Conversion of epidermal growth factor (EGF) into a stimulatory ligand for A431-cell growth by herbimycin A by decreasing the level of expression of EGF receptor

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We examined effect of the tyrosine kinase inhibitor herbimycin A on A431 human epidermoid carcinoma cells which over-express epidermal growth factor (EGF) receptor. Herbimycin A inhibited the autophosphorylation of EGF-stimulated receptors in intact cells both time- and dose-dependently. The inhibition was found to be due to a decrease in the level of expression of the receptor amount, because herbimycin A equally decreased the receptor quantity and EGF-stimulated receptor kinase activity in intact cells, but did not exhibit a direct inhibitory effect on EGF receptor kinase activity *in vitro*. The decrease of the level of EGF receptor was also confirmed by ¹²⁵I-EGF binding to herbimycin

A-treated cells, and Scatchard analysis showed that the decrease in the receptor number occurred in the major population of the low-affinity binding ones, whereas the number with high-affinity binding was unaffected. Interestingly, although the proliferation of A431 cells was inhibited by EGF, herbimycin A converted EGF into a stimulatory ligand for cell growth, as determined by both cell number and DNA synthesis. These findings indicated that herbimycin A decreased the level of expression of EGF receptor by a mechanism other than inactivation of the receptor kinase and reversed the transformed phenotype of A431 cells to a normal one in the proliferative response to EGF.

INTRODUCTION

It is known that receptor-type tyrosine kinases are deeply involved in signalling pathways for cell proliferation. Epidermal growth factor (EGF) receptor is a typical receptor-type tyrosine kinase (reviewed by Carpenter [1]); the primary structure of its cytoplasmic domain is homologous to that of the product of *v-erbB*, the transforming gene of avian erythroblastosis virus, and is thought to be the *c-erbB* proto-oncogene product. Many human tumour cells of ectodermal origin (glioblastoma, squamous carcinoma of lung, mammary gland, oesophagus and skin) are known to express extraordinarily high numbers of EGF receptors, suggesting a primary role of the receptor in the development of these neoplasias. Human epidermoid cells are good materials with which to investigate a possible causal relationship between the excess expression of EGF receptor and malignant cell growth.

Herbimycin A was found to reverse the transformation by *src* oncogene by inactivating the kinase activity of p60^{v-src} [2]. It also reversed the transformation of other tyrosine kinase-encoding oncogenes (*yes*, *fps*, *erb-B*, *abl*, *ros*), but did not reverse those of *raf*, *ras* or *myc*, suggesting that herbimycin A is a specific inhibitor of tyrosine kinases [3]. Herbimycin A has recently been demonstrated to inhibit tyrosine kinases specifically *in vitro* without affecting protein kinase A or protein kinase C [4]. The inhibition of the kinase activity appeared to be due to its binding to cysteine residue(s) of tyrosine kinase, like thiol reagent, and its direct binding has recently been confirmed by labelling v-Src and BCR-ABL tyrosine kinases with [¹⁴C]herbimycin A [5].

Although we have studied extensively the effect of herbimycin A on tyrosine kinases which belong to the non-receptor class, we have not determined its effect on the other type of tyrosine

kinases, which belong to the receptor class. We therefore investigated the effect of herbimycin A on A431 human epidermoid carcinoma cells that over-express EGF-receptor tyrosine kinase and found that it markedly decreased the autophosphorylation of EGF receptors in intact cells. Unexpectedly, however, it was found that the decrease in the receptor phosphorylation was due to the decrease in amount of receptor rather than to inactivation of kinase activity. Interestingly, herbimycin A converted EGF into a stimulatory ligand to proliferate A431 cells.

