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Epidermal Growth Factor Prolongs Survival Time of Tumor-bearing Mice

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We observed that human epidermal growth factor (hEGF) alone prolonged the survival time of mice bearing various murine syngeneic tumors as well as athymic nude mice bearing human xenografts. No changes in the subcutaneous solid tumor mass volume were observed. Prolongation of survival time by hEGF was observed in mice bearing murine epidermoid carcinoma (BSC) and human gastric carcinoma (KATO III), but not in murine epidermoid carcinoma (KLN205) or human epidermoid carcinoma (A431). Human tumor cells such as A431, KATO III, and murine tumor cells, KLN205, BSC had roughly 2×10^6, 3×10^4, 1.3×10^3 and 1×10^3 EGF receptors/cell, respectively. Although KLN205 and BSC tumor cells maintained nearly the same number of EGF receptors, the effects of hEGF were very different. Although A431 tumor cells had nearly 100 times more receptors than KATO III cells, the prolongation of survival time of mice bearing A431 by hEGF was no better than that of mice bearing KATO III. Accordingly, it appears that this prolongation of survival time by hEGF is independent of the number of EGF receptors on tumor cells. In addition, hEGF was shown to inhibit experimental pulmonary metastasis of murine BSC tumor, but was ineffective with murine KLN205 tumor. These results suggest that prolongation of survival time by hEGF may result from the inhibition of tumor cell metastasis and EGF may play a role in preventing the metastasis of certain malignant neoplasms unrelated to its effects through the EGF receptor on tumor cells.

Key words: Human epidermal growth factor — Survival time — Experimental pulmonary metastasis — Epidermal growth factor receptor — Laminin

Epidermal growth factor (EGF), a 53-amino-acid polypeptide, was originally isolated from mouse submandibular glands (mouse EGF),[1] and a homologous peptide, urogastrone, was obtained from human urine (human EGF).[2] EGF exerts various biological actions such as stimulation of proliferation of the epidermis and several epithelial tissues both in cell cultures and *in vivo*.[3–6]

Recently, we reported EGF-receptor-mediated selective cytotoxicity of antitumor agents toward human tumor xenografts in nude mice and murine syngeneic solid tumors.[7] Coadministration of EGF did not, however, enhance the toxicities of antitumor agents as estimated from the changes of LD_{50} values or body weight loss.[7] The above selective potentiation of efficacy of the antitumor agents by human EGF can be characterized as follows. Human EGF enhanced the efficacy of an antitumor agent, 5-fluorouracil (5-FU), against human epidermoid carcinoma A431 transplanted sc in athymic nude mice [ED_{50}=2.9 (0.2–49.7, 95% confidence interval) μg/kg, sc] in a dose-dependent manner. Various degrees of enhancement were also observed against other experimental tumors transplanted sc. With regard to 5-FU and cisplatin, the degrees of enhancement were directly proportional to the numbers of hEGF binding sites present on tumor cell plasma membrane (threshold of binding site density = 1.5×10^3 sites/cell), thus suggesting that the binding of EGF to the receptors on tumor cells is an essential process in enhancing the susceptibility of tumor cells to antitumor agents.[7] Normal cells such as intestinal epithelial and bone marrow cells are endowed with relatively few EGF binding sites; somewhat less than 10^3 sites/cell. This may explain in part the absence of EGF-enhanced cytotoxicity by antitumor agents toward normal cells. Here we have extended our study on the effect of hEGF alone on the survival time of mice bearing various solid tumors or human tumor xenografts.

Abbreviations: hEGF, human epidermal growth factor; MST, median survival time; YIGSR, Tyr-Ile-Gly-Ser-Arg.

MATERIALS AND METHODS

Materials Human EGF was produced through recombinant DNA techniques in quantities large enough for *in vivo* animal experiments by Wakunaga Pharmaceutical Co., Ltd.[8] Tyr-Ile-Gly-Ser-Arg (YIGSR)[9] was purchased from Peptide Institute Co., Ltd., Osaka. Human EGF and YIGSR were dissolved in phosphate-buffered saline, pH 7.4, containing 0.01% Tween 80, which was added to avoid the adsorption of hEGF on vessels.

