Epidermal Growth Factor Inhibits Growth of A431 Human Epidermoid Carcinoma in Serum-free Cell Culture

DAVID W. BARNES
Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

ABSTRACT A medium consisting of a rich basal nutrient mixture supplemented with bovine insulin (10 μg/ml), human transferrin (10 μg/ml), human cold-insoluble globulin (5 μg/ml), and ethanolamine (0.5 mM) supported the growth of the A431 human epidermoid cell line in the absence of serum with a generation time equal to that of cells in serum-containing medium. Addition of epidermal growth factor (EGF) to this culture medium at concentrations mitogenic for other cell types resulted in a marked inhibition of A431 cell growth. Inhibitory effects of EGF were observed at 1 ng/ml and near-maximal effects were observed at 10 ng/ml. The inhibitory effect of EGF could be reversed by the omission of EGF in subsequent medium changes and could be prevented by the addition of anti-EGF antibody to the culture medium. Inhibition of A431 cell growth by EGF also could be demonstrated in serum-containing medium.

Epidermal growth factor (EGF), a polypeptide originally isolated from mouse submaxillary gland by Cohen (see reference 1), is a potent mitogen for a number of cell types in culture or in vivo. The A431 human epidermoid carcinoma cell line has been used in recent years as a model for the study of early events after the interaction of EGF with its specific cell surface receptor. These cells express an unusually large number of high affinity cell membrane receptors for EGF (2, 3) and have been used to demonstrate internalization of EGF by whole cells (3–6) and enhancement of protein phosphorylation in membrane preparations treated with EGF (7–11). These cells also have been used in experiments designed to identify and isolate the EGF receptor (12–14), and in studies of rapid effects of EGF on cell morphology (15, 16) and pinocytotic activity (17). Although the A431 cell line is clearly an attractive model system for the study of EGF effects, no data have been reported regarding the mitogenic potential of EGF for these cells, and indications were that experiments designed to demonstrate stimulation of cell growth upon the addition of EGF to A431 cells under common culture conditions had been unsuccessful (11).

Recent work from the laboratories of Sato and others (18, 19, and references cited therein) has established that for many cell types it is possible to replace the usual serum supplement in culture medium with specific combinations of nutrients, hormones, and growth factors, binding proteins, and attachment factors, and that the biological effects of hormones or growth factors often may be better identified in such serum-free media than in serum-containing media. Using this approach, a serum-free medium was developed which supported the growth of A431 cells as well as serum-containing medium. It was expected that in this serum-free medium an enhancement of cell growth due to EGF might be demonstrated. However, the presence of EGF at concentrations mitogenic for other cell types was found to be markedly inhibitory for the growth of A431 cells in the serum-free medium. Subsequently, the inhibition also was found to be easily demonstrable in serum-containing medium. A preliminary report of this work appeared as an abstract in May 1981 (20).

MATERIALS AND METHODS

Materials

Powdered culture media were obtained from GIBCO Laboratories (Grand Island Biological Co., Grand Island, N. Y.). Tissue culture plasticware was obtained from Falcon International, Inc. (Chicago, Ill.). The following materials were obtained from Sigma Chemical Co. (St. Louis, Mo.): crude trypsin, EDTA, soybean trypsin inhibitor, bovine insulin, human transferrin, ethanolamine, and HEPES. Fetal calf serum (FCS) was obtained from Reheis. Affinity-purified rabbit anti-mouse EGF was obtained from LAREF. Mouse epidermal growth factor, human cold-insoluble globulin, bovine fibroblast growth factor and partially purified human platelet-derived growth factor were obtained from Collaborative Research, Inc. (Waltham, Mass.). Human cold-insoluble globulin obtained from Bethesda Research Laboratories, Inc. (Rockville, Md.) was also stimulatory for A431 cell growth in serum-free medium, if added either directly to the medium or after pretreatment of the culture dishes. Amino acid analysis by the vendor of the EGF used in the experiments described in this report (lot 1099-63) indicates an amino acid composition essentially identical to the reported amino acid composition of EGF (1). Six other lots of EGF obtained from Collaborative Research were also inhibitory for the growth of A431 cells, and no lots were found to be inhibitory at concentrations as high as 250 ng/ml for the

growth of a number of other human and nonhuman fibroblastic and epithelioid cell lines and strains in serum-free medium. EGF purified from mouse submaxillary gland in the laboratory of Dr. Ralph Bradshaw (Washington University) was as effective as the commercially available product at inhibiting A431 cell growth. This EGF preparation was a gift of Dr. Gordon Sato (University of California at San Diego) and was originally a gift to Dr. Sato from Dr. Bradshaw.

