

# RECEPTOR BINDING OF EPIDERMAL GROWTH FACTOR IN CULTURED HUMAN CHORIOCARCINOMA CELL LINES: EFFECTS OF ACTINOMYCIN-D AND METHOTREXATE

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## ABSTRACT

Binding of epidermal growth factor (EGF) to its receptor was evaluated in the four cultured choriocarcinoma cell lines BeWo, NaUCC-1, NaUCC-2, and NaUCC-3. Also, the effect of the anti-tumor drugs actinomycin-D (Act-D) and methotrexate (MTX) on the EGF receptor binding was investigated in these cell lines. Incubation of these cells with [<sup>125</sup>I]EGF at 37°C resulted in a higher binding than that at 22°C or at 4°C. These bindings were saturable during 30- to 60-min incubation, and were specific and reversible. Scatchard analysis showed that the maximal number of receptor binding sites was  $2.89 \times 10^3$ /cell in BeWo cells,  $2.04 \times 10^3$ /cell in NaUCC-1 cells,  $1.84 \times 10^3$ /cell in NaUCC-2 cells, and  $1.01 \times 10^3$ /cell in NaUCC-3 cells. Preincubation with Act-D or MTX for 24 hr decreased the number of receptor binding sites (26%–53%) and slightly increased the receptor binding affinities. Combination of the two drugs resulted in a further diminution of EGF receptor binding sites in BeWo, NaUCC-1, and NaUCC-3 cells, respectively, but reversed the Act-D effect in NaUCC-2 cells. These results indicated that choriocarcinoma tissue is rich in EGF receptors, that the anti-tumor drugs Act-D and MTX diminish the receptor binding sites in the tissue, and suggest that MTX might induce a drug resistance to Act-D in some choriocarcinoma tissue.

Key Words: Epidermal Growth Factor, Receptor Binding, Choriocarcinoma, Actinomycin-D, Methotrexate.

## INTRODUCTION

Epidermal growth factor (EGF), a single chain polypeptide of 6,040 daltons isolated from the mouse submaxillary gland<sup>1)</sup>, has shown several biological responses in human placenta, for example, the release of human chorionic gonadotropin (hCG) and the differentiation of trophoblastic tissue<sup>2,3,4)</sup>. Similar responses were also observed in choriocarcinoma cell lines<sup>4,5,6)</sup>. It has become evident that all these responses are effected through a specific binding of the EGF receptor on the surface of the cells<sup>7,8,9)</sup>, and the study of this receptor has come to the forefront of research on choriocarcinoma tissue<sup>10)</sup>. In recent years, several authors have reported that the activity and metabolism of EGF receptors are controlled by gonadotropin hormones, some sexual steroid hormones, and EGF itself<sup>11,12,13)</sup>. In the present study, we evaluated the distribution of EGF receptors in the four cultured choriocarcinoma cell lines BeWo, NaUCC-1, NaUCC-2, and NaUCC-3, respectively. And we investigated the effect of the anti-tumor drugs actinomycin-D (Act-D) and methotrexate (MTX) on the EGF receptors in these cell lines.

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## MATERIALS AND METHODS

EGF (BT-201, receptor grade) purified from mouse submaxillaries was purchased from Biomedical Technologie, INC. (Stoughton, MA). Iodine-125-labeled EGF was obtained from New England Nuclear Research Products (Boston, MA); its radioactivity was about 156–168  $\mu\text{Ci}/\mu\text{g}$  protein.

The human choriocarcinoma cell line BeWo was established by Pattillo *et al.* in 1968<sup>14</sup>). Human choriocarcinoma cell lines NaUCC-1, NaUCC-2, and NaUCC-3 were newly established in our laboratory, and their cytological characteristics have been reported by Sugiura *et al.*<sup>15</sup>). BeWo cells were grown in RPMI1640 (pH 7.4, Nissui Co., Tokyo); NaUCC-1, NaUCC-2, and NaUCC-3 cells, in minimal essential medium (pH 7.4 Nissui Co., Tokyo) at 37°C in a 95 %-air 5 %-CO<sub>2</sub> incubator (Forma Scientific, OH). The culture mediums contained 10 percent fetal calf serum (FCS).