Animals Male C57BL/6 mice weighing 18–25 g as well as BALB/c male nude mice weighing about 20 g were

used in these studies. C57BL/6 mice were obtained from Charles River Japan, Inc., Kanagawa. Athymic mice, BALB/c A-nu, were obtained from Clea Japan, Inc., Tokyo. Athymic mice had been maintained under specific-pathogen-free conditions, and were housed in sterilized cages. Animals were provided with sterilized food, drinking water, bedding, and filters. All cages were placed in a laminar-air-flow unit.

Tumors Murine epidermoid carcinoma (BSC), murine colon adenocarcinoma No. 38 (colon 38), or human gastric carcinoma (SC-6-JCK) were maintained by serial subcutaneous transplantation of 30–50 mg murine tumor fragments or 1–2 mm cubes of human tumor in syngeneic C57BL/6 mice or athymic nude mice. Murine squamous carcinoma (KLN205) cell line was grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (M. A. Bioproducts, MD), human epidermoid carcinoma (A431) cell line in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (M. A. Bioproducts) and human gastric carcinoma (KATO III) cell line in RPMI1640 medium containing 10% fetal calf serum. Murine leukemia (P388) was maintained by serial intraperitoneal passage every 10–14 days in male DBA/2N mice. The donors of these tumor cell lines were previously described.[7]

Evaluation of antitumor activity Solid tumor-bearing mice used were 7 weeks old at the beginning of treatment (8 weeks old in the case of athymic mice). Human EGF was given subcutaneously in the abdominal region at 4–14 days after inoculation. Antitumor activity was evaluated by two methods, the measurement of tumor diameters as previously described[7] and the recording of survival time. Median survival time (MST) defined as the median day of death for an EGF or control group, was determined. The results were expressed as follows:

$$\%\ \mathrm{T/C}=\frac{\text{MST of hEGF-treated animals}}{\text{MST of control animals}}$$

Each experimental group contained five to 13 animals.

Pulmonary metastasis counting The subcutaneous inoculation of 30–50 mg of fragmented BSC tumor cells led to 100% pulmonary metastasis and resulted in the formation of more than 23 pulmonary metastatic foci within 3 weeks of inoculation. To determine the extent of inhibition of pulmonary metastasis by hEGF and the cytotoxicity of hEGF in terms of solid tumor mass volume, a group of mice was injected subcutaneously with vehicle of hEGF (10 ml/kg). Other groups of mice received hEGF (0.1 mg/kg) or the pentapeptide, YIGSR (30 mg/kg) subcutaneously in the abdominal region. The treatment was given 4 times at intervals of 3 days (4, 7, 10, 13 days after inoculation). Three weeks after inoculation, mice were killed and the number of pulmonary tumors on the surface of the lungs was counted. The counts of pulmonary metastatic foci of mice bearing solid tumors were measured as above, summed, and arbitrarily classified into 8 categories as follows: a metastasis index value in a mouse of 0, 1, 2, 3, 4, 5, 6, 7, or 8 represents 0, 1–5, 6–10, 11–15, 16–20, 21–25, 26–30, or >31 pulmonary metastatic colonies, or death, respectively.

The subcutaneous inoculation of 5×10^5 suspended KLN205 tumor cells led to 100% pulmonary metastasis. To evaluate the extent of inhibition of pulmonary metastasis by hEGF and the cytotoxicity of hEGF in terms of solid tumor mass volume, the same treatments except for the schedule of administration were done with BSC tumor. The treatment was given 14 times at daily intervals (from 7 days after inoculation). Three weeks after inoculation, mice were killed and both of the lungs were weighed, since the degree of pulmonary metastasis was directly proportional to the lung weight.

Binding assay for EGF receptor In order to elucidate the number of EGF receptors on tumor cells, the cells were treated as follows. Solid tumors (BSC, colon 38) were carefully dissected, minced, suspended in Hanks' solution (pH 7.5) and treated with type I collagenase (0.1%), deoxyribonuclease I (0.025%) and trypsin inhibitor (0.05%) at 37°C for 30 min. Binding assay for EGF receptors was performed as previously described.[7] Binding assays for tumor cell monolayers (KLN205, P388, A431, KATO III, SC-6-JCK) were performed by the method of Richert *et al.*[10]

Results were measured with a gamma counter. Estimation of binding parameters was done by plotting the dose-displacement data, expressed as the bound-to-free ratio vs. bound EGF. The nonlinear Scatchard plots were analyzed using a two-site model[11] by an iterative nonlinear regression approach.

