Eur J Cancer, Vol. 28A, No. 12, pp. 1996–1999, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Effects of Human Recombinant Epidermal Growth Factor on the Growth of MKN-28 Human Gastric Carcinoma Transplanted into Nude Mice

Yoichi Murayama, Yasuyuki Onishi and Yoshio Mishima

The effects of human recombinant epidermal growth factor (EGF) on the growth of MKN-28 human gastric carcinoma, transplanted into nude mice were studied. Modulation of tissue cAMP and EGF receptor levels by EGF was also studied to reveal the mechanism of the growth inhibitory effects of EGF. EGF exhibited a dose-dependent growth inhibitory effect on MKN-28 human gastric carcinoma transplanted into nude mice. Local injection of 2 ng of EGF moderately inhibited the growth of MKN-28 gastric carcinoma, while injections of 20 ng, 200 ng and 2 μg of EGF significantly inhibited tumour growth. EGF decreased tissue cAMP levels in a dose-dependent manner within 24 h after EGF injection. On the other hand, EGF increased the EGF receptor levels up to two or three fold within 24 h after EGF injection. Conversely, the EGF receptor affinity for EGF decreased according to the increase in EGF receptor levels.
Eur J Cancer, Vol. 28A, No. 12, pp. 1996–1999, 1992.

INTRODUCTION

EPIDERMAL GROWTH factor (EGF) binds to its cell surface receptor and stimulates cell proliferation in various epithelial cells. EGF also inhibits cell growth *in vitro* of some types of breast cancer, epidermal cancer and oesophageal cancer which have high levels of EGF receptors [1, 2]. We recently reported the growth inhibitory effects of EGF on MX-1 and UM-1 human breast cancer and ES-4 oesophageal cancer transplanted into nude mice [3]. In this paper, we present an interesting relationship between EGF dose and tumour growth of MKN-28 gastric carcinoma transplanted into nude mice.

In addition to these investigations, the modulation of intracellular cyclic adenosine monophosphate (cAMP) and EGF receptor levels by EGF in MKN-28 gastric carcinoma transplanted into nude mice was studied to reveal the mechanism of the growth inhibitory effects of EGF on these carcinomas.

MATERIALS AND METHODS

Animals

Adult athymic nude mice (SPF/VAF Crj: CD-1(IcR)-nu: Charles River Breeding Laboratories Japan, Azugi, Japan), weighing 30–40 g were housed under specific pathogen-free conditions.

Tumours

MKN-28 human gastric carcinoma was obtained from a lymph node metastasis of gastric carcinoma in a 70-year-old female in 1975. The MKN-28 cell line is a well-differentiated adenocarcinoma. This cell line was obtained from Immuno-Biological Laboratory (IBL Inc., Fugioka, Gunma, Japan) [4].

Correspondence to Y. Murayama.
Y. Murayama and Y. Mishima are at the Second Department of Surgery, School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-Ku, Tokyo, Japan 113; and Y. Onishi is at the Central Institute for Experimental Animals, Tokyo, Japan.
Revised 22 Apr. 1992; accepted 22 June 1992.

Experimental protocol

MKN-28 cells were grown in RPMI-1640 supplemented with fetal bovine serum. Cell (5×10^7) in 0.3 ml of saline were transplanted into the subcutaneous tissue. 58 days after transplantation, the initially 2×3 mm tumour had grown to 15×15 mm and was transplanted into 15 nude mice. Tumour-bearing mice were divided into five groups 13 days after transplantation. Each group consisted of three experiments. For each group, 0.2 ml of buffer solution, containing 2 ng, 20 ng, 200 ng, 2 μg of EGF or no EGF, was injected subcutaneously around the tumour (EGF was dissolved in 0.2 ml of phosphate buffered saline, pH 7.4). The animals were examined on each of the subsequent 7 days for tumour growth and the diameter of the tumour was measured with calipers in two perpendicular directions (length and width). The estimated tumour weight, growth rate and growth curve were recorded to evaluate the effects of EGF on tumour growth. The estimated tumour weight (W) was calculated as follows: W = length (mm) × width $(\text{mm})^2/2(\text{mg})$.

