Response of A431 Experimental Human Solid Xenograft to Mitomycin C in Combination with Human Epidermal Growth Factor in Mice

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We previously demonstrated that the antitumor efficacy of various antitumor agents such as 5-fluorouracil and cisplatin against experimental solid tumors was enhanced by pre- or simultaneous administration of human epidermal growth factor (hEGF). In the present study, the combined therapy by hEGF and mitomycin C (MMC) as an antitumor agent was studied in A431 solid tumor-bearing mice to determine the dosage schedule of hEGF. When MMC alone was injected intraperitoneally (2 mg/kg) every 7th day to the tumor-bearing mice, tumor weights increased to 2138 ± 285 mg from 282 ± 41 mg during 22 d. Tumor weight in every day treatment of hEGF alone for 21 d increased to the same extent in the treatment by MMC alone. On the other hand, the increase of the solid tumor weight in the every day treatment and in the every 7th day treatment of hEGF, in combination with the every 7th day administration of MMC, were as follows; from 282 ± 41 mg to 1522 ± 357 mg (71.2 ± 16.7% of MMC alone) and from 280 ± 44 mg to 1245 ± 150 mg (58.2 ± 7.0% of MMC alone), respectively, demonstrating a greater antitumor potency of MMC in the combination with the every 7th day treatment of hEGF. Both combined therapies did not affect the toxicity of MMC as evaluated by decrease in nontumorous body weight. Single subcutaneous administration of hEGF to A431 tumor-bearing mice caused the decrease of the binding capacity of hEGF to A431 tumor cells by 80% 24 h after the administration. However, the decreased binding capacity recovered to the untreated control level 4 d after the administration of hEGF. In conclusion, the every 7th day treatment of hEGF was superior than the every day treatment in its function to increase the susceptibility of A431 solid tumor to MMC.

Keywords — human epidermal growth factor; adjuvant chemotherapy; mitomycin C; administration schedule; human tumor inhibition; biological response modifier; athymic nude mice; EGF receptor; down-regulation

Introduction

Recently, we demonstrated the enhanced antitumor efficacy of 5-fluorouracil and cisplatin against experimental solid tumors including A431 by pre- or simultaneous administration of human epidermal growth factor (hEGF) in mice.\textsuperscript{1} The degree of the enhancement was directly proportional to the number of EGF binding sites existing on the tumor cell plasma membrane (more than 1.5 × 10\textsuperscript{3} sites/cell), suggesting that the binding of EGF to the receptors on the tumor cells is an essential process in enhancing the susceptibility of tumor cells to antitumor agents. Also, coadministration of hEGF did not enhance the toxicity of antitumor agents as measured by LD\textsubscript{50} and body weight loss in normal rats, probably due to the fewer EGF binding sites (less than 10\textsuperscript{3} sites/cell) in normal cells including intestinal epithelial and bone marrow cells.\textsuperscript{1} In the previous study, it was suggested that the binding of hEGF to the receptors on tumor cells is an essential process in enhancing the susceptibility of tumor cells to antitumor agents. Based on this proposition a study on dosage of hEGF emerged.

The purpose of the present study is to show the effect of dosage levels of hEGF on the antitumor activity of mitomycin C (MMC). In the present study, hEGF and/or MMC were administered to A431 solid tumor-bearing mice every day or every 7th day for 21 d, whereas MMC was administered every 7th day. A431 was used because the tumor cell has plenty of EGF receptors (1.2 × 10\textsuperscript{6}/cell) and the combined therapy with hEGF and 5-fluorouracil or cisplatin
showed a great antitumor activity against the tumor cell as reported previously. Mice bearing A431 solid tumor received MMC alone or in combination with hEGF (1 mg/kg). MMC was administered at a dose of 2 mg/kg every 7th day. The treatment of hEGF was performed daily or weekly for 21 d. The antitumor efficacy of the combined therapy was evaluated by periodical changes of the weight of solid tumor and the nontumorous body weight (subtracted an estimated tumor weight from actually measured total body weight).

Materials and Methods

Materials — Human EGF used in the present study was produced through recombinant deoxyribonucleic acid (DNA) techniques by Wakunaga Pharmaceutical Co., Ltd., and the purity was more than 99.9%. MMC was purchased from Kyowa Hakko Co., Ltd. (Tokyo, Japan). Human EGF was dissolved in a phosphate buffered saline (pH 7.4) containing 0.01% Tween 80 which was added to avoid the adsorption of hEGF on experimental glass vessels. MMC was dissolved in a physiological saline. All preparations were found to be non-pyrogenic by the pyrogen tests (rabbits). Other chemicals were of analytical grade.