The [<sup>125</sup>I]EGF binding was tested by a modified procedure of Chen *et al.*<sup>16</sup>). First, FCS, which probably contained some growth factors, was removed from the culture medium 24 hr before the binding test. Well-cultured cells in monolayer-confluence in 50-mm dishes (Falcon) were incubated with [<sup>125</sup>I]EGF (5,000–50,000 cpm) in 2 ml of medium at 4°C, 22°C, or 37°C. Incubation was stopped at various time periods — 15, 30, 60, 90, and 120 min — by washing twice with medium, and twice with phosphate sodium buffer (0.1 M, pH 7.4) at 4°C. The radioactivity remaining in the dishes was measured with a gamma counter (ARC 500, Aloka Co., Tokyo). Control binding was measured by adding excess unlabeled EGF. To determine the number of EGF receptors per cell, the cell number was also calculated at the time of the [<sup>125</sup>I]EGF binding test.

In the next test, well-grown cells were further incubated in the presence of Act-D, MTX, or a combination of both at 37°C for 2, 4, 6, 12, 24 and 48 hr, and the cell numbers were counted. Then the [<sup>125</sup>I]EGF binding test was performed by the procedure described above. Since each choriocarcinoma cell line shows different sensitivity to Act-D and MTX, the dosages of Act-D and MTX used for each choriocarcinoma cell were chosen according to our previous study<sup>17</sup>). The sensitivity of each choriocarcinoma cell line to Act-D and MTX can be expressed as the 50-percent effective dose (ED<sub>50</sub>). The ED<sub>50</sub> of Act-D was  $5.0 \times 10^{-5}$   $\mu\text{g}/\text{ml}$  for BeWo,  $2.0 \times 10^{-6}$   $\mu\text{g}/\text{ml}$  for NaUCC-1,  $1.0 \times 10^{-5}$   $\mu\text{g}/\text{ml}$  for NaUCC-2, and  $2.0 \times 10^{-5}$   $\mu\text{g}/\text{ml}$  for NaUCC-3 cells; the ED<sub>50</sub> of MTX was  $2.0 \times 10^{-3}$   $\mu\text{g}/\text{ml}$  for BeWo,  $8.0 \times 10^{-3}$   $\mu\text{g}/\text{ml}$  for NaUCC-1,  $2.0 \times 10^{-3}$   $\mu\text{g}/\text{ml}$  for NaUCC-2, and  $1.5 \times 10^{-3}$   $\mu\text{g}/\text{ml}$  for NaUCC-3 cells.

As a positive control of EGF receptor-rich cell line, we used epidermoid carcinoma cell line A-431<sup>18</sup>) and investigated the effect of anti-tumor drugs on these cells. The above experiments were performed in triplicate for each sample.

Human placentas from four normal pregnant women (8 weeks, 1; term, 3) were also used in the study. The preparation of trophoblast cell membrane and the binding test were described previously by Chen *et al.*<sup>16</sup>).

Student's t-test was used to determine statistical significance, and Scatchard plot analysis<sup>19</sup>) was applied in analysing the EGF-receptor binding.

## RESULTS

Well-cultured choriocarcinoma cells were exposed to [<sup>125</sup>I]EGF in the presence or absence of unlabeled EGF at 4°C, 22°C, and 37°C. After 60 min of incubation, the most specific binding of [<sup>125</sup>I]EGF was found at 37°C incubation. At 22°C incubation, the [<sup>125</sup>I]EGF binding was about 85 % of that at 37°C; and at 4°C, it was lower than 30 % of that at 37°C. Binding could be inhibited gradually by increasing the amount of unlabeled EGF, and it was partially displaceable by unlabeled EGF (data not shown).