The tumour growth rate was defined as Wb/Wa where Wa is the estimated tumour weight on day 13 after transplantation (before treatment), and Wb is the estimated tumour weight on day 7 after treatment. The EGF receptor levels were also assayed in the control and EGF-treated tumours removed on day 7 after injection. In a further experiment, buffer solution, 2 ng of EGF or 2 μg of EGF was subcutaneously injected around the tumour (tumour weight: average range 684 mg, 550–936 mg). Tumour was removed 24 h after injection and stored at −80°C. EGF receptor and cAMP levels in the tumours were assayed to detect modulation of the EGF receptor and cAMP levels by EGF.

EGF receptor (EGF-R) assay

EGF-R binding capacity was determined using a modification of the competitive binding assay described by Fitzpatrick or Sainsbury [3, 5, 6]. Tumour specimens were minced and homogenised using several intermittent bursts of a Polytron in ice-cold buffer. The homogenate was then centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was centrifuged at 100 000 *g* for 35 min at 4°C. The membrane pellets were resuspended in

phosphate buffer. 100 μl of membrane fractions were incubated with [^{125}I]EGF (0.01 pmole/100 μl/tube) in the presence or absence of 200 μl of unlabelled EGF (0–80 nM) for 2 h at 25°C. The binding was stopped by addition of ice-cold phosphate buffer containing bovine serum albumin (2 mg/ml). To separate the bound and free fractions, samples were incubated with 500 μl of 0.6% γ-globulin and 500 μl of 32% polyethylene glycol 6000 for 30 min at 4°C, followed by centrifugation at 2000 *g* for 30 min at 4°C. The pellets were counted in a gamma counter. The results were expressed as fmol bound per mg of membrane protein (measured according to Lowry *et al.* [7]).

Dissociation constant (K_d) was determined using Scatchard plot of the binding data [8] and levels of EGF-R-EGF complex were determined at various concentrations of added EGF. A Scatchard plot of the ratio of concentrations of specifically bound [^{125}I]EGF to free hormone (B:U) vs. bound hormone concentration was prepared. Where a single binding component was present, the equilibrium constant of dissociation, K_d, was equal to the reciprocal of the slope of this curve, and the extrapolated intercept on the abscissa was equal to the concentration of the binder–hormone complex.

Binding capacity (BC) and K_d were calculated as follows:

$$BC = 4X/\text{concentration of protein}$$
$$K_d = X/Y,$$

where X is bound [^{125}I]EGF concentration and Y is the ratio of concentration of bound [^{125}I]EGF to free hormone (Fig. 2). K_d was calculated after eight measurements.

Tissue cAMP assay

Tissue cAMP was determined using a radioimmunoassay as described by Steiner *et al.* [9] Tissue was stored at −80°C prior to analysis. Tissue was homogenised in iced conditions after adding 1 ml of 0.1 N HCl and then centrifuged at 3000 rpm for 15 min at 4°C. 2 ml of supernatant was succinylated prior to tissue cAMP assay. The level of tissue cAMP was determined by radioimmunoassay using a Yamasa radioimmunoassay Kit [10].

Materials

Human recombinant EGF was obtained from Wakunaga COLT (Hiroshima, Japan). EGF was biochemically synthesised from the amino acid sequence reported by Gregory [11, 12].

Statistical analysis

Statistical analysis was performed using the Student's *t*-test.