Animals — Male nude mice with a BALB/c gene background, weighing about 20 g, were used. The athymic mice, BALB/c A-nu, were obtained from Clea Japan, Inc. (Tokyo, Japan). 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 sterilized laminar-air-flow unit.

Tumors — Human epidermoid carcinoma of vulva (A431) was used in this study. A431 cells were donated from Japanese Cancer Research Resources Bank (Tokyo, Japan). A431 cell line was grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (M. A. Bioproducts, MD).

Evaluation of Antitumor Activity — A431 tumor cells grown in vitro were subcutaneously inoculated (1–2 × 10⁶ cells/mouse) at the right axilla area of the mice. To minimize the variation of tumor weight in each group, mice with unusually small as well as large tumor nodules (by gross inspection) 1–2 weeks after inoculation were excluded. Tumor weight of each mouse was estimated as follows: The perpendicular diameters of the tumors were measured with vernier calipers and converted to the weight by the following equation: tumor weight (mg) = 0.5ab², with a = length in mm, and b = width in mm.

Administration of MMC and hEGF — Mice bearing A431 solid tumors were randomly divided into 6 groups of 8 mice each. Mice in each group received vehicle alone, hEGF alone in two different dosage schedules, MMC alone, or MMC with hEGF in two different dosage schedules, respectively. The dosage schedules of MMC and/or hEGF were as follows; 1st group, vehicle alone; 2nd group, hEGF alone every 7th day for 21 d (q7d×3); 3rd group, hEGF alone every day for 21 d (qd×21); 4th group, MMC alone every 7th day for 21 d (q7d×3); 5th group, MMC q7d×3 and hEGF q7d×3 for 21 d; 6th group, MMC q7d×3 and hEGF qd×21. MMC was administered intraperitoneally at a dose of 2 mg/kg and hEGF at a dose of 1 mg/kg was administered subcutaneously at abdominal area opposite to the solid tumor. The dose and dosage schedule of MMC were determined by considering antitumor effect and toxic effect. The dose of hEGF was fixed at 1 mg/kg, since the antitumor efficacy in the combination therapy by hEGF and 5-FU against A431 tumor depended on a dose of hEGF and a maximal potency was observed at 1 mg/kg as reported previously.

Binding Assay for EGF Receptors — Subcutaneous A431 tumors were carefully dissected out 1–2 weeks after inoculation. Minced tumor paste was suspended in a HANKS solution and treated with type I collagenase (0.1%), deoxyribonuclease I (0.025%) and trypsin inhibitor (0.05%) for 30 min at 37°C. The tumor cell suspension was centrifuged and the supernatant was discarded. The residue was resuspended in the binding medium (Medium 199 containing 0.25% bovine serum albumin), which was oxygenated in advance by bubbling with 95% O₂.
and 5% CO₂. These procedures were repeated twice and the final suspension was obtained. Binding assay was performed in a manner similar to that described previously with a small modification. Briefly, triplicated cell suspensions (2 × 10⁷ cells/ml, 0.5 ml) were incubated for 30 min at 4°C. ¹²⁵I-EGF (about 10 pCi, specific activity > 900 Ci/mmol, Amersham) was added and incubated for 90 min at 4°C. Non specific binding was measured in the presence of a 1000-fold excess of unlabelled human EGF. The cell suspensions were centrifuged and the precipitates were washed 4 times with ice-cold binding medium. Radioactivity of the resultant precipitate was measured with an Auto Well Gamma System (ARC-600, Aloka Co., Ltd., Tokyo, Japan).

Statistical Analysis — Student’s t-test or Aspin-Welch method after F-test was used for statistical analysis. Significance level of \( p < 0.05 \) or \( p < 0.10 \) was used as the criteria of significance.

