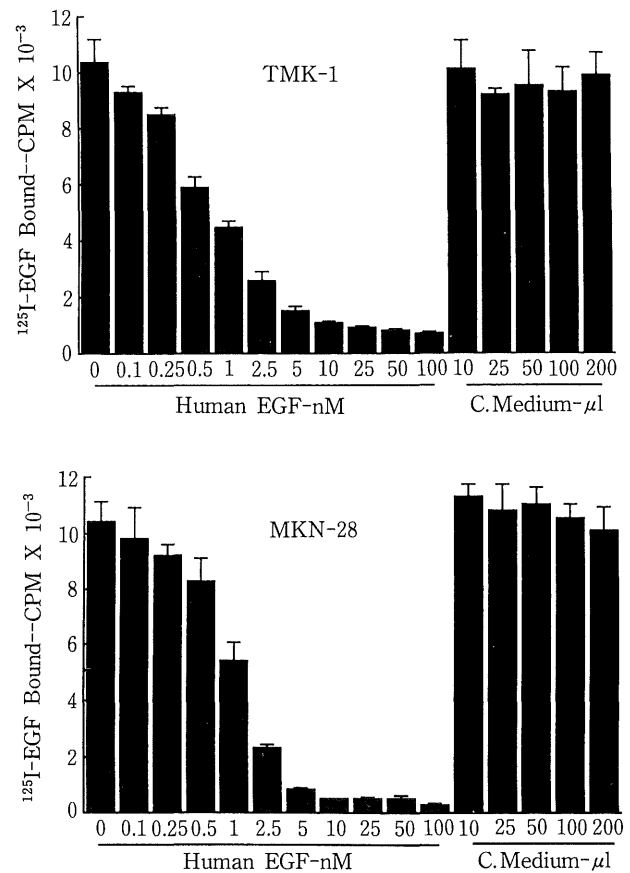

The Culture

Supernatatants

8000

6000

4000

2000

0

^{3}H-TdR Incorporation (CPM)

EGF   0   1   10

100pM - Supernatants (%v/v) -

Fig. 3. EGF replacing biological activity to KB of the cell lysates or the culture supernatants of TMK-1 and MKN-28 cells. KB were cultured for 24 hours in the presence of the cell lysates (*upper*) or the culture supernatants (*lower*) of TMK-1 (■) and MKN-28 (□) cells. The data indicate ^{3}H-TdR incorporation in the experimental groups and represent means ± SD of triplicate determinations. *, $P<0.005$, **, $P<0.025$, ***, $P<0.1$.

EGF replacing biological activity of the culture supernatants or the cell lysates of TMK-1 and MKN-28 cells

We examined whether hEGF produced by TMK-1 and MKN-28 cells has biological activities. KB human epidermoid carcinoma cell line, known to have 4×10^5 EGF receptors per cell, was used[31]. As shown in Fig. 3, the cell lysates of TMK-1 and

12

10

8

6

4

2

0

^{125}I-EGF Bound--CPM X 10^{-3}

TMK-1

0 0.1 0.25 0.5 1 2.5 5 10 25 50 100   10 25 50 100 200

Human EGF-nM   C.Medium-μl

12

10

8

6

4

2

0

^{125}I-EGF Bound--CPM X 10^{-3}

MKN-28

0 0.1 0.25 0.5 1 2.5 5 10 25 50 100   10 25 50 100 200

Human EGF-nM   C.Medium-μl

Fig. 4. EGF-radioreceptor assay of conditioned media obtained from TMK-1 and MKN-28 cells. Media were conditioned by TMK-1 and MKN-28 cell monolayers, immunoadsorbed, eluted, dialyzed, lyophilized, and reconstituted as described in Materials and Methods. The data represent means ± SD of triplicate determinations.
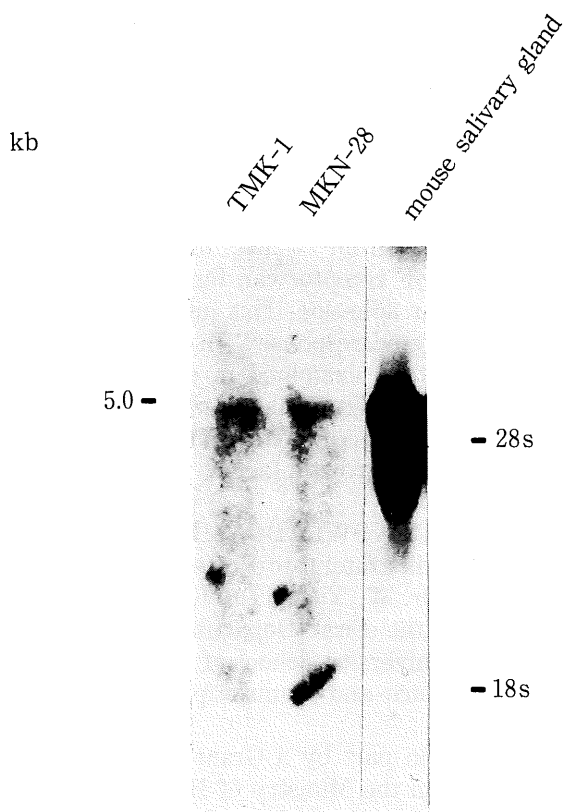
kb