RESULTS

As shown in Fig. 1, EGF depressed tumour growth in a dose-dependent manner. A decrease in tumour weight was noted after injection of 2 μg of EGF. Tumour growth rates of the control tumour and tumours treated with 2 ng, 20 ng, 200 ng and 2 μg of EGF were [mean (S.D.)] 4.6 (0.9), 3.0 (0.3), 1.5 (0.3), 1.8 (0.7) and 0.8 (0.7), respectively. Growth rate [mean (SE)] of tumours treated with 20 ng, 200 ng and 2 μg of EGF was 1.4 ± 0.7. This is significantly different from that of control tumour ($P < 0.001$). The mean growth rates of control tumour and tumour treated with 2 ng of EGF were also significantly different ($P < 0.05$). Doubling time of control tumours was 4.25 days and that of tumours treated with 2 ng of EGF was 6.22 days.

Modulation of cAMP levels and binding capacity of the EGF receptor for EGF was also investigated in these tumours. EGF increased the binding capacity of the EGF receptor for EGF from 100 to 487 fmol/mg of protein for 7 days after EGF treatment. Modulation of cAMP and EGF receptor binding capacity by EGF was also seen at 24 h after injection of EGF (Table 1). EGF decreased tissue cAMP in a dose-dependent manner within 24 h after EGF injection. Tissue cAMP levels in control tumours, and tumours treated with 2 ng and 2 μg of EGF were 1888, 1049 and 884 pmol/g of wet tissue, respectively. On the other hand, EGF increased EGF receptor levels within 24 h after EGF injection. EGF receptor levels of control tumour, and tumour treated with 2 ng and 2 μg of EGF were 100, 326 and 200 fmol/mg of protein, respectively. According to the increase in EGF receptor levels, affinity of the EGF receptor for EGF decreased as shown in Fig. 2.

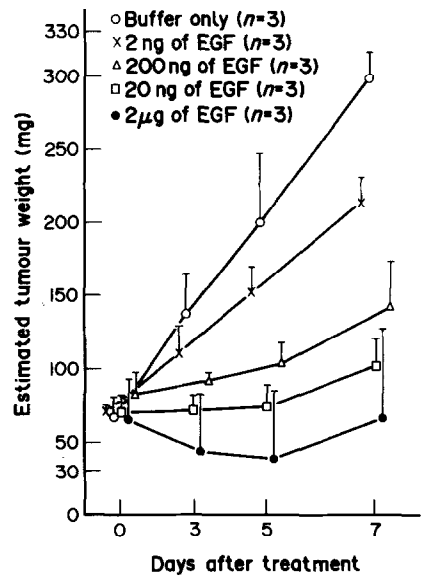

Fig. 1. Effect of EGF on tumour growth of MKN-28 gastric carcinoma.

DISCUSSION

Tumour growth and response to EGF treatment

It has been reported that the amount of EGF binding to the EGF receptor was 12.68 (1.98) fmol/mg of protein in gastric carcinoma and 5.72 (2.15) fmol/mg protein in non-neoplastic gastric mucosa, indicating that MKN-28 human gastric carci-

Table 1. Modulation of intra-cellular cAMP and EGF receptor by EGF in MKN-28 gastric carcinoma at 24 h after treatment with EGF

Tumours	Intra-cellular cAMP (pmole/g of wet tissue)	Binding capacity of EGF receptor (fmole/mg of protein)	*Kd* (PM)
Control	1888	100	319
Tumour treated with 2 ng of EGF	1049	326	411
Tumour treated with 2 μg of EGF	884	200	518