Results

Enhanced Antitumor Activity of MMC by hEGF

Tumor bearing mice 1—2 weeks after inoculation of A431 tumor cells received vehicle alone, hEGF alone in two different dosage schedules, MMC alone or MMC with hEGF in two different dosage schedules. These drug treatments were performed for 3 weeks. Results are summarized in Table I in terms of tumor weight and nontumorous body weight which was estimated by subtracting tumor weight from total body weight. Periodical changes in the tumor weight and nontumorous body weight during the drug treatment are also shown in Fig. 1. Tumor growth in the treatment with hEGF alone (2nd and 3rd groups) was similar to the treatment with vehicle alone (1st group). Every 7 th day treatment with MMC alone (4th group) also showed no significant difference in the gain of tumor weight compared to the treatment with vehicle alone (1st group) and hEGF alone (2nd and 3rd groups). On the other hand, the combined therapy with MMC and hEGF administered every 7th day (5th group) caused a remarkable inhibition of the tumor growth (\( p < 0.05 \)) and the tumor weight on the 22nd day was suppressed by 52% of that of MMC alone (4th group). The average suppression of the tumor weight on the 22nd day in every day treatment of hEGF combined with every 7th day MMC treatment (6th group) was 33% of the level of MMC alone at a significance level of \( p < 0.10 \). Thus, the combined therapy with hEGF and MMC showed a remarkable antitumor activity in the every 7th day treatment of hEGF.

The nontumorous body weight was also measured every 3—4 d. When the gain of nontumorous body weight was expressed as a ratio of that on day 21st to day 0 in each group, the combined therapy of hEGF and MMC (5th and 6th groups) resulted in significantly greater nontumorous body weights compared to that of MMC alone (4th group) as follows: 4 th group,

<p>| Table I. Effect of the MMC + hEGF Combined Treatment on the Growth of the A431 Transplantable Human Epidermoid Carcinoma of Vulva in Nude Mice, and on the Body Weight of Mice |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 21</th>
<th>Day 0</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline + vehicle</td>
<td>265 ± 41</td>
<td>2426 ± 403</td>
<td>28.2 ± 0.3</td>
<td>26.1 ± 0.7</td>
</tr>
<tr>
<td>Saline + hEGF q7d × 3</td>
<td>280 ± 46</td>
<td>2446 ± 183</td>
<td>27.4 ± 0.4</td>
<td>25.4 ± 0.6</td>
</tr>
<tr>
<td>Saline + hEGF qd × 21</td>
<td>273 ± 41</td>
<td>2175 ± 344</td>
<td>26.6 ± 0.7</td>
<td>28.7 ± 1.7</td>
</tr>
<tr>
<td>MMC q7d × 3 + vehicle</td>
<td>282 ± 41</td>
<td>2138 ± 285</td>
<td>26.2 ± 0.6</td>
<td>25.0 ± 1.1</td>
</tr>
<tr>
<td>MMC q7d × 3 + hEGF q7d × 3</td>
<td>280 ± 44</td>
<td>1245 ± 150 a)</td>
<td>27.2 ± 0.3</td>
<td>27.5 ± 0.6 b)</td>
</tr>
</tbody>
</table>
| MMC q7d × 3 + hEGF qd × 21 | 282 ± 41 | 1522 ± 357 b) | 27.6 ± 0.3 | 28.4 ± 0.5 a)

The values represent mean ±S.E. of 8 mice. MMC was administered 2 mg/kg, i.p., and hEGF was 1 mg/kg, s.c. a, b) indicate significant difference, \( p < 0.05, 0.10 \) vs. MMC alone, respectively.
Fig. 1. Periodical Changes in the Tumor Weight (A, C) and Nontumorous Body Weight (B, D) during the Treatment with MMC, hEGF, or MMC and hEGF in A431 Tumor-Bearing Mice

A, B: Groups were treated 3 times on day 0, 7, and 14. (○), vehicle alone (1st group); (△), hEGF alone (2nd group); (●), MMC alone (4th group); (▲), MMC and hEGF (5th group). C, D: MMC was administered every 7th day on day 0, 7, and 14. Vehicle or hEGF was administered daily from day 0 to 21. (○), vehicle alone (1st group); (△) hEGF alone (3rd group); (●), MMC alone (4th group); (▲), MMC and hEGF (6th group). MMC was administered intraperitoneally at a dose of 2 mg/kg each. Human EGF was administered subcutaneously at a dose of 1 mg/kg each. Vehicle and/or saline were administered subcutaneously at a dosing volume of 10 ml/kg. Arrows indicate the time of administration. Bars represent the S.E. of 8 trials. a, b) and c) indicate the significant differences at levels of p < 0.01, 0.05, and 0.10, respectively from the treatment of MMC alone.

95.3 ± 2.8%; 5 th group, 100.9 ± 1.9%, p < 0.10 vs. 4th group; 6th group, 102.7 ± 1.7%, p < 0.05 vs. 4th group.