MATERIALS AND METHODS

Materials

Herbimycin A was isolated as described previously [6] and was used from a 1.0 mg/ml stock solution dissolved in dimethyl sulphoxide (DMSO). Erbstatin was provided by Dr. T. Takeuchi, Institute of Microbial Chemistry (Tokyo, Japan). Tyrphostin (3,4,5-trihydroxybenzylidene malononitrile) was purchased from GIBCO BRL (Gaithersburg, MD, U.S.A.). EGF was from Takara Shuzo Co. (Kyoto, Japan). [³²P]Phosphoric acid ([³²P]P_i; > 370 MBq/ml) was purchased from the Japan Atomic Research Institute (Ibaraki, Japan), L-[³⁵S]methionine (680.7 Ci/mmol) was from NEN Research Products (Wilmington, DE, U.S.A.), [³²P]ATP (4500 Ci/mmol) was from ICN Biomedicals (Costa Mesa, CA, U.S.A.), and mouse ¹²⁵I-EGF (100 µCi/µg) was purchased from Amersham International (Amersham, Bucks., U.K.). Anti-EGF receptor monoclonal antibody (Ab-1) was purchased from Oncogene Science Inc. (Uniondale, NY, U.S.A.), and as a second antibody rabbit anti-mouse IgG was purchased from Organon Teknika Corp.–Cappel Products (West Chester, PA, U.S.A.).

Abbreviations used: EGF, epidermal growth factor; DMSO, dimethyl sulphoxide; DME, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; MEM, minimal essential medium.

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Cell growth and DNA synthesis

A431 human epidermoid carcinoma cells (obtained from The Japanese Cancer Research Resources Bank) were grown in Dulbecco's modified Eagle's medium (DME) containing 10% fetal-calf serum (FCS). For cell-proliferation experiments, cells were seeded in 24-well plates at two different cell densities (2×10^5 or 2×10^4 per 2 cm^2 well). At 24 h after cell seeding, the medium was changed, and herbimycin A and EGF were added at the concentrations indicated in the Figure legends. After 3 days of culture (70–80% of confluency at high density), the cell number was determined with a Coulter counter. For assay of DNA synthesis, the cells were plated at 2×10^5 per well and cultured for 48 h. The medium was then replaced with 0.5%-serum-containing medium and incubated for another 48 h. The medium was again replaced with serum-free medium, herbimycin A and EGF were added, and 24 h later the cells were pulse-labelled with [^3H]thymidine for 1 h. Radioactivity was determined by solubilizing cells with 1 M KOH, and counted for radioactivity in a scintillation counter.

Cell labelling and herbimycin A and/or EGF treatment

Cultured A431 cells were washed with phosphate-free minimal essential medium (MEM), then placed in the same fresh medium containing [^{32}P]P_i (0.75 mCi/ml), and cultured for 4 h with or without EGF (50 ng/ml) and herbimycin A. For pretreatment, cells were incubated with herbimycin A for 20 h, washed and resupplied with the labelling medium containing herbimycin A.

For steady-state labelling of the protein, A431 cells were cultured and washed with methionine-free MEM, then incubated in 1.5 ml of MEM with decreased methionine (20% of the normal concentration in DME) and 5% FCS containing [^{35}S]methionine (25 $\mu\text{Ci}/\text{ml}$) for 24 h. The medium was removed, new labelling medium was supplied and the cells were further incubated for 20 h. For treatment of cells with herbimycin A, it was added in the second incubation.

Immunoprecipitation

Labelled cells were washed with PBS and solubilized with RIPA buffer (20 mM Tris/HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 100 kallikrein-inactivator units/ml aprotinin, 100 μM Na₃VO₄, 1 mM phenylmethanesulphonyl fluoride, and 20 $\mu\text{g}/\text{ml}$ each of antipain, pepstatin and leupeptin), or for [^{35}S]methionine-labelled cells in accordance with the conditions for the kinase reaction, with HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 100 kallikrein-inactivator units/ml aprotinin, 100 μM Na₃VO₄, 1 mM phenylmethanesulphonyl fluoride, and 20 $\mu\text{g}/\text{ml}$ each of antipain, pepstatin and leupeptin) on ice for 30 min. Lysates were transferred to Eppendorf tubes, and centrifuged to remove debris. The supernatants, containing a fixed amount of protein, were incubated with anti-(EGF receptor) antibody for 1 h at 0 °C, followed by treatment with the second antibody. Next, immune complexes were collected with formaldehyde-fixed Protein A (Pansorbin; Calbiochem, San Diego, CA, U.S.A.) and washed with RIPA buffer. The immunoprecipitates were suspended in 30 μl of electrophoresis sample buffer (42 mM Tris/HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 0.002% Bromophenol Blue), boiled for 3 min, and spun down. The supernatants were analysed by SDS/PAGE in 7.5% gels.