Cell Culture

A431 cells were a gift of Dr. T. Hunter (The Salk Institute). The cell line was originally established in the laboratory of Dr. G. Todaro (National Institutes of Health) (2). Cultures were incubated in a 5% CO_2–95% air atmosphere at 37°C in a one-to-one mixture of Ham's F12 and Dulbecco-Modified Eagle's Medium supplemented with 1.2 g of sodium bicarbonate per liter, 15 mM HEPES, and antibiotics. Other medium supplements were as indicated in the text and figure legends. For the initiation of experiments, cells were detached from stock flasks with a solution of 0.1% crude trypsin and 0.9 mM EDTA in phosphate-buffered saline (PBS), suspended in an equal volume of 0.1% soybean trypsin inhibitor in medium, centrifuged out of suspension, and replated at 2.0×10^4 cells/35-mm diameter plate in the appropriate media. Where indicated, bovine insulin, human transferrin, human CIg, and ethanolamine were added directly to culture plates containing 1 ml of medium as small volumes of sterile, 100-fold concentrated stocks shortly before adding cells in a second milliliter of medium. Final concentrations were 10 μg/ml insulin, 10 μg/ml transferrin, 5 μg/ml CIg, and 0.5 mM ethanolamine. EGF was added as small volumes of sterile, concentrated stocks to give indicated final concentrations. FCS was added to give a final concentration of 10%. For determination of cell number, cells were detached from plates with the trypsin-EDTA-PBS solution and suspensions were counted in a Coulter particle counter (Coulter Electronics, Inc. Hialeah, Fla.). Data shown are the average of determinations from duplicate plates. Individual samples did not vary >10% from the average.

RESULTS

Growth of A431 Cells in Serum-free Medium

The A431 cell line in culture usually is grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FCS (2, 3, 12–14). Because other basal nutrient media, particularly Ham's F12 or mixtures of F12 and DME, are superior to DME in supporting growth of some cell lines (18, 19), an initial survey of cell growth was carried out in F12, DME, or a one-to-one mixture of F12 and DME (F12:DME) supplemented with 0 to 10% human, horse, calf, newborn calf, or fetal calf serum. Best growth was seen in F12:DME supplemented with 10% FCS (F12:DME+FCS), and all stock cultures used in these experiments were grown in this medium. 6 d after seeding plates at a cell density of $2.5 \times 10^3/cm^2$, cell number in plates containing F12:DME+FCS was ~10% higher than cell number in plates containing DME supplemented with 10% FCS.

Using F12:DME as the basal nutrient medium, about 40 supplements found to be stimulatory for growth of one or more cell types in serum-free media (18, 19) were assayed for growth stimulatory activity on A431 cells. Although most were inactive, a few stimulatory supplements were identified. The most active factors in promoting A431 cell growth in the absence of serum were bovine insulin (2–20 μg/ml), human transferrin (2–20 μg/ml), human cold-insoluble globulin (CIg) (2–10 μg/ml), and ethanolamine (0.1–1 mM). Insulin, transferrin, and CIg are common supplements in serum-free media (18, 19); ethanolamine and phosphoethanolamine previously have been reported to be stimulatory for cell growth in culture (21). Small increases in cell number were also seen in serum-free medium supplemented with glycyl-histidyl-lysine (2 μg/ml), and small, inconsistent increases in cell number were sometimes seen upon supplementation with parathyroid hormone (5 ng/ml) or somatostatin (100 ng/ml). These three factors were not included in the serum-free media used in the studies described here.

CIg acts as an attachment factor for a number of cell lines in serum-free media (18, 19). As previously reported for other cell types (22–24), CIg also was effective if the plates into which A431 cells were seeded were pretreated for 4 h with CIg-containing medium and then washed with fresh medium before adding cells. Under these conditions, CIg presumably adheres to the culture dish and mediates the proper attachment of cells to the substratum. Serum spreading factor, a glycoprotein isolated from human serum which is immunologically distinct from CIg but which produces biological effects similar to those of CIg in serum-free cell culture (25), also was effective on A431 cells under these circumstances. Pretreatment of culture dishes with polylysine (~350,000 mol wt) by the method of McKeehan and Ham (26) was not stimulatory for A431 cell growth in serum-free medium.

In F12:DME supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, 5 μg/ml CIg, and 0.5 mM ethanolamine (F12:DME+4F), A431 cells grew with a generation time of ~30 h, a rate essentially identical to that of cells in F12:DME+FCS (Fig. 1). After 6 d in culture, cell number in plates containing F12:DME+4F was 90%–120% of cell number in plates containing F12:DME+FCS. No further increase in cell number beyond that of plates containing F12:DME+4F was seen in plates containing F12:DME+4F supplemented with 10% FCS. Even in plates containing F12:DME, without supplementation with the four factors, A431 cells were capable of growth in the absence of serum, although the generation time was longer (~48 h), and the time lag before onset of growth after plating was greater than that seen for cells in plates containing F12:DME+4F. Medium changes at 72-h intervals did not decrease the generation time of A431 cells in plates containing F12:DME+4F compared to that seen under the conditions of Fig. 1, but did extend the period of exponential growth, so that a density of 6×10^5 cells/cm^2 could be obtained in 12 d from cultures seeded at 2.5×10^3 cells/cm^2 (Fig. 2). A431 cultures grown to confluence in F12:DME+4F could be trypsinized and replated repeatedly in the same medium and have been carried as long as 5 wk in this manner in the complete absence of serum.