Statistical analysis All statistical analyses were performed on a personal computer, model PC-9801 VX21 (NEC Corporation, Tokyo). Student's *t* test or the Aspin-Welch method after the F-test was used for statistical analysis of differences in tumor size, nontumorous body weight or lung weight. Mann-Whitney's U-test was employed in the case of pulmonary tumor metastatic colonies, and life span. A value of $P<0.05$ was used as the criterion of significance.

RESULTS

Prolongation of survival time of mice bearing colon 38 solid tumor by hEGF alone The survival rate of animals given inoculations of murine colon 38 was determined (Fig. 1B). In this experiment, median survival time (MST, days), when 50% of the untreated or hEGF-treated animals had succumbed to the tumor burden, was 33 or 65 days following tumor inoculation. The MST of the animals receiving hEGF was significantly (197%)

longer than that of non-treated mice ($P<0.01$ by Mann-Whitney's U-test). However, there was no significant effect of hEGF alone on subcutaneous solid tumor mass volume as shown in Fig. 1A. Nontumorous body weights were also measured every 3–4 days. Body weights (ranging from 14 to 19 g) were randomized to obtain the control group (16.6±0.3 g) and hEGF group (16.5±0.4 g). Treatments with hEGF prevented a significant reduction in body weight of tumor-bearing animals (93.7±3.8%) as compared to the control group (73.2±4.2%).

Effect of hEGF alone on survival time of mice bearing various solid tumors Inoculation of tumor cells used in this study into the right axillary region (sc) of mice on day 0 led to 100% tumor take. The survival rate of animals given inoculations of various tumor cells was determined. As shown in Table I, hEGF prolonged the survival time of mice bearing various tumors.

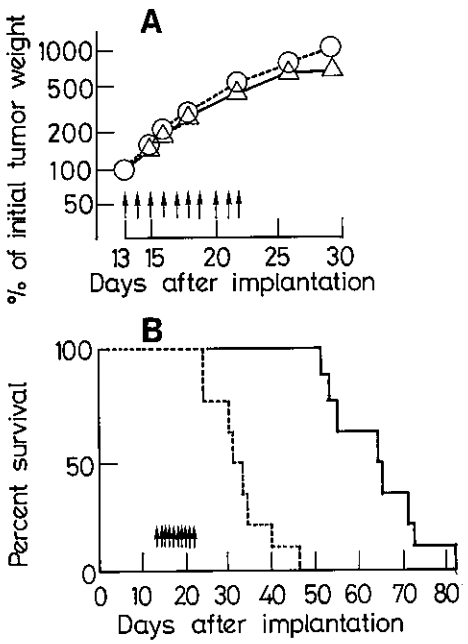

Fig. 1. Effect of hEGF on the growth of the colon 38 transplantable murine colon adenocarcinoma in syngeneic C57BL/6 mice (A), or on life span (B). Inoculation of 30–50 mg of fragmented colon 38 tumor cells in the right axillary region (sc) on day 0 led to 100% tumor take. The variation of tumor weight in each group was minimal 13 days after inoculation as follows; control group (464.0 ± 57.7 mg), hEGF group (456.1±50.3 mg). Groups were treated 10 times from 13 to 22 days after tumor inoculation with hEGF (1 mg/kg, sc) (—△—) and PBS with 0.01% Tween 80 (10 ml/kg, sc) (···○···). Arrows indicate the time of administration. Points and bars represent means and ±SE of 8 animals in each group. Mice were identified as being alive or dead every day. Survival time of mice treated with hEGF was significantly longer than that of non-treated mice ($P<0.01$ by Mann-Whitney's U-test).