5.0 ▬

▬ 28s

▬ 18s

Fig. 5. Expression of EGF mRNA in TMK-1 and MKN-28 cells. Poly (A) $^+$RNA (15 μg) from each cell line was electrophoresed in 0.8% agarose, transferred to nylon membrane, and probed with a hEGF cDNA (2 × 10^6 cpm). RNA size (in kilobase, kb) was determined by comparison with ribosomal RNA markers.

Fig. 6. Inhibition of spontaneous ^{3}H-TdR incorporation by MAb specific to EGF. TMK-1 and MKN-28 cells were cultured for 24 hours in the presence of 0, 100 or 1000 μg per ml of anti-hEGF MAb (KEM-10, ■), anti-hTGF MAb (WA-3, ▨), anti-hIgE MAb (25B4, ☐). The data indicate ^{3}H-TdR incorporation in the experimental groups as a percentage of spontaneous ^{3}H-TdR incorporation and represent means ± SD of triplicate determinations.

creased by progestins[22]. EGF mRNA was detected in five gastric carcinoma tissues (33.3 %) among 15 gastric carcinamas, but interestingly it was not detected in normal gastric mucosae[34].

Inhibition of spontaneous ^{3}H-TdR uptake of TMK-1 and MKN-28 cells

To demonstrate clearly that EGF produced by cancer cells plays a role in the generation of TMK-1 and MKN-28 cells, we examined whether EGF specific MAb inhibits ^{3}H-TdR uptake of TMK-1 and MKN-28 cells. Anti-EGF MAb inhibited the spontaneous ^{3}H-TdR uptake of TMK-1 and MKN-28 cells (Fig. 6). In the case of TMK-1 cells, anti-EGF MAb reduced ^{3}H-TdR incorporation by 80 % at a concentration of 1000 μg per ml, but the reduction of anti-TGFα Mab was of the same level as that of anti-hIgE MAb (negative control). On the other hand, in the case of MKN-28 cells, anti-EGF MAb reduced ^{3}H-TdR incorporation by 70 % at a concentration of 1000 μg per ml, and the reduction of anti-TGF MAb was at the same level as that of anti-EGF MAb. This inhibitory effect was not observed in the case of KATO-III which was immunofluorescence negative and not detected for EGF mRNA (data not shown).
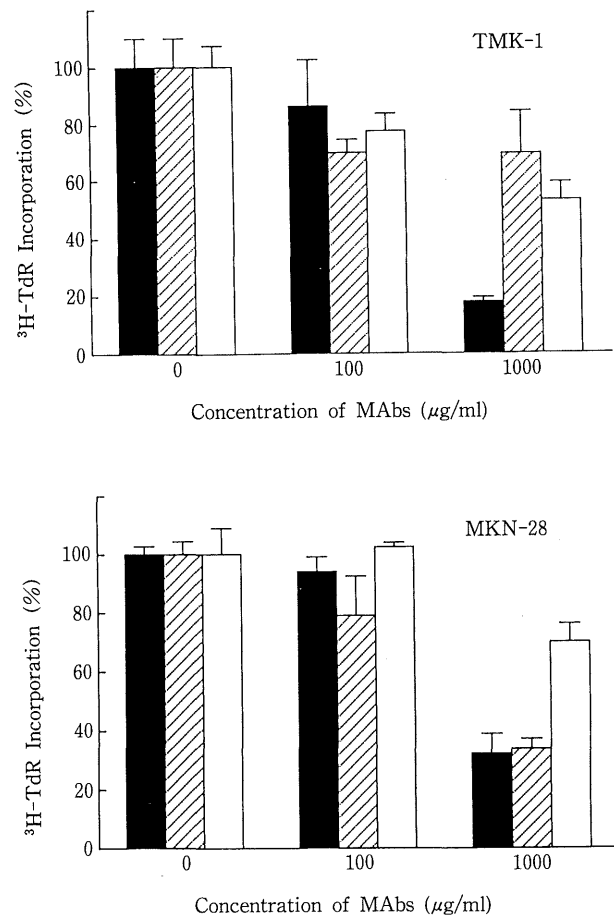
DISCUSSION

In this study, I examined whether EGF produced by gastric carcinoma cell lines, TMK-1 and MKN-28, act as an autorine growth factor.