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Figure 1 shows the time-dependent binding of [ $^{125}$ I]EGF to the four cultured choriocarcinoma cells. The rate of binding of [ $^{125}$ I]EGF increased immediately after the start of incubation, became saturated during 30–60 min of incubation, and then declined gradually thereafter. The four choriocarcinoma cell lines showed a similar pattern of time-dependent [ $^{125}$ I]EGF binding, though the time of saturation (the peak binding) differed among them. The time course of [ $^{125}$ I]EGF binding of the epidermoid carcinoma cell line A-431 was similar to those of the choriocarcinoma cell lines (Fig. 1). In contrast to these cultured cell lines, the membrane preparation of normal human trophoblast showed no decline in [ $^{125}$ I]EGF binding even after long exposure to EGF at either 37°C or 22°C (Fig. 2).

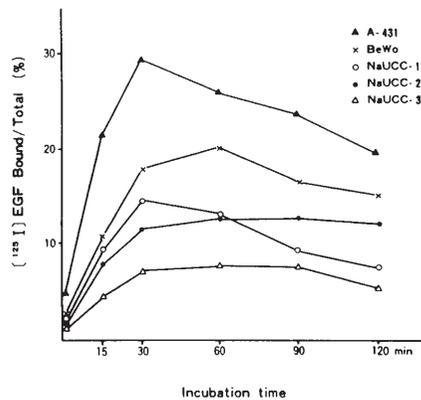


Fig. 1. Time-dependent [ $^{125}$ I]EGF-receptor binding pattern on four cultured choriocarcinoma cells lines and on epidermoid carcinoma cell line A-431.

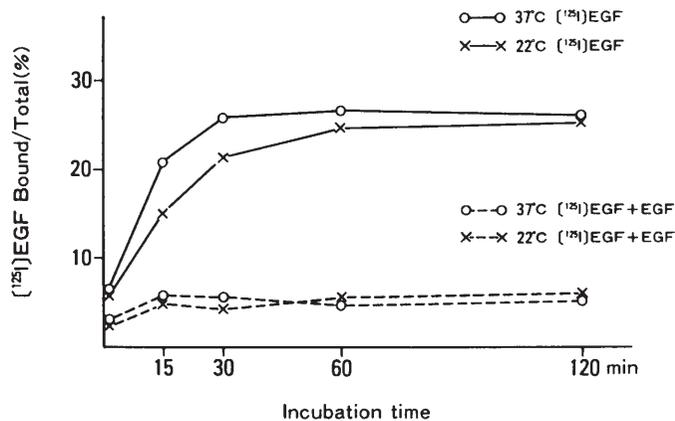


Fig. 2. Time-dependent [ $^{125}$ I]EGF-receptor binding pattern on the membrane preparation of normal chorionic tissue.

We then investigated the effect of the two anti-tumor drugs on [ $^{125}$ I]EGF binding in the cultured choriocarcinoma cells. Figure 3 shows the Scatchard plot analysis of [ $^{125}$ I]EGF binding to the four choriocarcinoma cell lines following 24-hr preincubation with Act-D or MTX or the combination of both. In the normal control group (without Act-D and MTX), the maximal binding sites (Bm) of EGF were highest in BeWo,  $2.89 (\pm 0.24) \times 10^3/\text{cell}$ , and least in NaUCC-3,  $1.01 (\pm 0.14) \times 10^3/\text{cell}$ . In NaUCC-1, the Bm was  $2.04 (\pm 0.22) \times 10^3/\text{cell}$ , and in NaUCC-2,  $1.84 (\pm 0.15) \times 10^3/\text{cell}$ . Twenty-four hour preparation with Act-D reduced approximately 42 % of the binding sites in BeWo ( $P < 0.01$ ), 35 % in NaUCC-1 ( $P < 0.05$ ), 31 % in NaUCC-2 ( $P < 0.05$ ), and 26 % in NaUCC-3 ( $P < 0.005$ ). Twenty-four-hour preincubation with MTX eliminated about 53 % of the binding sites in BeWo cells ( $P < 0.01$ ), and 31 % of the binding sites in NaUCC-3 cells ( $P < 0.05$ ). Reduction in the binding sites in NaUCC-1 and NaUCC-2 cells was not statistically significant ( $P > 0.05$ ). A combination of Act-D and MTX further diminished the EGF binding sites in BeWo (72 % of the control group,  $P < 0.01$ ), NaUCC-1 (47 % of the control group,  $P < 0.01$ ), and NaUCC-3 cells (36 % of the control group,  $P < 0.05$ ), but reversed the Act-D effect in NaUCC-2 cells (124 % of the Act-D treated group,  $P < 0.05$ ). The changes in the number of EGF receptors in the four cell lines are summarized in Figure 4.