Table I. Effect of hEGF on Survival of Mice Bearing Various Solid Tumors

Tumor	hEGF			n	MST (day)	[T/C] (%)
	Dose (mg/kg, sc)	Schedule	Times			
P388	1	daily	7	7	13.3	[101.5]
BSC	1	every 3 days	4	5	40.5	[120.9]*
KLN205	0.1	daily	14	13	57.5	[107.5]
	1	daily	14	13	57.5	[107.5]
Colon 38	1	daily	10	8	65.0	[197.0]**
KATO III	0.1	weekly	6	5	147.5	[335.2]**
SC-6-JCK	1	weekly	4	7	115.5	[153.0]
A431	1	daily	14	8	138.5	[105.6]
	1	weekly	5	8	137.5	[101.9]

CDF1 mice (7 mice/group) were inoculated sc with a suspension of 1×10^6 cells of P388 murine leukemia cells on day 0. C57BL/6 mice (5–13 mice/group) were inoculated sc with 30–50 mg of fragments of BSC, colon 38 or with a suspension of 5×10^5 cells of KLN205. BALB/c A-nu nude mice (5–8 mice/group) were inoculated sc with 1–2 mm cubes of SC-6-JCK or with a suspension of 1×10^6 cells of KATO III or A431 tumor cells on day 0. P388-, KLN205-, colon 38-, or A431-bearing mice or nude mice received 7–14 administrations of vehicle or hEGF (0.1 or 1 mg/kg, sc) daily from day 7–14. BSC-bearing mice received 4 administrations every 3 days from day 4. KATO III-, SC-6-JCK- or A431-bearing nude mice received 4–6 administrations weekly from day 12–16. Each value represents median survival time, MST (day) and % T/C. The probabilities are indicated as: **, $P<0.01$, *, $P<0.05$ vs. vehicle (control) by Mann-Whitney's U-test.

Mice bearing BSC, colon 38 or KATO III tumor had a median survival time of 33.5, 33 or 44 days, respectively, after tumor inoculation when treated with sterile phosphate-buffered saline with Tween 80 as the vehicle of hEGF. Human EGF showed antitumor activity against colon 38, BSC or KATO III when administered as repetitive sc injections as indicated in Table I. Mice bearing BSC, or colon 38 tumor had a median survival time of 40.5 days (% T/C=120.9), or 65.0 days (% T/C=197) when treated with hEGF at a dose of 1 mg/kg. Even at a dose of 0.1 mg/kg, hEGF caused a remarkable prolongation (MST=147.5 days; % T/C=335.2) of the survival period in mice bearing KATO III tumor. But the survival times of mice bearing P388, KLN205, SC-6-JCK and A431 were not prolonged significantly by hEGF (1 mg/kg). The hEGF treatments did not have any effect on the tumor growth or non-tumorous body weight change relative to the control group (data not shown).

Non-correlation between the amount of EGF receptors and prolongation of survival time of tumor-bearing mice by hEGF The correlation between the number of EGF receptors on the tumor cells and prolongation of survival time in tumor-bearing mice was examined. Cancer cell lines used in this study had various numbers of EGF receptors on their plasma membranes. Estimated numbers/cell were: BSC, 1×10^3; KLN205, 1.4×10^3; KATO III, 3×10^3; colon38, 1×10^4; SC-6-JCK, 1.4×10^5; A431, 1.2×10^6. We, however, failed to detect EGF receptor on mouse P388 leukemia cells. As shown in Table I, degrees of hEGF-induced prolongation of survival time of mice bearing tumors were determined for various sc transplantable tumors including human tumors (A431, SC-6-JCK and KATO III), and murine tumors (BSC, KLN205, colon 38 and P388). Survival times were expressed as % T/C. Human EGF prolonged the survival time of mice bearing BSC, colon 38, or KATO III tumors, but not that of mice bearing P388, KLN205, SC-6-JCK or A431 tumors. As shown in Fig. 2, there was no correlation between the number of EGF receptors and prolongation of survival time in tumor-bearing mice.