To estimate phosphorylation of the receptor, the ^{32}P radioactivity in cut bands was measured with a scintillation counter.

Alternatively, radioactivities in Figure 2 and Table 1 were analysed by using a Fujix BAS2000 bio-image analyser. To quantify the [^{35}S]methionine incorporation into protein, the bands revealed by fluorography were cut out, solubilized, and radioactivity was measured with a scintillation counter.

Protein kinase assay

To measure EGF-receptor kinase activity after cells were treated with herbimycin A, cells were solubilized with HNTG buffer and cell debris was removed. A 5 μl portion of the lysate, containing 10–20 μg of protein, was incubated with 10 μl of kinase buffer (20 mM Hepes, pH 7.4, 5 mM MnCl₂) with or without 4 $\mu\text{g}/\text{ml}$ EGF for 30 min at room temperature, and reaction was started with 5 μl of kinase buffer containing [γ - ^{32}P]ATP (5 $\mu\text{Ci}/\text{assay}$; 15 μM) and incubated at 0 °C for 10 min. Reaction was stopped by addition of RIPA buffer, and the mixture was immunoprecipitated with anti-(EGF receptor) antibody and applied to SDS/PAGE.

For kinase assay examining the direct effect of inhibitors, the assay was conducted as described above, except that 2 μl of 10-fold-concentrated inhibitor dissolved in 10% DMSO was included throughout the incubation.

^{125}I -EGF-binding assay

^{125}I -EGF binding to A431 cells was performed principally by the method of Carpenter [7], but at 4 °C, for 2 h. Cells seeded in 24-well plates (1.5×10^5 cells per 2 cm^2 well) were incubated in DME containing 10% FCS for 1 day and were treated with or without herbimycin A for 20 h. After removing the medium, the cells were washed with DME (without serum), and placed in 0.25 ml of binding medium (20 mM Hepes, pH 7.4, and 1% BSA in DME). ^{125}I -EGF (diluted with unlabelled EGF) was added and the cells were incubated on ice for 4 h. Non-specific binding was measured by adding a 100-fold excess of unlabelled EGF. After removing the medium, cells were washed with PBS and solubilized with 0.5 ml of 0.1 M NaOH. The amount of radioactivity was determined in a γ -radiation scintillation counter (Packard MINAXI γ). Assays were carried out in duplicate.

RESULTS

Effect of herbimycin A on EGF-stimulated EGF-receptor kinase activity

We first examined the ability of herbimycin A to inhibit EGF-stimulated EGF-receptor autophosphorylation in intact cells. A431 cells were treated with herbimycin A for 4 or 24 h and were labelled with [^{32}P]P_i for the last 4 h in the absence or presence of EGF (50 ng/ml). Figure 1 shows that EGF stimulated the EGF-receptor autophosphorylation. Treatment of cells with up to 10 $\mu\text{g}/\text{ml}$ herbimycin A for 4 h had no effect on EGF-receptor phosphorylation, but significant inhibition of phosphorylation was found after 24 h of treatment (Figure 1, lanes f–h). With 24 h treatment, phosphorylation of the EGF receptor was inhibited in a dose-dependent manner, and with 1.0 $\mu\text{g}/\text{ml}$ herbimycin A the level of EGF-receptor phosphorylation was about 26% of the control.

