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Epidermal Growth Factor Receptor-mediated Selective Cytotoxicity of Antitumor Agents toward Human Xenografts and Murine Syngeneic Solid Tumors

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Severe toxic side effects of antiproliferative agents limit their clinical usefulness as antitumor drugs. Recently we observed that the antitumor efficacy of various antitumor agents (5-fluorouracil, tegafur, adriamycin, mitomycin C, cyclophosphamide, and cisplatin) against experimental solid tumors was enhanced by prior or simultaneous administration of human epidermal growth factor (EGF). However, coadministration of EGF did not enhance the toxicity of antitumor agents as measured by LD_{50} and body weight loss. The above selective potentiation of efficacy of the antitumor agents by human EGF can be characterized as follows. In a dose-dependent manner, human EGF enhanced the efficacy of an antitumor agent (5-FU) treatment 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]. Various degrees of enhancement were also observed against other experimental tumors transplanted sc. The degrees of enhancement were directly proportional to the numbers of human EGF binding sites present on tumor cell plasma membrane (threshold of binding site density=1.5×10^3 sites/cell) using 5-FU or cisplatin as an antitumor agent, 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. Normal cells including intestinal epithelial and bone marrow cells are endowed with fewer EGF binding sites (less than 10^3 sites/cell). This may explain partially the absence of EGF-enhanced cytotoxicity by antitumor agents toward normal cells.

Key words: Human EGF — Adjuvant chemotherapy — Human tumor inhibition — Biological response modifier — EGF receptor

One of the problems in cancer chemotherapy is the low therapeutic indices of antiproliferative-type agents; the dose sufficient to eradicate tumor cell populations is generally close to the lethal dose. Antitumor drugs such as 5-fluorouracil (5-FU), tegafur, mitomycin C, adriamycin, cyclophosphamide, and cisplatin have been widely used in the treatment of various human solid tumors, but their strong toxicities, mainly gastrointestinal toxicity and myelosuppression, sometimes hamper their clinical application.

Metabolically active cells are more vulnerable to most anticancer drugs than quiescent cells, because they offer a larger number of target sites which relate to the lethal interaction with anticancer drugs.[1]

Epidermal growth factor (EGF), consisting of 53 amino acids, was originally isolated from the mouse submandibular glands (mouse EGF),[2] and a homologous peptide, urogastrone, was subsequently identified in human urine (human EGF).[3] The binding of EGF to its plasma membrane receptor initiates a variety of stepwise biochemical changes in the target cells such as increased uptake of potassium ion,[4] sugar,[5] amino acids,[6] and uridine[7] during the first 10 min to 2 h following EGF addition, followed by increased glycolysis with the activation of phosphofructokinase for 1–2 h.[8,9] Stimulation of hyaluronic acid synthesis[10] and increased membrane activity, such as macropinocytosis and ruffling, are seen within 6 h.[11] Enhanced protein[12] and RNA[13] synthesis follow by the 12th h, and entry into the S phase of the cell cycle with maximal DNA synthesis occurs 20 h after the EGF addition.[13,14] Since considerably more EGF binding sites were found on the tumor cell surface than on the plasma membrane of normal cells, we thought that EGF might specifically enhance the cytotoxicity of commonly used anticancer drugs toward tumor cells.

Therefore, we compared the efficacy of antitumor agents alone and in combination with EGF, and confirmed EGF-induced potentiation of the cytotoxicity of antitumor agents.

Abbreviations: hEGF, human epidermal growth factor; 5-FU, 5-fluorouracil; TFR, tegafur [1-(2-tetrahydrofuryl)-5-fluorouracil]; ADM, adriamycin/doxorubicin; MMC, mitomycin C; CPA, cyclophosphamide; CDDP, cisplatin, *cis*-dichlorodiammineplatinum.

MATERIALS AND METHODS

Materials Six antitumor agents [5-fluorouracil (5-FU), 1-(2-tetrahydrofuryl)-5-fluorouracil (tegafur, TFR), adriamycin (ADM), mitomycin C (MMC), cyclophos-

phamide (CPA), and *cis*-dichlorodiammineplatinum (cisplatin, CDDP)] were used. Human EGF (hEGF) has been produced through recombinant DNA techniques in quantities large enough for *in vivo* animal experiments by Wakunaga Pharmaceutical Co., Ltd.[15] 5-FU was purchased from Sigma Chemical Co., St. Louis, Mo. Tegafur was from Taiho Pharmaceutical Co., Ltd., Osaka; MMC and ADM were from Kyowa Hakko Co., Ltd., Tokyo; CPA was from Shionogi Pharmaceutical Co., Ltd., Osaka; CDDP was from Nippon Kayaku Co., Ltd., Tokyo. 5-FU was dissolved in 3.44% Tris-HCl buffer solution adjusted to pH 8.4. MMC and CPA were dissolved in physiological saline. Human EGF was dissolved in phosphate-buffered saline, pH 7.4, containing 0.01% Tween 80, which was added to avoid the adsorption of hEGF by vessels. All preparations were found to be non-pyrogenic in pyrogen tests (rabbits).