Effect of hEGF on the formation of pulmonary metastasis As shown in Fig. 3, hEGF (0.1 mg/kg, sc) significantly ($P<0.05$) inhibited the pulmonary metastasis of subcutaneous BSC tumor compared to control mice (27% reduction from the control in the pulmonary metastatic index). A similar degree of inhibition of extrapulmonary metastasis formation was also observed in the YIGSR (30 mg/kg, sc) group ($P<0.01$) showing that inhibitory effect of

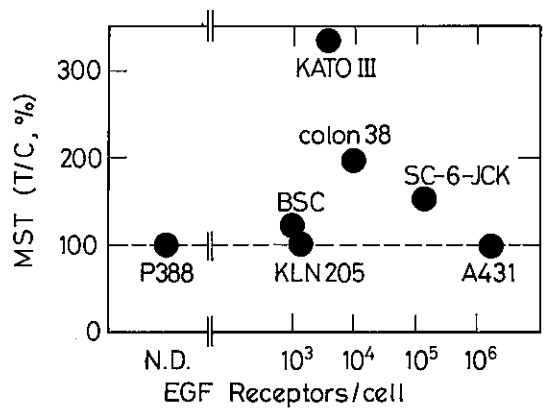

Fig. 2. No correlation between the amount of EGF receptors and prolongation of survival time of tumor-bearing mice by hEGF. MST (T/C, %) values of mice bearing various sc transplanted tumors are shown as a measure of the prolonging effect of hEGF on survival time. All 7 cancer cell lines used except P388 have EGF receptors. BSC and colon 38 tumor cells were suspensions of cells from subcutaneous solid tumors. Other tumor cell lines (P388, KLN205, KATO III, SC-6-JCK, A431) were from monolayer cultures. Number of EGF receptors was estimated by Scatchard analysis (see "Materials and Methods"). Human EGF (0.1 or 1 mg/kg) was given sc 1–14 times on various schedules.

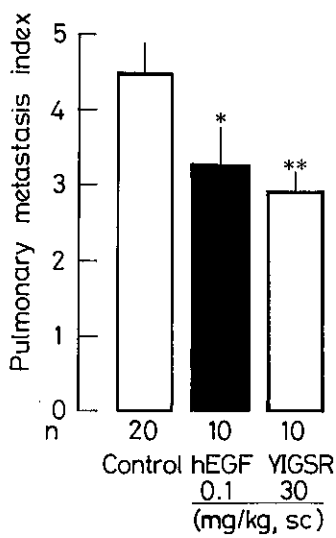

Fig. 3. Inhibitory effect of hEGF on the formation of pulmonary metastasis. C57BL/6 mice (10–20 mice/group) were inoculated sc with 30–50 mg of fragments of BSC tumor cells on day 0. Mice bearing solid BSC tumor received four administrations of vehicle, hEGF (0.1 mg/kg, sc), or YIGSR (30 mg/kg, sc) on days 4, 7, 10, and 13. Human EGF and YIGSR were solubilized in PBS and injected sc into tumor-bearing mice. Control mice received the same amount of cells and PBS containing 0.01% Tween 80. Three weeks after inoculation, mice were killed and the number of pulmonary tumors on the surface of the lungs was counted. Each value represents the mean±SE of pulmonary metastasis index (see "Materials and Methods"). The probabilities are indicated as: **, $P<0.01$; *, $P<0.05$ vs. control by Mann-Whitney's U-test.

hEGF is 300 times as potent as that of YIGSR. Human EGF and YIGSR did not cause any significant inhibitory effect on subcutaneous tumor growth as compared to the control group (data not shown). Treatments with hEGF or YIGSR also caused no reduction in nontumorous body weight as compared to the control group (data not shown).

In contrast, the pulmonary metastasis of KLN205 tumor, which has almost the same density of hEGF receptors on its plasma membrane as BSC tumor does, was not affected by the treatment with hEGF. Human EGF (0.1 mg/kg, sc) as well as YIGSR (30 mg/kg, sc) did not inhibit the pulmonary metastasis of KLN205 tumor in mice. The weights of both lungs of mice bearing KLN205 tumor treated with hEGF and YIGSR for 14 days were 0.44 ± 0.05 and 0.46 ± 0.06 g, respectively, compared to the value of 0.33 ± 0.05 g in the control group treated with the vehicle of hEGF. Human EGF (0.1 mg/kg) was administered sc every day from 7 days after inoculation. Human EGF and YIGSR did not cause any significant effect on the subcutaneous tumor growth or nontumorous body weight compared to the control values (data not shown).