Figure 2 shows the time course of the effect of herbimycin A on EGF-stimulated EGF-receptor tyrosine kinase activity. A431 cells were treated with 1.0 $\mu\text{g}/\text{ml}$ herbimycin A for 10 min to 20 h. The cell lysates were then pulsed with EGF (50 ng/ml) and [γ - ^{32}P]ATP. As shown in Figure 2, EGF stimulated EGF-receptor kinase activity by 6-fold, and the inhibitory effect of herbimycin

Figure 1 Effect of herbimycin A on the phosphorylation of EGF receptor in A431 cells

Cells were untreated (lanes a–e) or pretreated (lanes f–h) without (C) or with the indicated concentrations of herbimycin A (H) for 20 h, then washed, refed with phosphate-free MEM, [32 P]P_i was supplied, and incubation was continued for another 4 h in the absence (lane a) or presence (lanes b–h) of 50 ng/ml EGF with another addition of herbimycin A. The cells were lysed, immunoprecipitated with anti-(EGF receptor) antibody, and analysed by SDS/PAGE (7.5% gel) and autoradiography. Shown are the results of gel exposure after treatment with 1 M KOH at 55 °C for 2 h to enrich phosphotyrosine [33]. The values at the bottom are 32 P radioactivities of each receptor band, in c.p.m. and as percentages of that of the control. Positions of the 170 kDa EGF receptor (arrow) and of size markers (kDa) are shown at the left.

Figure 2 Effect of herbimycin A on kinase activities of EGF receptor in A431 cells

Cells were untreated (C) or treated with 1.0 µg/ml herbimycin A (H) for indicated lengths of time from 10 min to 20 h, and lysates were prepared and used for kinase reaction without (lane a) or with (lanes b–h) EGF (2 µg/ml) stimulation. The kinase reaction was stopped with RIPA buffer, and the receptor was immunoprecipitated and analysed by SDS/PAGE (7.5% gel) and autoradiography. Values at the bottom are 32 P radioactivity as a percentage of that of the control. Positions of 170 kDa EGF receptor (arrow) and size markers (kDa) are shown at the left.

A was revealed when the treatment of the cells extended over 4 to 20 h. The decrease in the receptor kinase activity was time-dependent, and significant inhibition (26% of the activity remained in treated cells as compared with the control) was

Figure 3 Effect of herbimycin A on protein amount (a) and kinase activity (b) of EGF receptor in A431 cells

Cells were labelled for 24 h with [35 S]methionine as described in the Materials and methods section. The cells were unexposed (C) or exposed to herbimycin A (H) at the indicated concentrations during the period of another 20 h of labelling. The cells were lysed, and 20 µg of each lysate was subjected either to immunoprecipitation with an anti-(EGF receptor) antibody for the determination of [35 S]methionine-labelled receptor (a) or to the receptor kinase reaction with or without 200 ng/ml EGF (b). Autophosphorylated EGF receptor was then immunoprecipitated for analysis. Values at the bottom represent radioactivity in each receptor band, as c.p.m. and as a percentage of the control. Positions of 170 kDa EGF receptor (arrows) and size markers (kDa) are shown at the left.

Table 1 Direct effect of tyrosine kinase inhibitors on EGF-receptor kinase activity

Inhibitors were dissolved in 10% DMSO and added into the reaction mixture at $\frac{1}{10}$ vol. as described in the Materials and methods section. Percentage inhibition was the extent of decrease in 32 P radioactivity, measured by bio-image analyser, compared with the control containing 1% DMSO instead of inhibitor.

Inhibitor	Concn. (µg/ml)	Inhibition (%)
Herbimycin A	1.0	0
	10	0
	100	0
Erbstatin	10	87
	100	99
Tyrphostin	10	49
	100	96

observed 20 h after treatment with the inhibitor. Longer treatment for 48–72 h caused almost complete inhibition (results not shown), whereas short-period treatments of less than 1 h caused no significant decrease in the receptor kinase activity. These results indicated that herbimycin A caused a decrease in phosphorylation or kinase activity of EGF receptor in a dose-dependent manner, and that time is required to achieve the maximal inhibitory effect.