Animals Male C57BL/6, DBA/2N mice, weighing 18–25 g as well as male nude mice with a BALB/c genetic background, weighing about 20 g, were used in these studies. C57BL/6 mice were obtained from Charles River Japan, Inc., Kanagawa. DBA/2N and the athymic mice, BALB/c A-nu, were obtained from Clea Japan, Inc., Tokyo. The athymic mice, which had been maintained under specific-pathogen-free conditions, were housed in sterilized cages and provided with sterilized food, drinking water, bedding, and filters. All these cages were placed in a laminar-air-flow unit.

Tumors Human epidermoid carcinoma of vulva (A431) and nasopharynx (KB), human gastric carcinoma (SC-6-JCK, KATO III, MKN45), human colon carcinoma (HCT8), murine colon adenocarcinoma 38 (colon38), murine epidermoid carcinoma (BSC), murine squamous carcinoma (KLN205) and P388 murine leukemia were used in this study. A431 cells were donated by Dr. H. Mizusawa, Japanese Cancer Research Resources Bank, Tokyo. KB cells were purchased from Dainippon Pharmaceutical Co., Ltd., Osaka. SC-6-JCK cells were donated by Dr. H. Sumiyoshi, Hiroshima University School of Medicine, Hiroshima. KATO III and MKN45 cells were donated by Dr. H. Ohkura, National Cancer Center, Tokyo. HCT8 cells were a gift from Dr. J. R. Bertino, Memorial Sloan-Kettering, NY. Colon 38 and P388 were donated by Dr. T. Tashiro, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. BSC was a gift from Dr. T. Furuse, Laboratory of Radiation Pathology, National Institute of Radiological Sciences, Chiba. KLN205 was purchased from the American Type Culture Collection (ATCC), MD. A431, KB and KLN205 cell lines were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (M. A. Bioproducts, MD). KATO III and MKN 45 cell lines were grown in RPMI1640 medium containing 10% fetal calf serum. SC-6-JCK, BSC and colon38 tumors were maintained by serial sc transplantation of 20–40 mg tumor fragments in athymic nude mice, male C57BL/6 mice and male C57BL/6 mice, respectively. P388 was maintained by serial ip passage every 10–14 days in male DBA/2N mice.

Evaluation of antitumor activity Mice bearing solid tumor, 7 weeks old at the beginning of treatment, were used (8 weeks old in the case of athymic mice). Antitumor agents were administered ip or sc in the back region, and human EGF was given through 3 different routes; sc in the abdominal region, ip, and iv into the tail vein at 1–2 weeks after inoculation. The perpendicular diameters of the tumors were measured with vernier calipers and converted to weight by applying the following equation; tumor weight (mg) $=0.5ab^2$, a=length and b=width, where a and b are both in mm.[16] Tumor weights were recorded and animals were randomized into groups. To minimize the variation of tumor weight in each group, mice with unusually small as well as large tumor nodules (by gross inspection) were excluded. Each tumor weight after the treatment was expressed as % of initial tumor weight; $(W_n/W_0)\times 100$, where W_n is the tumor weight at day n and W_0 is the initial tumor weight when the treatment was started (day 0).

Acute toxicity studies The lethal toxicity of antitumor agents was assessed alone and in combination with hEGF (0.1, 1, 10 mg/kg, sc) by Probit analysis[17] or Van der Waerden analysis.[18] Seven to eight male ICR mice per dose were used. The observation period was 14 days. All drugs were administered sc.

Cell preparations for EGF-receptor binding assay Bone marrow cells: the marrow of the ICR male mice (6 weeks old) was harvested by snipping the tip of the femur and flushing it with M199 containing 0.25% BSA (binding medium) with a tuberculin syringe. The bone marrow cells were isolated by centrifugation in the binding medium. Intestinal epithelial cells: mice (6-week-old ICR males) were killed, and the intestinal mucosa was scraped from a 15-cm segment. The collected mucosa was washed with 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. The epithelial cells were isolated by centrifugation in the binding medium. Tumor cells were obtained from sc transplanted solid tumor (BSC, A431); the tumors were carefully dissected out, and minced tumor paste was suspended in Hanks solution. Subsequent procedures were the same as in the preparation of intestinal epithelial cells.