The EGF receptor binding affinity (Kd) after preincubation with Act-D, MTX or the combination of both, slightly increased in the NaUCC-1, NaUCC-2, and NaUCC-3 cells, respectively, but was not affected in the BeWo cells (Fig. 3).

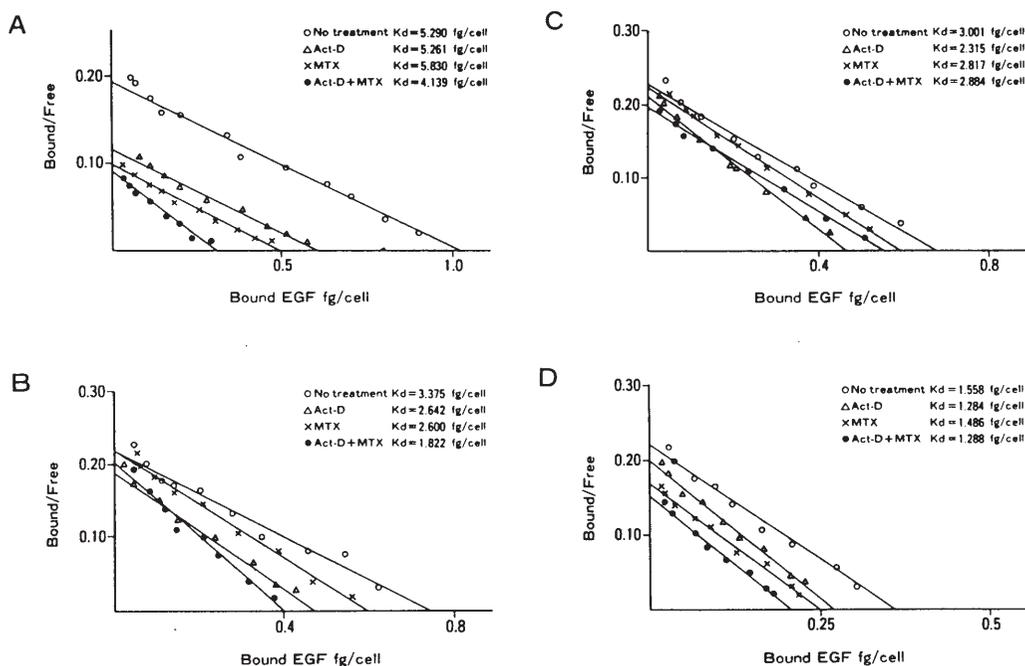


Fig. 3. Scatchard analysis of EGF receptor binding with BeWo cells (A), NaUCC-1 cells (B), NaUCC-2 cells (C), and NaUCC-3 cells (D) after 24-hr preincubation with Act-D, MTX, and a combination of the two drugs, respectively. Kd, the dissociation constant; Bm, the maximal number of binding sites.

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The number of EGF receptor binding sites of the epidermoid carcinoma A-431 cell line was about  $2.80 (\pm 0.41) \times 10^4/\text{cell}$ , and it was not significantly affected by the treatment of Act-D or MTX even at the highest dosage as used in the choriocarcinoma cell lines.