We next determined the amount of EGF-receptor protein, to clarify whether the inhibitory effect of herbimycin A was due to a decrease in the amount of receptor or inactivation of the receptor kinase activity, as was observed with v-Src kinase [2,8]. As shown in Figure 3(b), EGF-stimulated receptor kinase activity decreased to 40 and 30% of the control with treatment of herbimycin A at

Figure 4 Scatchard plot of EGF binding in herbimycin A-treated A431 cells

Scatchard-plot analysis was done from the data of EGF binding assay with control (○) and herbimycin A-treated (●; 1.0 µg/ml, 20 h) cells. The binding assay was carried out with [³⁵S]-EGF (radioactivity 2236 c.p.m./ng). Each data point represents the average value from two wells.

0.3 and 1.0 µg/ml respectively. In accordance with this, the steady-state level of EGF-receptor protein that was assessed by labelling cells metabolically to equilibrium with [³⁵S]methionine was decreased to 40 % of the control (Figure 3a). These results suggested that the decrease in the receptor kinase activity was due to the decrease in receptor amount, rather than to inhibition of the specific activity of the receptor kinase.

We next examined the direct effect of herbimycin A on EGF-receptor kinase activity, using cell lysates from A431 cells in a protein kinase assay. We found that, under the conditions used, herbimycin A at 1–100 µg/ml was unable to inhibit EGF receptor kinase activity (Table 1). For comparison, the kinase assay was performed in the presence of either erbstatin or tyrphostin, well-characterized inhibitors of EGF-receptor kinase activity [9–11]. In contrast with herbimycin A, these inhibitors significantly inhibited the receptor kinase activity (Table 1). In a separate experiment, we also examined the direct effect of herbimycin A, using an A431-cell membrane fraction, but no inhibitory effect was observed (results not shown).

Effect of herbimycin A on EGF-receptor expression on the cell surface as determined by ¹²⁵I-EGF binding

To examine the effect of herbimycin A on expression of the EGF receptor on the cell surface, we next determined the number of binding sites for EGF. The binding assay was performed with ¹²⁵I-labelled EGF after A431 cells were treated with 1.0 µg/ml herbimycin A for 20 h. In agreement with the previous reports [12–14], a Scatchard-plot profile showed the existence of two affinity classes of EGF receptors (Figure 4). The dissociation constants were estimated to be $K_{d1} = 2.4 \times 10^{-8}$ M and $K_{d2} = 6 \times 10^{-10}$ M, and the receptor numbers to be 1.0×10^6 and 0.72×10^5 per cell, for low- and high-affinity receptors respectively. The analysis for herbimycin A-treated cells still indicated the existence of two affinity classes (K_d values 7.7×10^{-9} M and 1.1×10^{-9} M). However, the number of low-affinity receptors was significantly decreased to about 30–40 % of the control, whereas that for high-affinity binding receptors was unchanged. Thus the ¹²⁵I-EGF-binding capacity of herbimycin A-treated cells correlated well with the decreased level of the receptors as quantified by immunoprecipitation. The decreased EGF-binding capacity was not due to a direct inhibition of EGF binding to the cells, because herbimycin A even at 10 µg/ml had no effect on the binding of ¹²⁵I-EGF to the cells (results not shown).

Figure 5 Effect of herbimycin A on the growth of A431 cells in response to EGF

The cells were seeded either at low (1×10^4 cells/cm²) (a) or high (1×10^5 cells/cm²) cell density (b). After incubation overnight, the medium was replaced and incubation was continued for 3 days in the presence of different concentrations of EGF without (○) or with herbimycin A (+H: □, 0.1 µg/ml; ■, 0.3 µg/ml; ▲, 1.0 µg/ml). Cell numbers from EGF-untreated control runs at each concentration of herbimycin A were taken as 100%.