Binding assay for EGF receptor Unlabeled hEGF was added to the isolated bone marrow, intestinal epithelial or tumor cell suspension (2×10^7 cells/ml, 500 μl). Triplicate cell suspensions were incubated at 4°C for 30 min. ^{125}I-EGF (about 10 pCi, specific activity >900 Ci/mmol,

Amersham) was added and incubated at 4°C for 90 min. Non specific binding was measured in the presence of a 1,000-fold excess of unlabeled hEGF. The cells were washed 4 times with ice-cold binding medium. Binding assays for tumor cell monolayers were performed by the method of Richert *et al.*[19] 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 non linear Scatchard plots were analyzed according to the two-site model[20] by an iterative process for nonlinear regression.

Statistical analysis All statistical analyses were performed on a personal computer model PC9801 VX21 (NEC Corporation, Tokyo). Student's t test or the Aspin-Welch method after the F-test was used, with $P<0.05$ as the criterion of significance. The ED_{50} was evaluated by the Litchfield-Wilcoxon method.[21]

RESULTS

Synergistic enhancement of 5-FU antitumor activity by hEGF To determine whether or not the cytotoxicity of 5-FU on A431 could be enhanced by coadministration of hEGF, 4 groups of tumor-bearing mice were compared. The first control group of mice was injected ip with 5-FU (100 mg/kg) and sc in the abdominal region with the vehicle of hEGF. The experimental group of mice received both hEGF (1 mg/kg, sc) in the abdominal region and 5-FU (ip). The second control group of mice was given hEGF and the vehicle of 5-FU. The third control group of mice received the vehicles only. The treatments were given twice on day 0 (14 days after tumor inoculation) and day 7 (q7d). Tumor weights of the above four randomized groups on day 0 were as follows; 3rd control group (265±39 mg), hEGF group (294±62 mg), 5-FU group (293±64 mg), and hEGF plus 5-FU group (262 ±55 mg). After 10 days, 5-FU treatment alone hardly reduced the tumor weight (708±183 mg) as compared to that in the 3rd control group (808±127 mg). Human EGF alone also caused no significant change on tumor growth (713±102 mg). However, the combination of these two agents caused a remarkable inhibition of the tumor growth (361±106 mg; $P<0.05$ vs. 3rd control; $P<0.05$ vs. 5-FU alone). The percentage of initial tumor weight was used as the parameter in Fig. 1A. The combination of the two agents (129±17% compared to the control of 312±39%; $P<0.005$ vs. control) was again clearly the most effective. This inhibition was significantly greater than that obtained with 5-FU alone of 240±22% ($P<0.005$ vs. 5-FU). Nontumorous body weight was measured every 3–4 days. Mice with body weights ranging from 20 to 28 g were randomized into the control group (24.1±1.8 g), 5-FU group (24.1±1.0 g), hEGF group (24.9±1.0 g), and 5-FU plus hEGF group (24.1±1.0 g) on day 0. Treatment with 5-FU or 5-FU plus hEGF caused a reduction in body weight (87.9±2.0%, 86.8±1.1%, respectively), $P<0.05$ as compared to the control group (101.8±4.8%). But there was no significant difference between the 5-FU alone and 5-FU plus hEGF groups, suggesting that hEGF did not enhance the cytotoxicity of 5-FU against normal cells in dose-limiting organs (Fig. 1B).