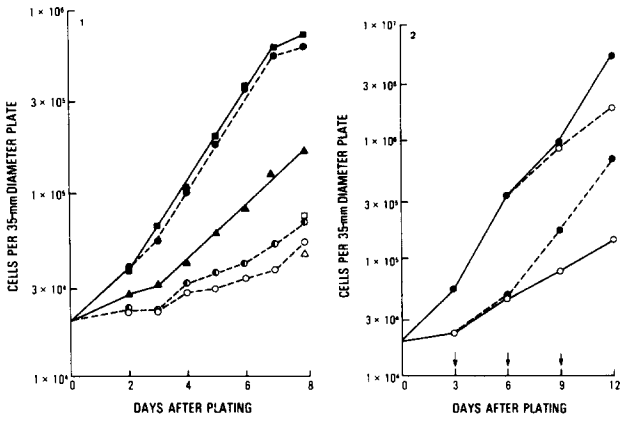

FIGURE 1 Growth of A431 cells in serum-containing or serum-free medium in the presence or absence of EGF. F12:DME+FCS (■——■); F12:DME+4F (●- -●); F12:DME (▲——▲); F12:DME+4F + 10 ng/ml EGF (◐- -◐); F12:DME+4F + 100 ng/ml EGF (○- -○); F12:DME+FCS+ 100 ng/ml EGF (□); F12:DME + 100 ng/ml EGF (△).

FIGURE 2 Reversal of EGF inhibition of cell growth. Arrows indicate days on which medium was changed for all conditions. F12:DME+4F (●——●); F12:DME+4F + 10 ng/ml EGF (○——○), F12:DME+4F changed to F12:DME+4F + 10 ng/ml EGF on day 6 (○- -○); F12:DME+4F + 10 ng/ml EGF changed to F12:DME+4F on day 3 (●- -●).

Inhibition of A431 Cell Growth by EGF

Addition of 10 ng/ml mouse EGF to F12:DME+4F at the time of cell plating resulted in an increase in both the generation time and the time lag before an increase in cell number could be detected (Fig. 1). Addition of EGF at 100 ng/ml resulted in an even greater inhibition of growth; the cell number 6–8 d after seeding in plates containing F12:DME+4F with 100 ng/ml EGF was ~10% of the cell number in plates containing F12:DME+4F. Inhibition of growth in the presence of EGF also could be demonstrated with cells in plates containing F12:DME, without the supplements, or in plates containing F12:DME+FCS.

It was not necessary to add EGF to the culture medium at the time of plating to demonstrate inhibition of growth: 10 ng/ml EGF added to cells upon medium change 6 d after plating in F12:DME+4F resulted in a decreased growth rate in the subsequent days of culture (Fig. 2), although there was a lag of several days before significant inhibition of cell growth due to the addition of EGF could be observed, suggesting that under the conditions of this experiment the effect of EGF on cell growth was not rapid. Similarly, cells incubated for the first three days after seeding in F12:DME+4F containing 10 ng/ml EGF and then changed to F12:DME+4F without EGF resumed growth with a generation time near that of cells not exposed to EGF, but only after a lag of ~3 d, during which the effect of EGF was still quite evident.

An inhibitory effect of 1 ng/ml EGF could be demonstrated on A431 cell growth; a near-maximal inhibitory effect was seen at 10 ng/ml EGF (Fig. 3). At 10–100 ng/ml, morphological effects of EGF were observed; cells were more rounded and granular and assumed unusual, less uniform shapes than in medium in the absence of EGF. Effects of EGF on A431 cell morphology have been reported previously by Chinkers et al. (15, 16). Several laboratories have explored the relationship between cell shape and cell proliferation (25, 30, 31) and it may be that the inhibitory effect of EGF on A431 cell growth may be in some way related to the ability of EGF to induce cell rounding.