The amounts of EGF protein in hte lysates and the culture supernatants of these cells were determined by ELISA and EGF specific immunofluorescence was found in these cells. However, EGF protein was not detected in the supernatants of these cells by EI ISA and EGF-redioreceptor assay. Therefore, it is likely that TMK-1 and MKN-28 cells synthesized EGF protein but did not secrete into the medium.

A MAb against hEGF was developed by use of recombinant hEGF as immunogen. The MAb could block the binding of labeled hEGF to its receptors and neutralized the biological activity of hEGF by blocking the binding of hEGF to its receptors. The lysates of TMK-1 and MKN-28 cells augmented ^{3}H-TdR uptake in KB cells used as target cells.

However, the culture supernatants of these cells did not affect ^{3}H-TdR uptake in KB. These findings directly corresponded with the amounts of EGF protein detected in the lysates and culture supernatants of these cells.

Anti-EGF MAb inhibited ^{3}H-TdR uptake of TMK-1 and MKN-28 cells. The possibility of non-specific toxic effects of MAbs on these cell lines was considered but ruled out. Since the same does of anti-hIgE MAb neither inhibited cell growth, nor the growth of KATO-III which did not express EGF mRNA (data not shown). Interestingly, in the case of MKN-28 cells, the same dose of anti-hTGFα MAb inhibited cell growth. Because TMK-1 and MKN-28 also expressed TGFα mRNA, and MKN-28 was higher rather than TMK-1 at its expression level (data not shown). These observations raise the possibility that EGF and TGFα produced by tumor cells act as autocrine growth factors[33,34].

Evidence has been accumulating to support the hypothesis, originally proposed by Sporn and Todaro[27,28], that autocrine stimulation by growth factors is a mechaism of cancer cell growth. For example, that PDGF[7], bombesin[12], TGFα[26], TGFβ[3], insulin-like growth factor I[18], interleukin 2[14], interleukin 6[20], and gastrin[17] act as an autocrine growth factors for various human cancers. In these studies, interleukin 6 was most clearly indicated. On the other hand, it was suggested that EGF-like domain in laminin, or other extracellular matrix proteins and in the extracellular portions of some mombrane proteins are signals for cellular growth and differentiation[15]. In addition, membrane-bound proTGFα is biologically active in the absence of processing, and can interact with EGF receptors on contiguous cells[8,35]. Furthermore, in recent studies, it has been reported that autocrine growth, as non-typical autocrine mechanism can occur as a result of the intracellular action of a growth factor, COOH-terminal-modified interleukin 3[13] or v-sis oncogene product, p28[v-sis5].

The results obtained in this study revealed that anti-hEGF MAb inhibited spontaneous ^{3}H-TdR of TMK-1 and MKN-28 cells which did not secrete EGF into the culture supernatants. If the interaction between EGF and its receptor occurs only at the cell membrane level as a typical autocrine loop, EGF could be detected in the culture supernatants. On the other hand, if the interaction takes place in the intracellular protein export apparatus including Golgi apparatus and exocytosis vesicles, anti-EGF MAb could not inhibit the growth of these cells. Therefore, EGF antibody might bind to EGF protein at the cell surface, leading to the inhibition of cell growth. Indeed, Hannink and Donoghue have shown, using an inducible v-sis expression system, that treatment of cells with monensin, which inhibits transport of proteins to the cell surface, prevents PDGF receptor function[16]. This suggests that growth factor and receptor must interact at

the cell surface. In other words, there are two possibilities. One is that a part of the ligand-receptor interaction might have taken place in the intracellular protein export apparatus, and the other is that EGF expressed on the tumor cell surface might bind to the EGF receptor on an adjacent tumor cell surface, leading to signal transduction. Additionally, Anklesaria proposed the term "juxtacrine" to designate the from which membrane-anchored TGFα and EGF receptor can function as mediators of intercellular adhesion. This interaction may promote a mitogenic response[2]. In future, these possibilities must be examined in detail.

The present findings allow us to suggest, therefore, that EGF produced by tumor cells may act as an autocrine growth factor.

ACKNOWLEDGEMENTES

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