Effect of herbimycin A on EGF-mediated growth of A431 cells

We next examined the effect of herbimycin A on cell growth, because the A431 cell line is unusual in that its growth is inhibited by EGF, and the growth-inhibitory effect of EGF has been postulated to be due to the expression of an extremely large number of EGF receptors [15]. We undertook this experiment using two different cell densities, since the level of EGF-receptor expression on the cell surface was reported to be altered according to cell density [16]. As shown in Figure 5, A431-cell growth was inhibited by EGF in a dose-dependent manner, and this was more obvious at a lower cell density, whereas additions of herbimycin A at 0.1–0.3 µg/ml blocked the inhibition of cell growth (Figure 5a). At a higher cell density, the addition of herbimycin A actually caused a stimulation of cell growth (Figure 5b). The conversion of EGF into a stimulatory ligand for A431-cell growth was also observed in an assay determining DNA synthesis monitored by [³H]thymidine incorporation. Figure 6 shows the DNA synthesis in A431 cells exposed to herbimycin A in the absence or presence of several concentrations of EGF. The ordinate of the Figure shows that low concentrations of EGF (1–10 ng/ml) stimulated DNA synthesis, whereas a high concentration of EGF (100 ng/ml) inhibited the synthesis, roughly agreeing with the data shown in Figure 5(b). Increasing concentrations of herbimycin A inhibited DNA synthesis in the absence or presence of 1 ng/ml EGF. However, when herbimycin A was added along with 10 or 100 ng/ml EGF, DNA synthesis was

Figure 6 Effect of herbimycin A on DNA synthesis

(a) A431 cells seeded at 1×10^5 cells/cm² were cultured with different concentrations of EGF (○, 0; ●, 1 ng/ml; ■, 10 ng/ml; ▲, 100 ng/ml) in the presence of the indicated concentrations of herbimycin A (+H) for 24 h, and [^3H]thymidine uptake was measured as described in the Materials and methods section. (b) The relative values of DNA synthesis in the presence of the indicated concentrations of EGF compared with EGF-untreated controls is expressed from the data shown in (a).

stimulated. As shown in Figure 6(b), maximal stimulation of DNA synthesis occurred at 0.3 $\mu\text{g/ml}$ herbimycin A, and a nearly 20-fold increase in the rate of synthesis over the EGF-untreated control was demonstrated at 10–100 ng/ml EGF. These results indicate that herbimycin A altered A431-cell growth behaviour toward that of a normal cell phenotype in the proliferative response to EGF.

DISCUSSION

Previous studies indicated that the ansamycin antibiotic herbimycin A blocked many biological events mediated by tyrosine kinases [17–23]. It selectively decreased tyrosine kinase activities both in cells and in cell-free systems [2–4,8]. All these effects, however, have been demonstrated with non-receptor-type tyrosine kinases such as Src, Abl, Lck, Fyn etc., and no studies with receptor-type tyrosine kinases have been reported. In the present study, we examined the effect of herbimycin A on EGF receptors, using human epidermoid carcinoma A431 cells, which over-express this typical receptor-type tyrosine kinase. We found that

herbimycin A had no direct inhibitory effect on EGF-receptor kinase activity, but decreased the level of expression of EGF-receptor protein in A431 cells and converted EGF into a stimulatory ligand for cell growth.

One of the major points found in this study was that the decrease in EGF-stimulated phosphorylation and the receptor kinase activity in herbimycin A-treated A431 cells was due to the decrease in amount of EGF receptor. This decrease in the level of expression was quantified by both immunoprecipitation of metabolically labelled receptor and growth-factor binding, and results reasonably in agreement were obtained. Although the degree of inhibition varied slightly from experiment to experiment (60–80 % decrease at 1.0 $\mu\text{g/ml}$ for 20–24 h), the levels of receptor kinase activity were fairly consistently identical with those of receptor protein amount. Thus we concluded that the decrease in the receptor kinase activity was due to a decrease in receptor amount, rather than to inhibition of the specific activity of the receptor kinase.