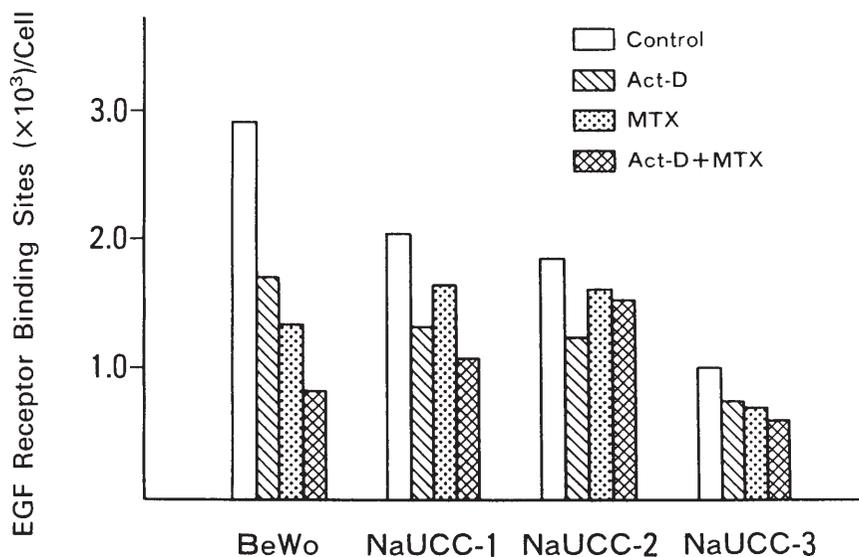


Fig. 4. Number of EGF receptor binding sites in choriocarcinoma cells and the effects of Act-D, MTX, and a combination of the two drugs ( $n=6$  for each group).

## DISCUSSION

The present study showed that choriocarcinoma is rich in EGF receptors. The number of receptor binding sites in these cells was five to ten percent of that in the epidermoid carcinoma cell line A-431. These values agree with those previously reported for normal and malignant chorionic tissues<sup>8,9,10</sup>.

$[^{125}\text{I}]\text{EGF}$  bound rapidly and reversibly to choriocarcinoma cells. The fate of the bound  $[^{125}\text{I}]\text{EGF}$ -receptor complex is not yet clear. At least, it cannot be explained only by the insertion or endocytosis of the complex, the process known as down-regulation<sup>20</sup>. The reduction of membrane-bound  $[^{125}\text{I}]\text{EGF}$  following the peak binding (Fig. 1) suggests that long incubation with  $[^{125}\text{I}]\text{EGF}$  leads to a dissociation of EGF from its receptor by the inactivation of the receptor itself, and/or a diminution of the number of receptors on the membrane surface<sup>20</sup>. It seems that in these living cells, there is a rapid regulation of EGF receptors after they are activated by EGF. This view was supported by the fact that the membrane preparation of trophoblastic tissue showed a different pattern of time-course binding, in which no decline was observed after the binding was saturated (see Fig. 2).

Twenty-four hour preincubation with Act-D or MTX diminished the EGF receptor binding sites in all choriocarcinoma cell lines. These effects were not observed after preincubation of 12 hr or less, and 48 hr of preincubation led to a further diminution of the receptor binding sites (data not shown).

It is believed that the EGF receptor binding activity and metabolism are related to the bioactivity of these cells<sup>21)</sup>. The variation of receptor binding sites may be reflected by the different stages of cell differentiation. Therefore, the diminution of EGF receptor binding sites by Act-D and MTX might be a consequence of the inhibition effects of the two anti-tumor drugs on tumor cell activity. However, it is difficult to explain the increase of EGF receptor binding affinity after preincubation with Act-D and MTX (see Fig. 3).

It should be noted that the combination of Act-D and MTX reversed the Act-D-induced diminution of the EGF receptor binding sites in NaUCC-2 cells but not in the other cells. In view of our previous report that a combined treatment of Act-D and MTX reversed the growth-inhibition effect of Act-D in NaUCC-2<sup>22)</sup>, the present study suggests that MTX induces a drug resistance to Act-D in NaUCC-2 cells. Although the mechanism of this drug resistance is poorly understood, we wish to suggest that the most widely used combination chemotherapy for choriocarcinoma, Act-D and MTX, may not always be the best choice in all cases.

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