Specific Binding of hEGF to A431 Tumor Cells

The effect of single administration of hEGF on the EGF receptors on A431 tumor cells in vivo was examined by determining the specific binding of $^{125}$I-EGF, since it is well known that exposure of cells to EGF causes the down-regulation of EGF receptors. Figure 2 shows the reduction and recovery of specific hEGF binding to its receptors on A431 tumor cells. Twenty-

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![Graph showing specific binding of hEGF over days after administration]

Days after administration

- Fig. 2. Changes in the Specific Binding of hEGF to Its Receptors on A431 Tumor Cells after Single Administration of hEGF Subcutaneously at a Dose of 1 mg/kg. Bars represent the S.E. of 4 trials.
- a) Significantly different from days 0, \( p < 0.05 \).

four hours after the single treatment with hEGF at a dose of 1 mg/kg, the specific binding to receptors decreased by 80% of untreated mice, although complete recovery of specific binding was found 4 days after the single treatment.

Discussion

Previously, we demonstrated that the combined therapy of hEGF and antitumor agents might be a feasible approach for the treatment of cancer, provided that tumor cells are endowed with a high density of EGF receptors. Results showed that the enhancing effect of hEGF for the antitumor activity requires the binding of EGF to its receptor and that the binding seems to render tumor cells more susceptible to the antitumor agents. Also, the combined therapy did not affect the median lethal dose for each antitumor agent up to the dose of hEGF at 10 mg/kg. The above results suggest that hEGF does not modify the original cytotoxicity of each antitumor agent.

In the present study, the combined effect was examined from a viewpoint of dosage schedule of hEGF using MMC as antitumor agent and A431 solid tumor, which has many EGF receptors on the cell surfaces. The dosage schedule of daily or weekly treatment of human EGF was compared. With respect to the effect of EGF alone on the growth of tumor cells, it has been reported that EGF inhibits the proliferation of A431 tumor cells at a very low concentration \( 10^{-11} - 10^{-10} \) M, depending on the cell densities in vitro. In another report, the development of epidermal tumors by daily repetitive administration of EGF (2-5 mg/kg, subcutaneous administration) for several months has also been reported. However, as shown in Fig. 1A and 1C, the administration of hEGF alone to A431 tumor bearing mice did not affect the growth of A431 tumor compared to the administration of vehicle alone (1st group) in the present study. On the other hand, coadministration of hEGF with MMC enhanced remarkably the efficacy of the antitumor agent as shown in Fig. 1A and 1C. Greater effect was observed in the every 7th day treatment of hEGF compared to the every day administration of hEGF.

It will be necessary to establish an in vitro system which closely mimics in vivo animal study to elucidate the mechanism of the combined therapy. However, as described already, the action of hEGF alone on A431 tumor cells in vivo was very different from the reported results demonstrating the inhibition of the growth of A431 cells by hEGF in vitro. We also tried the in vitro experiment to confirm the enhancing effect of hEGF for the antitumor activity of MMC using A431 monolayer culture cells. However, the in vitro experimental system did not work well because that hEGF alone inhibits the growth of A431 tumor cells in vitro. As another possible mechanism, the inhibitory action of hEGF for soluble immune response suppressor activity was also considered as reported by Anue. Soluble immune response suppressor is a protein produced by mitogen- or interferon-activated suppressor T cell that inhibits antibody secretion by B lymphocytes and cell segmentation in neoplastic cell lines. However, the contribution of the inhibitory action of hEGF to the soluble immune response suppressor activity in the combined therapy was not considered as important in an in vivo animal study employed, if any, since no significant action on the A431 tumor cells was observed when hEGF alone was
administered to the A431 tumor-bearing mice (Fig. 1A and 1C).

Previously we examined the effect of single administration of hEGF on the pharmacokinetics of hEGF in rats. The plasma concentration of immunoreactive hEGF was kept at significantly higher levels in the hEGF pretreated rats compared to untreated rats, in association with the decrease of the accessible EGF receptors on the cell surfaces. Therefore, repeated administration of hEGF (6th group) must cause a marked down-regulation of EGF receptors and reduction in the enhancing effect of hEGF because of the decrease in its binding sites. In fact, we observed more enhanced efficacy of MMC toward A431 solid tumor by weekly treatment of hEGF (5th group) than by daily treatment (6th group).

In conclusion, it was found that an appropriate dosage schedule for hEGF was required to enhance the antitumor potency of MMC in the combined therapy with hEGF for the treatment of A431 solid tumors rich in EGF receptors of more than $1.5 \times 10^9$/cell. Every 7th day administration of hEGF showed a greater effect compared to that of every day treatment.

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References