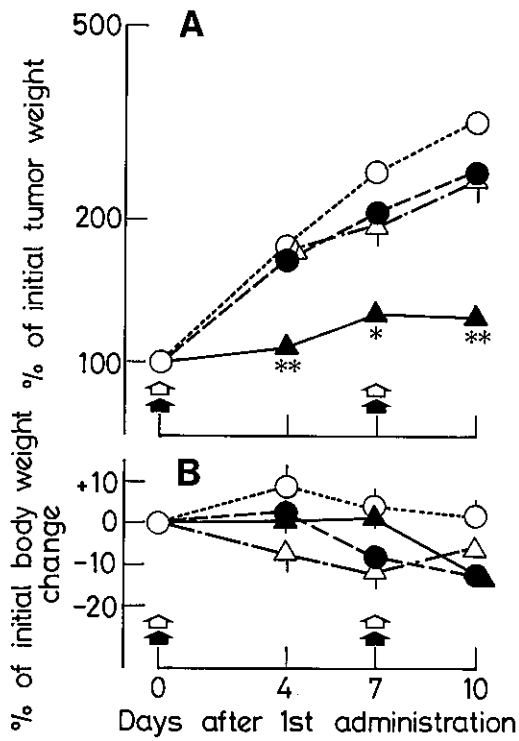

Fig. 1. Effect of the 5-FU+hEGF combined treatment on the growth of A431 transplantable human epidermoid carcinoma of the vulva in nude mice (A). Groups were treated twice on day 0 (14 days after tumor inoculation) and day 7 with 5-FU at 100 mg/kg, ip (●), hEGF at 1 mg/kg, sc (△), 5-FU (100 mg/kg, ip) plus hEGF (1 mg/kg, sc) (▲) and saline (10 ml/kg, ip) plus PBS with 0.01% Tween 80 (10 ml/kg, sc) (○). Arrows indicate the time of administration. Points and bars represent means and ±SE of 5 animals in each group; ** and * indicate significant differences between the 5-FU+hEGF and 5-FU control groups ($P<0.01$ and 0.05, respectively). (B) Effects of 5-FU with and without hEGF on nontumorous body weight. Treatments and symbols were the same as in A.

Effect of hEGF on the efficacy of various antitumor agents in experimental human and murine tumor models In order to determine whether this combination effect with hEGF is specific to 5-FU or common to antiproliferative antitumor agents, we examined the effect of hEGF on the efficacy of various antitumor agents, which have different modes of action, towards human A431

Table I. Synergistic Enhancement of Antitumor Activity of Various Antitumor Agents by Coadministered hEGF against Experimental Human and Murine Solid Tumors

Drug	% of the initial tumor weight[a]							
	A431[b]				colon38[c]			
	Dose[d] (mg/kg)	Tmax[f] (day)	EGF (1 mg/kg, sc)		Dose[e] (mg/kg)	Tmax (day)	EGF (0.1 mg/kg, sc)	
			−	+			−	+
5-Fluorouracil	100×2	10	240±21	129±17**	150	4	84±7	25±6**
Tegafur					400	2	91±18	34±12**
Adriamycin					10	6	112±12	59±6**
Mitomycin C	2×2	14	527±63	323±48*	5	1	127±14	97±5*
Cyclophosphamide	150×8	56	634±112	100±37**	200	6	102±30	24±5*
Cisplatin	5×3	20	998±68	702±116*	10	1	140±18	85±10*

a) See "Materials and Methods."
b) BALB/c A-nu athymic mice (5–12 mice/group) were inoculated sc with 1×10^6 A431 human tumor cells on day −10∼−14.
c) C57BL/6 mice (6–12 mice/group) were inoculated sc with 50–70 mg of fragmented colon38 murine tumor on day −7∼−12.
d) Administration schedule of drugs on q7d (ip).
e) Single administration (sc).
f) See "Results."
**, $P<0.01$; *, $P<0.05$ vs. antitumor agent alone.

Table II. Effect of hEGF on the Lethal Toxicity of Antitumor Agents to Normal Mice

hEGF (mg/kg) (sc)	LD_{50} [95% confidence limits] (mg/kg, sc)					
	Tegafur	5-Fluorouracil	Cyclophosphamide	Adriamycin	Mitomycin C	Cisplatin
0	826 [622–1080]	209 [160–256]	443 [415–473][a]	17.0 [13.1–19.1]	7.3 [6.7–7.8]	15.6 [14.7–16.7]
0.1	886 [689–1133]	198 [140–246]	425 [403–449]	19.2 [7.3–21.1]	7.3 [6.4–7.8]	14.7 [13.8–15.6]
1	970 [850–1094]	194 [155–230]	425 [403–449]	18.3 [17.0–22.9]	7.2 [3.6–7.9]	13.6 [12.4–14.4]*
10	925 [771–1112]	171 [129–201]	435 [410–468]	18.1 [16.3–19.7]	7.5 [7.0–8.0][a]	13.5 [11.8–14.4]*

Five groups of 7–8 male ICR mice received individual antitumor agents (sc) with and without coadministration of hEGF (0.1, 1, or 10 mg/kg, sc). Incidence of mortality after antitumor agent administration was observed for 2 weeks.
a) Van der Waerden analysis. All values except these two were analyzed by using the Probit model.
*, $P<0.05$ vs. EGF free control by Gehan's generalized Wilcoxon test.

carcinoma or murine colon adenocarcinoma (Table I). Tegafur (a prodrug of 5-FU), adriamycin, mitomycin C, cyclophosphamide or cisplatin was used as an antitumor agent. Tumor weights were expressed as the relative tumor weight (see "Materials and Methods") on the day when the difference of tumor weights between antitumor agent alone and combined treatment groups became maximum. In a murine colon tumor, a single dose combined treatment resulted in 29–76% reduction of tumor weight compared to the same antitumor agent alone. In a human tumor, a single dose combined treatment did not show any significant promotion of the efficacy of antitumor agents. However, repetitive combined treatments caused a significant reduction (17–70%) of tumor weight compared to an antitumor agent alone. In all cases, no significant effect of combined treatment on the body weight of these animals was observed (data not shown), which is consistent with the data in Table II showing that hEGF did not increase the toxicity of antitumor agents. Even though each antiproliferative antitumor agent has a different mode of action, hEGF enhanced the efficacies of all the antitumor agents tested in Table I. In particular, the efficacies of CPA, 5-FU and TFR were remarkably enhanced by hEGF both in human and murine models.