DISCUSSION

Metastatic dissemination accounts for most cancer deaths. The capacity for metastasis has been described as the most insidious and the single most important characteristic of neoplasma. Tumor cell metastasis is a complex process that depends in part on tumor cell adhesion to components of basement membranes and the extracellular matrix. Previous studies have indicated that the experimental metastasis of murine melanoma cells can be inhibited by *ex vivo* pretreatment of cells with purified adhesion-promoting fragments of laminin or fibronectin prior to administration.[9] The peptides Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR) and Tyr-Ile-Gly-Ser-Arg (YIGSR), which are partial structures of laminin, a basement membrane-specific glycoprotein, were recently identified as major sites for cell binding, and these peptides were selected for study based on their homology to EGF.[12] But, in studies with A431 cells, whole laminin did not compete with EGF for the EGF receptor,[12] demonstrating that CDPGYIGSR does not exert its activity at the EGF receptor. In our study, although laminin competed with ^{125}I-laminin at its own receptor on the murine BSC tumor cells, both EGF and YIGSR failed to compete with whole laminin (data not shown). CDPGYIGSR is also quite distinct from Arg-Gly-Asp (RGD), which serves as an important fragment in fibronectin and in vitronectin as a major cell attachment site.[13] The RGD sequence does not occur in EGF or the laminin B1 chain, nor does the sequence encompassed by CDPGYIGSR occur in fibronectin. Though these factors probably serve to explain the selective effects that these proteins exert on different cell lines, they are not sufficient to explain the mechanism of the inhibition of metastasis by hEGF.

Receptors for EGF are found in a greater proportion in metastatic tumors than in primary tumors in human breast and bladder tumors according to clinical findings and histological data.[14–16] However, our study failed to indicate that there was any clear correlation of the amount of receptor for EGF on tumor cells with either antimetastatic activity or prolongation of survival time in tumor-bearing mice after hEGF treatment. Thus, the effects of hEGF seem to be independent of the number of EGF receptors.

In this study, EGF not only inhibited experimental pulmonary metastasis formation but also prolonged survival time in tumor-bearing mice without *ex vivo* pretreatment of cells. EGF may be effective in preventing metastasis of certain malignant neoplasms. These results suggested that combined therapy with hEGF and antitumor agents might be a feasible approach in the treatment of cancer. Nevertheless, this study revealed the complexity of tumor metastasis and suggests that multiple strategies may have to be developed to inhibit hematogenous metastasis formation.

The present studies were not designed to examine the exact mechanism of the observed prolongation of survival time of tumor-bearing mice. Yet, it is clear that the observed prolongation of survival time of mice depends upon inhibition of pulmonary metastasis. There are several plausible mechanisms for the observed prolongation of life span of tumor-bearing mice, as follows. 1) EGF might render tumor cells more susceptible to the endogenous immune system. EGF plays an essential role in the regulation of cell division and differentiation.[3–6] Activated B cells and some transformed B-cell lines have been shown to bind EGF.[17] EGF interferes with both the immunosuppressive and growth-inhibitory properties of oxidized soluble immune response suppressor in both heterogeneous and homogeneous cell populations.[18] Moreover, EGF-induced 2′,5′-oligoadenylate synthetase activity,[19] an enzyme that has previously been implicated in the antiproliferative effect of interferon, might render tumor cells more susceptible to the immune attacks. 2) Human EGF might compete with plasminogen activator (PA) for its receptor. As with previous studies, the mechanism of inhibition appeared to involve an increased clearance rate of tumor cells from the pulmonary microcirculation. One of the many actions of EGF is to stimulate PA activity.[20] PA is found in the membranes of cells and its release can be mediated through other mitogens. It has been associated with tumor invasion. Antibody to human PA can block the metastasis of experimental

tumors.[21] The region of PA responsible for receptor binding resides in the amino terminal fragment.[22] This region of PA is referred to as the growth factor module since it shares partial amino acid sequence homology with EGF. Furthermore, this region of EGF is responsible for binding of EGF to its receptor. In order to determine the mechanism in detail, it will be necessary to establish an *in vitro* system which closely minics *in vivo* animal experiments.

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