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Growth-inhibitory and Growth-stimulatory Effects of Epidermal Growth Factor on Human Breast Cancer Cell Line, MDA.MB.436: Dependence on Culture Conditions

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Abstract—*Epidermal growth factor (EGF) is growth inhibitory for some cell lines, especially those having over-expressed EGF receptors. We have examined the effects of murine EGF on the growth of the human breast cancer cell line, MDA.MB.436, which has low numbers of EGF receptors. In the presence or absence of serum a 6 day exposure to 0.1 ng/ml EGF causes inhibition of growth if the culture medium is left unchanged during the course of the experiment but the same concentration of EGF causes stimulation above control if the EGF-containing medium is replaced daily. A 1 day exposure to 0.1 ng/ml followed by return to control medium has no effect on subsequent growth. The cells do not synthesize EGF receptor binding activity and added EGF is degraded within 2 days, suggesting that the inhibitory effects of EGF persist in its absence.*

INTRODUCTION

EPIDERMAL GROWTH FACTOR (EGF) has been shown to be mitogenic for some, but not all, human breast cancer cells [1–6]. Although EGF stimulates the proliferation of primary cultures of human breast cancer specimens in the presence of 10% serum [3], long-term tissue cultured breast cancer cells are stimulated by EGF only in the absence of serum or in the presence of sub-optimal concentrations of serum (<1%) [4, 6, 7]. Growth effects of EGF on breast cancer cell lines in routine passaging media (i.e. in the presence of 5–10% foetal calf serum) have not been fully reported.

Inhibition of cancer cell growth by EGF has been demonstrated both *in vitro* and *in vivo* [8–11]. It has been postulated that inhibition of proliferation of certain cell lines by physiological levels of EGF occurs because EGF receptors are overexpressed (i.e. $>10^6$ receptors per cell) due to an amplified receptor gene [10, 11]. The human breast cancer cell line MDA-468, which has an amplified EGF receptor gene, is inhibited by nanomolar concentrations of EGF; clonal variants which are not amplified are not inhibited [12]. Similarly, A-431 (human epidermoid carcinoma) cells with over-expressed EGF receptors are inhibited by physiological levels of EGF whilst non-amplified variants are not [10]. A recent report suggests that this simple relationship may not always hold true, as an A-431 variant has been isolated which retains high EGF binding capacity but is growth-stimulated by physiological levels of EGF [13], whereas the parental cells are stimulated only by picomolar concentrations [14]. A survey of the growth effects of EGF in several breast cancer cell lines failed to produce a correlation with their EGF receptor levels [5, 6].

We have examined the effects of EGF on growth of the human breast cancer cell line, MDA.MB.436, which has low levels of EGF receptor (200 sites per cell [5]).

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MATERIALS AND METHODS

Tissue culture

MDA.MB.436 cells, obtained from the American Tissue Culture Collection, were cultured in Liebowitz 15 medium containing 10% foetal calf serum (FCS), Phenol Red, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Tissue culture media and serum were obtained from Flow Labs., Ricksmanworth, U.K. The same batch of serum was used throughout this study.

Cell proliferative assays

Murine EGF (receptor grade) was obtained from Biogenesis, Bournemouth, U.K. Growth studies were carried out in Liebowitz-15 (L-15) medium in the presence or absence of 10% FCS. In serum-free experiments, 200 μg/ml bovine serum albumin (Sigma) was substituted for the FCS. MDA.MB.436 cells in L-15 medium containing 10% FCS were inoculated in 24-well tissue culture plates (Costar, Northumbria Biologicals Ltd, Cramlington, U.K.) at 5×10^4 per well. After 24 h (day 0), plating efficiency was determined and was found to be 90%. Medium was then removed and replaced with fresh medium (control) or medium containing EGF (treatment), as indicated. Control and EGF-containing media were either left unchanged or were changed daily during the course of study. In other experiments cells were exposed to control or EGF-containing media for 1 day (day 0 to day 1) after which control and EGF-containing media were removed and replaced with control medium, which was left unchanged during the remainder of the experiment. On days 1, 3 and 6, cells from replicate wells were removed by trypsinization and counted using a Coulter counter (model ZBI, Coulter Electronics, Luton, U.K.).