Effect of hEGF treatment on the acute toxicities of various antitumor agents Antiproliferative antitumor drugs are known to show mitotoxicity toward rapidly dividing normal cell populations, such as those present in the epidermis, gastrointestinal tract, and bone marrow.[22,23] In order to determine whether or not hEGF also enhances the cytotoxicity of antitumor agents toward normal cells in the above-noted dose-limiting

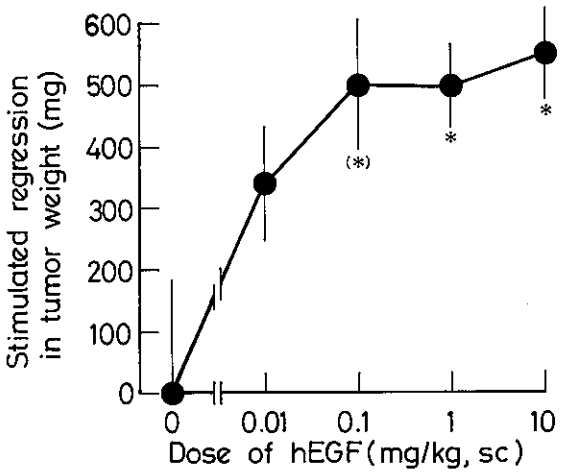

Fig. 2. The helper function of hEGF toward 5-FU in relation to dose. BALB/c A-nu nude mice bearing A431 human epidermoid carcinoma were given a single administration of 5-FU (100 mg/kg, ip) with or without various doses of hEGF (sc) on day 0 (14 days after tumor inoculation). Accelerated regression expressed as tumor weight difference at each hEGF dose (mean of the differences in tumor weight between the 5-FU+hEGF and 5-FU groups on day 14). Eight animals were in each group. * indicates significance at $P<0.05$ and (*) at $P<0.10$.

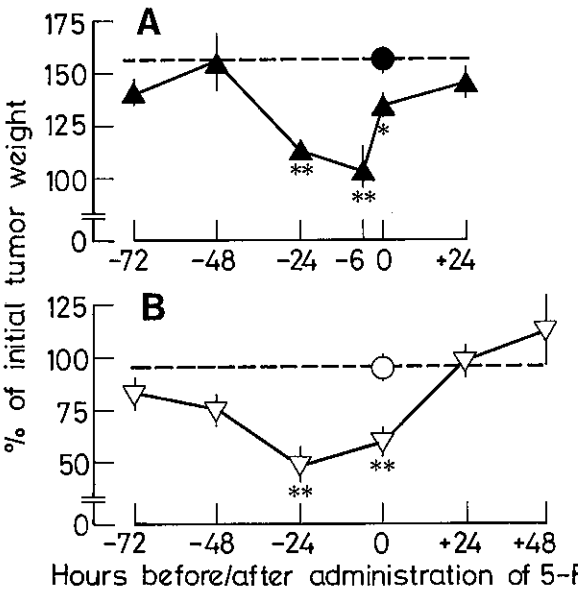

Fig. 3. Duration of potentiation by hEGF of A431 (A) or colon38 (B) to antitumor activity of 5-FU. BALB/c A-nu nude mice bearing A431 human epidermoid carcinoma were given a single administration of 5-FU (100 mg/kg, ip) on day 0, 11 days after tumor inoculation. Human EGF (1 mg/kg, iv) was given on different days as indicated. Each value represents % of initial tumor weight on day 4. The corresponding value for the 5-FU group is shown as the dotted line with one solid circle. The probable significances are indicated as: **, $P<0.01$; *, $P<0.05$. B: The same as A431 except that the tumor used was colon38. C57BL/6 mice bearing colon38 murine adenocarcinoma received a single administration of 5-FU (150 mg/kg, sc) on day 0, 22 days after tumor inoculation, and hEGF (0.1 mg/kg, sc) on different days.

organs, the values of median lethal dose (LD_{50}) for antitumor agents (TFR, CPA, 5-FU, ADM, CDDP, MMC) with and without coadministration of hEGF (up to 10 mg/kg, sc) were estimated using the Probit or Van der Waerden model. The effect of hEGF on the LD_{50} values of antitumor agents is shown in Table II. There was no significant difference between in the presence and absence of hEGF coadministration, suggesting that hEGF did not enhance the cytotoxicity of the antitumor agents toward normal cells in dose-limiting organs. In the case of cisplatin, the recommended dose of hEGF was considered to be 0.1 mg/kg, since higher doses of hEGF enhanced the cytotoxicity of cisplatin.