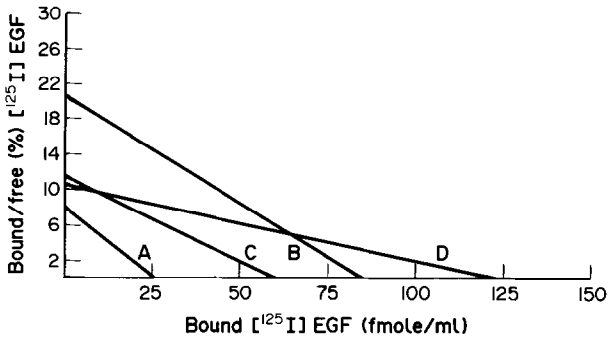

Fig. 2. Scatchard plot of the binding.
A: Control tumour binding capacity (BC) = 100 fmole/mg of protein, K_d = 319 PM. B: Tumour at 24 h after treatment with 2 ng of EGF: BC = 326 fmole/mg of protein, K_d = 411 PM. C: Tumour at 24 h after treatment with 2 μg of EGF: BC = 200 fmole/mg of protein, K_d = 518 PM. D: Tumour on day 7 after treatment with EGF: BC = 487 fmole/mg of protein, K_d = 1167 PM.

noma has a high level of EGF receptor binding [13]. We can explain the strong growth inhibitory effect of EGF on growth of MKN-28 human gastric carcinoma transplanted into nude mice from the high EGF receptor level of this tumour based on the results of EGF treatment with 20 ng, 200 ng and 2 μg of EGF. We can also explain the weak growth inhibitory effect of 2 ng of EGF for the same reason.

The relationship between EGF receptor status and response to EGF is very important. Squamous cell carcinoma such as ES-4 oesophageal cancer have high levels of EGF receptors on the cell surface and this tumour transplanted into nude mice responded very well to EGF as previously reported [3]. There is a positive correlation between response to EGF and EGF receptor status based on the *in vivo* results using MX-1 and UM-1 human breast cancer and the *in vitro* results using other breast cancer cell lines (AlAb 496, MCF-7, T-47, HBL-100, BT-474, SK-Br-3, BT-20, DU4475, and Lev III) [1]. For example, A-431 epidermoid cancer cells and BT-20 breast cancer cells which exhibit amplification of the EGF receptor gene respond very well to EGF. These cells respond to both high and low concentrations of EGF. On the other hand, DU4475 and Lev III, which do not have detectable EGF receptor binding sites, do not respond to EGF. These results indicate that the EGF receptor may have discriminatory power of response to EGF.

Modulation of EGF receptor and cAMP by EGF

The mechanism of action of EGF in the cell is not currently adequately understood. The first part of the process involves binding to the EGF receptor. The second involves internalisation through the G protein, which is an oncoproten of the *ras* oncogene. The third process involves activation of the so-called effector which causes activation of adenylate(A) cyclase and production of cAMP. The next signal-transduction is mediated through A-kinase. Finally, EGF activates many types of oncogenes, including *ras*, *myc*, and *fos* [4, 14]. EGF also activates transcription of EGF receptor gene mRNA [4, 14, 15]. To elucidate a detailed mechanism of the growth inhibitory action of EGF, we investigated the modulation of tissue cAMP levels and EGF receptor by MKN-28 human gastric carcinoma transplanted into nude mice, followed by treatment with EGF.

We demonstrated that 2 μg of EGF increased the binding capacity of the EGF receptor for EGF in MKN-28 human gastric carcinoma transplanted into nude mice. This modulation phenomenon is an effect of EGF on day 7 after EGF injection, and was also found in MKN-28 tumour removed at 24 h after treatment with EGF. There have been other papers which have reported the EGF-induced modulation of the EGF receptor. Johansson and Andersson [16] reported that a two-fold increase in the level of EGF receptor mRNA was observed 2–4 h after intraportal EGF injections. Cohen *et al.* [17] reported EGF receptor–protein kinase interactions and EGF stimulated phosphorylation activity of the EGF receptor in A-431 human epidermoid carcinoma cells.

Tahara *et al.* [4] reported that the 170 kDa EGF receptor was markedly phosphorylated by 10 and 100 nmol/l of EGF in an *in vitro* study. In addition to the modulation of the EGF receptor level, the decreased affinity of the EGF receptor for EGF is also important. It is suggested that decreased affinity of the EGF receptor for EGF binding associated with the increased EGF receptor level may be an important mechanism in the growth inhibitory effect of EGF in MKN-28 gastric carcinoma.