It was unexpected that the kinase activity of the EGF receptor was not inactivated by herbimycin A, because recent studies suggested that two conserved cysteine residues in the tyrosine kinase domains are the most probable binding sites for herbimycin A [5,24], and these residues are also present in the kinase domain of the EGF receptor [25]. Although we do not yet know the biological basis differentiating the insensitivity to herbimycin A of EGF-receptor kinase and cytoplasmic type tyrosine kinases, the finding raised the possibility in that different protein tyrosine kinases might exhibit different sensitivities to herbimycin A. We are currently examining the selective actions of the inhibitor on a wide variety of protein tyrosine kinases, including other receptor-type tyrosine kinases.

As to the mechanism of decrease in the receptor number, we speculate that herbimycin A specifically affected the synthesis and/or the degradation of EGF receptor, because protein synthesis in general was negligibly inhibited by herbimycin A, as determined by incorporation of [^3S]methionine after treatment at 10 $\mu\text{g/ml}$ for 24 h (results not shown). Moreover, herbimycin A, under the conditions described, stimulated cell proliferation and DNA synthesis, suggesting that the biochemical machinery involving DNA synthesis remains intact, and thus the effect of herbimycin A is highly specific. Another recent study suggested that herbimycin A enhanced degradation of 160 kDa EGF-receptor precursor in the endoplasmic reticulum of A431 cells, and details of this are reported in the accompanying paper [26].

It is well known that A431-cell growth is inhibited by high concentrations of EGF [15]. Interestingly, however, we found in the present study that herbimycin A converted EGF into a stimulatory ligand for A431-cell growth. Kawamoto et al. [27] previously investigated the relationship between the concentration of EGF receptor and effects of the EGF on cell proliferation, using variant A431 cells. They found that several clonal variants that required EGF-mediated growth inhibition expressed 2–4-fold more EGF receptor than did their respective parental variant. Thus it appears that the decreased concentration of EGF receptor that was induced by herbimycin A altered the proliferative response to EGF. The stimulatory effect of herbimycin A in the proliferative response of A431 cells to EGF was demonstrated with both high and low cell densities; since a more obvious effect was obtained at the higher cell density (Figure 5b), DNA synthesis was examined under this condition, and significant stimulation was observed. The EGF-binding assay was conducted at this high cell density, and some light was shed on cell growth stimulation; this is discussed below.

Characterization of the cell-surface EGF receptor by ^{125}I -EGF binding showed that the decrease in the receptor number occurred

in the major population with low-affinity binding, whereas the number with high-affinity binding was unaffected. The effects of monoclonal anti-(EGF receptor) antibodies on cell growth and EGF-receptor activity of the following are interesting. Gregoriou and Rees [28] reported that a monoclonal antibody showed a specific inhibition of low-affinity receptor of A431 cells, and growth of A431 cells treated with this antibody was stimulated at high concentrations of EGF. Behzadian and Shimizu [14] reported a similar result, in that an antibody that interacted preferentially with low-affinity binding blocked 70% of the EGF binding, but did not inhibit EGF-stimulated DNA synthesis in normal human fibroblasts (HFK). Thus the present data, together with these observations, strongly suggest that high-affinity binding of the receptor mediates the stimulation of DNA synthesis by EGF, and that over-expression of low-affinity EGF receptor plays a role in cell-growth inhibition by EGF, although the molecular state and precise roles of these two classes in relation to signal transduction remain unknown. Herbimycin A may therefore become a useful biochemical reagent to elucidate these points.

Some cancer cells, especially squamous-cell carcinoma of oesophagus, lung and skin, are known to express extremely high levels of EGF receptor [29,30]. There is a correlation between EGF-receptor levels and the malignancy [31]: the growth of such cell lines when planted *in vivo* was stimulated by the administration of EGF to mice [32]. These results suggested that amplification of the EGF receptor is attributable to progression of the tumour. Therefore, inhibitors such as herbimycin A that lower EGF-receptor concentration may be beneficial in treatment of these types of cancers.

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