Relationship between hEGF dose and synergistic enhancement of 5-FU activity The helper function of hEGF was characterized using 5-FU as an antitumor agent since the efficacy of 5-FU was remarkably enhanced by hEGF. While human EGF alone (1 mg/kg, sc) or 5-FU alone (100 mg/kg, ip) caused an insignificant loss of tumor weight (Fig. 1A), as shown in Fig. 2 hEGF enhanced the efficacy of 5-FU against A431 solid tumor in a dose-dependent manner. BALB/c A-nu nude mice were inoculated sc with 1×10^6 A431 human tumor cells 14 days before the initiation of treatments, and received a single administration of 5-FU (100 mg/kg, ip) with or without hEGF (0.01, 0.1, 1, 10 mg/kg, sc) on day 0. Mean difference in tumor weights between the 5-FU+hEGF treatment group and 5-FU only group on day 14 is plotted as loss of tumor weight in Fig. 2. ED_{50} of hEGF was determined as 2.9 μg/kg (0.2–49.7, 95% confidence limit) by the Litchfield-Wilcoxon method.

Duration of hEGF-induced susceptibility of tumor cells to 5-FU In order to determine the duration of the helper function of hEGF, hEGF was given to athymic nude mice bearing A431 epidermoid carcinoma either at the same time (0 h) as 5-FU, or at various time intervals before or after 5-FU injection, as shown in Fig. 3 (▲). When hEGF, 1 mg/kg, iv was given at the same time as 5-FU (100 mg/kg, ip), 20% regression ($P<0.05$) on day 4 on average was observed (% of initial tumor weight; see "Materials and Methods"). In contrast, administration of 5-FU alone hardly inhibited the tumor growth. When hEGF was given 24 or 6 h before the 5-FU treatment, 40 or 45% regression on day 4 was observed, respectively. However, no potentiation by hEGF was

observed when hEGF was given 24 h after the 5-FU administration. Since elimination of 5-FU (100 mg/kg, ip) from circulation was very rapid ($t_{\frac{1}{2}}$=8.1 min, data not shown), there would have been no 5-FU left in the plasma 24 h after the 5-FU administration. The above results showed that the prior or simultaneous (−24~0 h) exposure of tumor cells to hEGF was essential to enhance the efficacy of 5-FU. Similar results were also obtained on transplanted murine adenocarcinoma colon-38 system (▽). When hEGF (0.1 mg/kg, sc) was given at the same time as 5-FU (150 mg/kg, sc), 36% regression ($P<0.01$) on day 4 on average was observed. In contrast, administration of 5-FU alone merely kept the tumor weight at the initial level. When hEGF was given 24 h before the 5-FU treatment, 44% regression on day 4 was observed. The administration of hEGF at 72 and 48 h before 5-FU was not as effective, showing only slight regression (13 and 18%, respectively). Again no potentiation by hEGF was observed when hEGF was given 24 or 48 h after the 5-FU administration [elimination of 5-FU (150 mg/kg, sc) was also very rapid ($t_{\frac{1}{2}}$= 9.4 min, data not shown)]. Plasma concentration of hEGF (1 mg/kg, iv or 0.1 mg/kg, sc) 12 h after hEGF administration was estimated to be as low as the physiological level (<0.1 ng mEGF/ml of plasma) from extrapolation of the pharmacokinetics (data not shown). Since 5-FU administered 24 h after EGF caused a remarkable regression of solid tumors, co-circulation of EGF at the pharmacological level with 5-FU is not required for promotion of 5-FU cytotoxicity. In other words, administered EGF made tumor cells more susceptible to 5-FU and this enhanced susceptibility continued for 24 h. It should be emphasized that even though completely different types of tumor were used, almost the same duration of the helper function was observed.

Correlation between the numbers of EGF receptors on tumor cells and synergistic enhancement by EGF of anticancer activity of 5-FU and cisplatin Cancer cell lines used in this study had various numbers of EGF receptors on their plasma membranes; estimated numbers/cell were; A431, 1.2×10^6; KB, 1.5×10^5; SC-6-JCK, 1.4×10^5; HCT8, 1.4×10^4; KATO III, 3×10^3; MKN45, 2.4×10^3; KLN205, 1.4×10^3; BSC, 1×10^3. We, however, failed to detect EGF receptor on mouse P388 leukemia cells as well as on normal bone marrow cells. As shown in Table III, degrees of hEGF-induced potentiation of susceptibility to 5-FU and cisplatin were determined on various sc transplantable tumors including human tumors (A431, KB, SC-6-JCK, KATO III, MKN-45 and HCT8), and murine tumors (BSC, KLN205 and P388). Tumor weights were expressed as % of initial tumor weight estimated from measurements of tumor diameters (see "Materials and Methods") on the day when the difference of tumor weights between the two treatments (antitumor agent alone and antitumor agent +hEGF) became maximum. The maximally effective doses of 5-FU and cisplatin were roughly equal to LD_{25} of healthy ICR mice. These doses did not cause any death