On the other hand, we noted significant modulations of cAMP by EGF. EGF caused a dose-dependent 53% decrease in intracellular cAMP within 24 h. This modulation causes inhibition of cellular metabolism which influences metabolism of glucose, amino acids, nucleic acids, lipids and synthesis of ATP, and will result in inhibition of cellular proliferation. There are few reports on the relationship between EGF and cAMP from *in vitro* and *in vivo* experiments. Some reports showed a modulation of cAMP by EGF. Greil *et al.* [18] reported the inhibition of cAMP formation by EGF in thyroid follicles. They concluded that EGF inhibits cAMP formation by a raise of intracellular Ca^{2+}, as well as by the direct activation of proteinkinase C, indicating that a phosphorylated product could be a mediator for the inhibition of adenylate cyclase. Our data in modulation by EGF on cAMP in MKN-28 gastric carcinoma is the first report *in vivo*. Our results indicate that the decrease in intracellular cAMP by EGF causes growth inhibition in MKN-28 human gastric carcinoma transplanted into nude mice.

In conclusion, 20 ng to 2 μg of recombinant human EGF exhibited inhibitory effects on tumour growth in MKN-28 human gastric carcinoma transplanted into nude mice. These growth inhibitory effects of EGF may be mediated through modulation of the EGF receptor and intracellular cAMP by EGF.

1. Imai YL, Clement KH, Friesen HG, Shiu RP. Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in longterm tissue culture. *Cancer Res* 1982, **42**, 4394–4398.
2. Kamata N, Chida K, Rikimaru K, Horikoshi M, Enomoto S, Kuroki T. Growth-inhibitory effects of epidermal growth factor and overexpression of its receptors on human squamous cell carcinomas in culture. *Cancer Res* 1986, **46**, 1648–1653.
3. Murayama Y. Growth-inhibitory effects of epidermal growth factor on human breast cancer and carcinoma of the esophagus transplanted into nude mice. *Ann Surg* 1990, **211**, 263–268.
4. Yoshida K, Tsujino T, Yasui Y, *et al.* Induction of growth-factor receptor and metalloproteinase genes by epidermal growth factor and/or transforming growth factor in human gastric carcinoma cell line MKN-28. *Jpn J Cancer Res* 1990, **81**, 793–798.
5. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS. Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progenstin receptor. *Cancer Res* 1984, **44**, 3448–3454.
6. Sainsbury JRC, Farndon JR, Harris AL, Sherbet GV. Epidermal growth factor receptors on human breast cancers. *Br J Surg* 1985, **72**, 186–188.
7. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein

measurement with the folin phenol reagent. *J Biol Chem* 1951, **193**, 265–275.
8. Scatchard G. The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 1949, **51**, 660.
9. Steiner AL, Parker CW, Kipnis DM. Radioimmunoassay for cyclic nucleotides. *J Biol Chem* 1972, **247**, 1106–1113.
10. Sohn HE, Furukawa Y, Yumita S, Miura R, Yoshinaga K, Yamane R. Scrutinization of the direct assay method for plasma cyclic AMP and clinical applications of nephrogenous cyclic AMP. *Folia Endocr* 1985, **61**, 912–923.
11. Nakagawa S, Yoshida S, Hirao Y, Kasuga S, Fuwa T. Biological effects of biosynthetic human EGF on the growth of mammalian cells *in vitro*. *Differentiation* 1985, **29**, 284–288.
12. Gregory H. Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature* 1975, **257**, 325–327.
13. Yasui W, Sumiyoshi H, Hata J, *et al.* Expression of epidermal growth factor receptor in human gastric and colonic carcinoma. *Cancer Res* 1988, **48**, 137–141.
14. Muller R, Bravo R, Burckhardt J, Curran T. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature* 1984, **312**, 716–720.
15. Fernandez-Pol JA, Klos DJ, Hamilton PD, Talked VD. Modulation of epidermal growth factor receptor gene expression by transforming growth factor in a human breast carcinoma cell line. *Cancer Res* 1987, **47**, 4260–4265.
16. Johnasson S, Andersoon G. Similar induction of the hepatic EGF receptor *in vivo* by EGF and partial hepatectomy. *Biochem Biophy Res Commun* 1990, **166**, 661–666.
17. Cohen S, Carpenter G, King L Jr. Epidermal growth factor-receptor-protein kinase interactions. *J Biol Chem* 1980, **255**, 4834–4842.
18. Greil W, Niedernhuber G, Stubner D, Gartner R. Inhibition of cMAP formation by EGF in thyroid follicles is mediated by intracellular Ca. *Acta Endocr* 1987, **281** (suppl.), 267–269.