Addition of affinity-purified rabbit anti-mouse EGF antibody to the culture medium was effective at preventing the inhibition of cell growth caused by the addition of EGF (Fig. 4). This antibody in the same concentration range also was effective at inhibiting the previously reported mitogenic effect of EGF for HeLa cells in serum-free medium (data not shown) (32). Addition of the preparation of anti-EGF antibody used for the experiment of Fig. 4 to A431 cells at concentrations >10 μg/ml resulted in considerable inhibition of cell growth in F12:DME+4F. The remaining small inhibitory effect of EGF

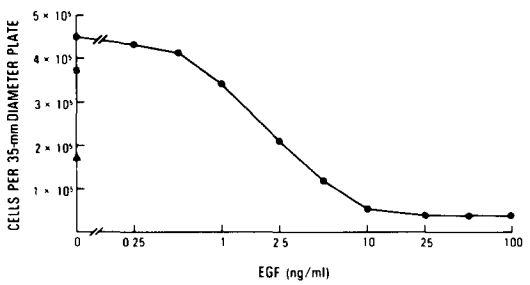

FIGURE 3 Concentration dependence of EGF inhibition of A431 cell growth. Cells were counted after incubation for 6 d in plates containing F12:DME+4F with the indicated concentrations of EGF (●——●); F12:DME (▲); F12:DME+FCS (■).

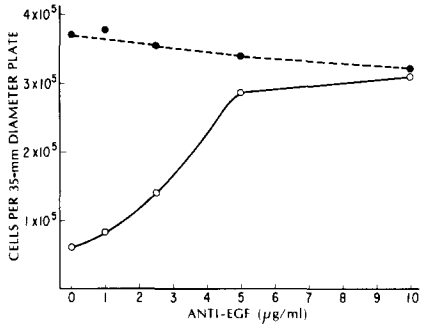

FIGURE 4 Prevention of EGF inhibition of A431 cell growth by anti-EGF antibody. Cells were plated in F12:DME+4F in the presence of anti-EGF at the indicated concentrations and the presence or absence of EGF at 10 ng/ml. Cells were counted 6 d after plating. F12:DME+4F without EGF (●- -●); F12:DME+4F with EGF (○——○).

in Fig. 4 in the presence of 10 μg/ml of the antibody presumably was due to the presence of residual, free EGF which was not bound to the antibody under the conditions of the experiment. It is also possible that the EGF-antibody complex retained some inhibitory activity for A431 cells.

DISCUSSION

EGF has been reported previously to be inhibitory for the growth of GH_3D_6 and GH_4 rat pituitary tumor cells in culture (27, 28). It is interesting that, for both the A431 and GH lines, EGF lengthened the generation time but was not toxic, because incubation with EGF never resulted in fewer cells than were present initially. Fibroblast growth factor (FGF) and thyrotropin releasing hormone (TRH) are also inhibitory for growth of GH4 cells (28). No effect of FGF at 50 ng/ml or TRH at 10 ng/ml was detected on A431 cells in serum-free medium, but some inhibition of A431 growth was seen upon incubation of cells in serum-free medium containing a partially purified preparation of platelet-derived growth factor or 1 mM dibutryl cyclic AMP. Because EGF is also capable of mediating differentiated functions of some cell types (1, 27, 28), it is reasonable to speculate that EGF may induce differentiative changes in A431 cells in culture and that such effects may be associated with the observed inhibition of growth. No gross changes which might be indicative of differentiation of an epidermoid cell type, such as whole cell keratinization, were detected after treatment of A431 cells with EGF. However, no detailed biochemical or microscopy investigations of more subtle markers of differentiation have been attempted.

The striking inhibition of A431 cell growth by EGF suggests that future attempts to relate effects of EGF on these cells to mitogenic effects of EGF on other cell types in vitro or in vivo should be approached with caution. However, it is unlikely that the data of any of the existing reports dealing with the biochemistry or cell biology of EGF effects on A431 cells represent responses unrelated to the mitogenic effects of EGF, because qualitatively similar effects may be demonstrated in other cell culture systems in which the mitogenic potential of EGF is well established.

Recently, Anderson et al. (29) have reported that A431 cells express unusually large numbers of receptors for low density lipoprotein (LDL) compared to other cell types examined in culture. Furthermore, these investigators found that receptor-mediated endocytosis of LDL by A431 cells is significantly altered in several respects when compared with that of other cell types studied in culture, such as diploid human fibroblasts.

The availability of the serum-free medium described here for the growth of the A431 cell line allows the design of experiments in which the external environment of healthy, growing cells may be controlled completely on a long-term basis. The ability to manipulate the nature and concentration of nutrients, hormones, lipoproteins or other components of the culture medium in the absence of undefined serum components should provide numerous advantages to investigators interested in studies related to the several useful and peculiar properties of the A431 line (18, 19).

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Note Added in Proof: A report by G. N. Gill and C. S. Lazar (1981. Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. *Nature (Lond.).* 293:305–307) of inhibition of proliferation in EGF-treated A-431 cells in serum-containing medium appeared after this paper had been accepted for publication.

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