Table III. Divergent Sensitivities of 9 Different Solid Tumors to the Combined 5-FU plus hEGF, or CDDP plus hEGF Treatment

Tumor[a]	% of initial tumor weight[b]							
	5-FU				CDDP			
	Dose (mg/kg, ip) [Schedule]	Tmax (day)	EGF (1 mg/kg, sc)		Dose (mg/kg, ip) [Schedule]	Tmax (day)	EGF (1 mg/kg, sc)	
			−	+			−	+
A431	100×2 [q7d]	10	240±21	129±17**	5×3 [q7d]	20	997±68	702±115*
SC-6-JCK	100×2 [q7d]	14	463±63	311±34*				
KB	50×2 [q4d]	8	242±55	140±27(*)				
HCT8	100×1	7	182±30	125±27	5×1	7	160±36	166±14
KATO III	56×2 [q7d]	14	276±34	274±14				
MKN45	56×1	7	272±26	292±20				
KLN205					10×1	8	213±35	223±15
BSC	100×1	2	203±18	194±6				
P388	100×1	2	157±27	144±14				

a) BALB/c A-nu nude mice (3–8 mice/group) were inoculated sc into the axillary region with 1–2×10^6 cells of A431, KB, KATO III, HCT8, MKN45, or with a 1–2 mm^2 cube of SC-6-JCK human tumor cells. C57BL/6 mice (6 mice/group) were inoculated sc into the axillary region with 1×10^6 cells of KLN205, or 50–70 mg of fragmented BSC murine tumor. CDF_1 mice (7 mice/group) were inoculated sc into the axillary region with 2×10^6 P388 murine leukemia cells.
b) See "Materials and Methods."
**, $P<0.01$; *, $P<0.05$; (*), $P<0.10$ vs. antitumor agent alone.

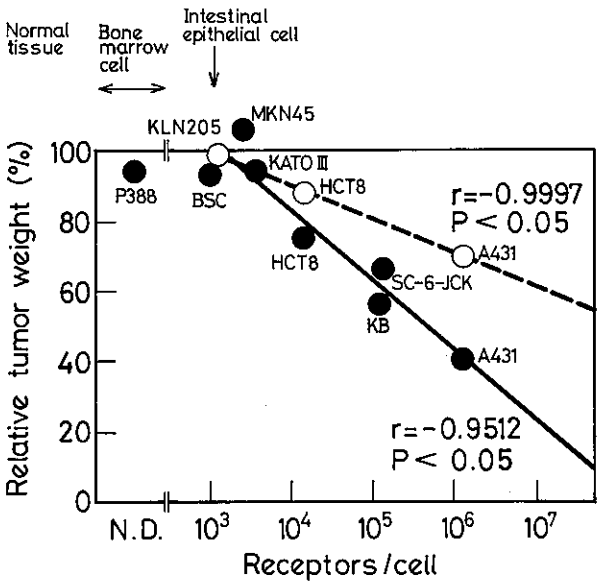

Fig. 4. Correlation between the number of EGF receptors on the tumor cells and the helper function of hEGF toward 5-FU (solid line), and CDDP (broken line). Relative tumor weights (see "Materials and Methods") on the day when the maximal effect of hEGF was observed were shown as the effect of hEGF on cytotoxicity of 5-FU or CDDP to various sc transplanted tumors in mice. The number of EGF receptors was estimated by Scatchard analysis (see "Materials and Methods"). Human EGF (1 mg/kg, sc) was given with 5-FU (50–100 mg/kg, ip), or CDDP (5–10 mg/kg, ip) simultaneously. *r*, Correlation coefficient.

in tumor-bearing mice. Strong potentiation by hEGF was seen in animals bearing EGF receptor-rich tumors. In sc transplanted tumors which possessed less than 1.5×10^3 receptors per cell, hEGF was totally ineffective. Among tumors possessing higher density of EGF receptors than the value noted above, the helping function of hEGF increased in proportion with the number of EGF receptors present on the tumor cell plasma membrane. In A431 human epidermoid carcinoma cells having 1.2×10^6 receptors per cell, the combined treatment was most effective, resulting in roughly 60 or 30% reduction of tumor weight compared to 5-FU or cisplatin alone, respectively. Figure 4 shows this receptor-efficacy correlation. Synergistic enhancement of anticancer activity of antitumor agents by hEGF was expressed as relative tumor weight: Relative tumor weight $=(W_{e+a}/W_a)\times 100$, where W_{e+a} is tumor weight after both hEGF and an antitumor agent and W_a is that after the antitumor agent alone. In this figure, 0% represents complete tumor regression, and 100% represents the antitumor-agent-alone level. At levels above 1.5×10^3/cell, potentiation of both 5-FU or cisplatin cytotoxicity by human EGF is directly correlated with the density of EGF receptors on tumor cells. Even different types of antitumor agents showed the same threshold.