Eur J Cancer, Vol. 28A, No. 12, pp. 1999–2002, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Constitutive Tissue Factor Expression of Human Breast Cancer Cell Line MCF-7 is Modulated by Growth Factors

Conrad Flössel, Thomas Luther, Sybille Albrecht, Matthias Kotzsch and Martin Müller

Expression of tissue factor, the initiator of the extrinsic coagulation protease cascade, is a feature of certain malignant tumours. To study the modulation of tissue factor expression we incubated the breast cancer cell line MCF-7 with several growth factors. Epidermal growth factor (EGF), transforming growth factor α (TGFα) and interleukin-1 (IL-1) rapidly increased tissue factor expression of MCF-7 cells peaking at 6–8 h after starting point of incubation, as determined by clotting test, enzyme linked immunosorbent assay and flow cytometry. The data presented support the hypothesis that modulation of constitutive tissue factor expression in tumour cells by TGFα and IL-1 could also occur *in vivo* possibly resulting from interactions of stromal and cancer cells. The meaning for tumour biology, however, remains unclear.
Eur J Cancer, Vol. 28A, No. 12, pp. 1999–2002, 1992.

INTRODUCTION

TISSUE FACTOR (TF) is a 47 kDa transmembrane glycoprotein receptor and the essential cofactor for factors VII/VIIa, thereby triggering the cell surface assembly of coagulation protease cascade, finally leading to formation and deposition of fibrin [1–3]. The human TF gene has been cloned [4]. Its molecular biology of expression and function has been reviewed and discussed recently [5]. The constitutively expressed TF is preferentially detectable in cells corresponding to biological boundary layers (skin, epithelia of respiratory and gastrointestinal tract, cerebral cortex and adventitia of vessels) as described by Drake *et al.* [6] and Fleck *et al.* [7]. Within the vasculature, endothelia and monocytes can be induced by endotoxins or cytokines to start synthesis of TF (for review, see [5]), a phenomenon which is commonly accepted as a link between haemostasis and inflammation [5]. TF can be also detected in cells of certain malignant tumours [8, 9]. Furthermore, fibrin deposits have been localised in several tumours and metastases (10, 11], and clotting abnormalities are frequently observed in tumour patients [12, 13]. But until now, there is no clear evidence what role, if any, TF expression and activation of blood coagulation cascade proteases would play in tumour growth.

Recent studies from our laboratory have shown that human primary breast cancer tissues express TF in a highly individual manner [14]. The amount of immunohistochemically detectable TF apoprotein showed a close correspondence to the procoagulant activity (PCA) of those tumour tissues. Besides the individual variations of TF in 115 primary cancers, a highly heterogeneous distribution of TF-positive cancer cells was the predominant pattern. The latter could reflect genomic heterogeneity of the cancer cell populations, as well as different

Correspondence to C. Flössel.
The authors are at the Institute of Pathology, Medical Academy Dresden, Fetscherstrasse 74, O–8019 Dresden, F.R.G.
Revised 15 June 1992; accepted 22 June 1992.