DISCUSSION

These results demonstrated that combined therapy with human EGF and antitumor agents might be a feasible approach in the treatment of cancer, provided that tumor cells are endowed with a high density of EGF receptors (Fig. 4).

While enhancing the effectiveness of the antitumor agents toward tumor cells, this combination did not increase the toxicity of the antitumor agents as measured by nontumorous body weight as well as LD_{50} of the antitumor agents (Fig. 1B and Table II). The observed absence of undesirable side effects may be explained by the fact that EGF receptors on normal cells of the dose-limiting organs are few in number; e.g., less than 10^3/cell for intestinal epithelia,[24] and even fewer for bone marrow cells (Fig. 4).

It has previously been reported that EGF receptor levels are higher in malignant as compared to normal tissues in many cases. In human squamous carcinomas of lung,[25] stomach,[26] and breast,[27] increased levels of EGF receptors have been reported. In contrast, EGF receptors were undetectable on lung small cell carcinoma.[28, 29] These squamous carcinomas with above the normal density of EGF receptors are thought to be the most appropriate targets of this combination therapy.

Exploitation of the *in vivo* effects of interferon (IFN), a biological response modifier (BRM), to achieve clinically detectable antitumor activity has not been easy. Since there does not seem to be a significant dose-response relationship in some malignancies,[30] IFN does not confer any advantage. Yet, human EGF dose-dependently enhanced the antitumor activity of 5-FU against human epidermoid carcinoma A431 xenografts in athymic mice (Fig. 2). In practice, hEGF began to exerts its maximal effect at the concentration of 0.1 mg/kg (sc). Inasmuch as the fully effective range was from 0.1 to 10 mg/kg, the determination of an appropriate hEGF dose in clinical trials should not be too difficult.

The binding of EGF to its receptor is followed by the accumulation of numerous EGF-EGF receptor complexes in discrete clusters throughout the cell membrane. Ligand-receptor clustering is a prelude to internalization or decrease in binding sites on the cell surface and intracellular processing, and is known to occur in a variety of cell types exposed to EGF.[26–29, 31] Therefore, rapidly repeated doses of hEGF may result in a reduction in helper function because of the decrease in binding sites. In fact, we observed greater enhancement of the efficacy of MMC action against A431 solid tumor by

every 7 day (q7d) treatment with hEGF than by every day (qd) treatment (data not shown).

As shown in Fig. 1A, single or double administration (every 7 days) of hEGF (1 mg/kg, sc) did not affect the growth A431 tumor or any other tumor used in this study (data not shown). But this acute administration of human EGF enhanced remarkably the efficacy of antitumor agents, as shown in this study. However, it has been reported that the development of epidermal tumors[32] was considerably enhanced by daily administration of EGF (2–5 mg/kg, sc) for several months. Even in the combination with antitumor agents, careful studies would be needed before chronic clinical application.

The present studies were not designed to examine the exact mechanism of the observed synergistic interaction between hEGF and antitumor agents. Yet, it is clear that this helper function requires the binding of hEGF to tumor cells via its specific receptor (Fig. 4) and that this binding renders tumor cells more susceptible to the antitumor agents for the duration of 24 h (Fig. 3).

There are several plausible mechanisms for the observed helper function, as follows. 1) EGF-induced cell cycle arrest of tumor cells which is known to occur in A431,[33] might render tumor cells more susceptible to the antitumor agents. 2) EGF receptor-mediated endocytosis might increase antitumor agent uptake. 3) EGF-induced 2′,5′-oligoadenylate synthetase activity,[34] which has previously been implicated in the antiproliferative effect of interferon, might render tumor cells more susceptible to the antitumor agents. 4) EGF-induced increase in the growth fraction of solid tumors may render target tumor cell populations more susceptible to the antitumor agents. 5) EGF-induced lack of cellular energy, which is needed to protect tumor cells from antitumor agents, because of hyperbiosynthesis of EGF receptors after their degradation[31] following their internalization. In order to elucidate the mechanism in detail, it will be necessary to establish an *in vitro* system which closely mimics *in vivo* animal experiments.

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