Assay of EGF receptor binding activity

EGF-like activity was assayed in the serum, cells and conditioned media using a commercial radioreceptor assay kit (Biomedical Technologies, Inc.) which is based on competitive binding to A-431 cell membranes. The FCS batch used in this investigation was assayed directly, the cells and conditioned media were assayed by acid–ethanol extraction, as described previously [15]. Briefly, cells were grown in 80 cm^2 flasks (Costar, U.K.) with 10 ml L-15 containing 10% FCS plus or minus EGF (10 ng). At the indicated times medium was removed and protein was precipitated from it by addition of 1 ml of acidified ethanol (95% in 1 M HCl). Cells were extracted with 1 ml acidified ethanol. Both cell and medium extracts were centrifuged and the supernatants were evaporated to dryness. Before assay the cell extracts were reconstituted in 0.2 ml and the medium extracts in 1 ml of diluent from the kit (Tris/saline solution) and neutralized. Extraction of EGF-'spiked' medium was >80% efficient.

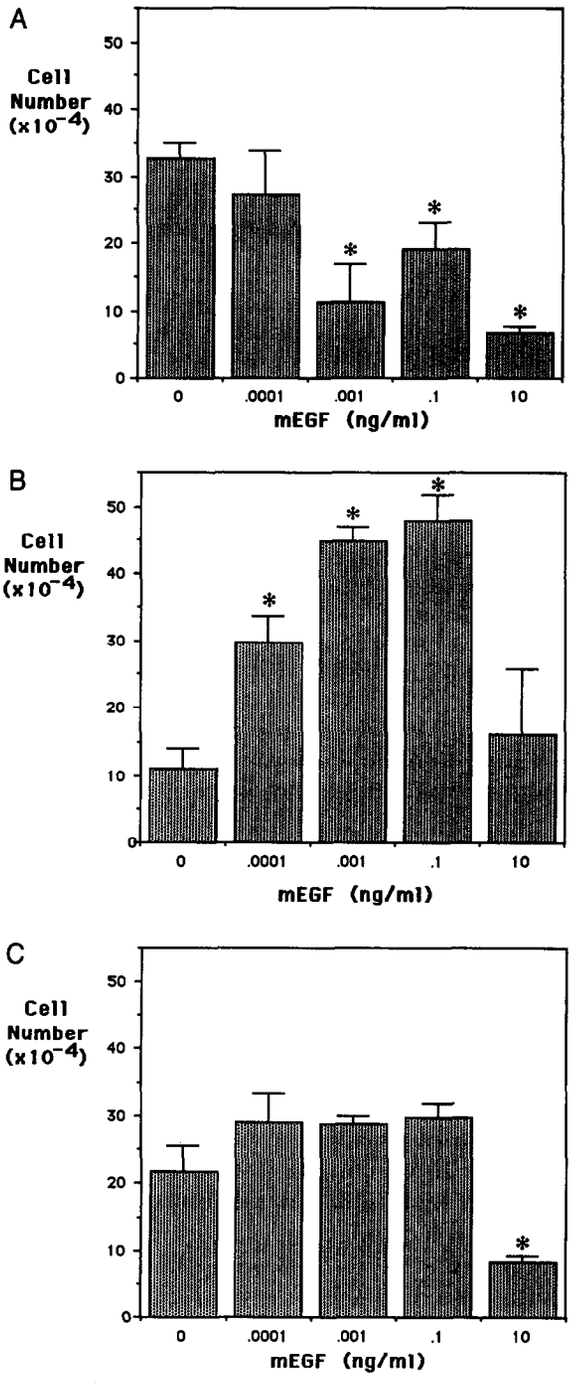

*Fig. 1. Effect of EGF on MDA.MB.436 cell proliferation in the presence of 10% FCS. (A) Control and treatment media were not changed during course of experiment. (B) Control and treatment media were changed daily. (C) Control and treatment media removed after 1 day (day 0–1) and replaced with control medium in each case. Results are mean cell numbers on day 6 ± S.D. (bars) of three or four wells. Significant difference between treatment and control are indicated (*P < 0.01, Student's t test).*

RESULTS

Representative experiments are presented (Figs 1 and 2). Results have been confirmed in at least two independent determinations.

In initial experiments 10 ng/ml EGF was found to inhibit MDA.MB.436 cell proliferation under standard conditions of culture (i.e. grown in the presence or absence of 10% FCS, medium changed every third day). Different cultural conditions were therefore examined to determine if there were circumstances under which EGF could stimulate MDA.MB.436 cell proliferation.

In the presence of 10% FCS, cells were inhibited by a one day exposure to 10 ng/ml EGF but 0.0001,

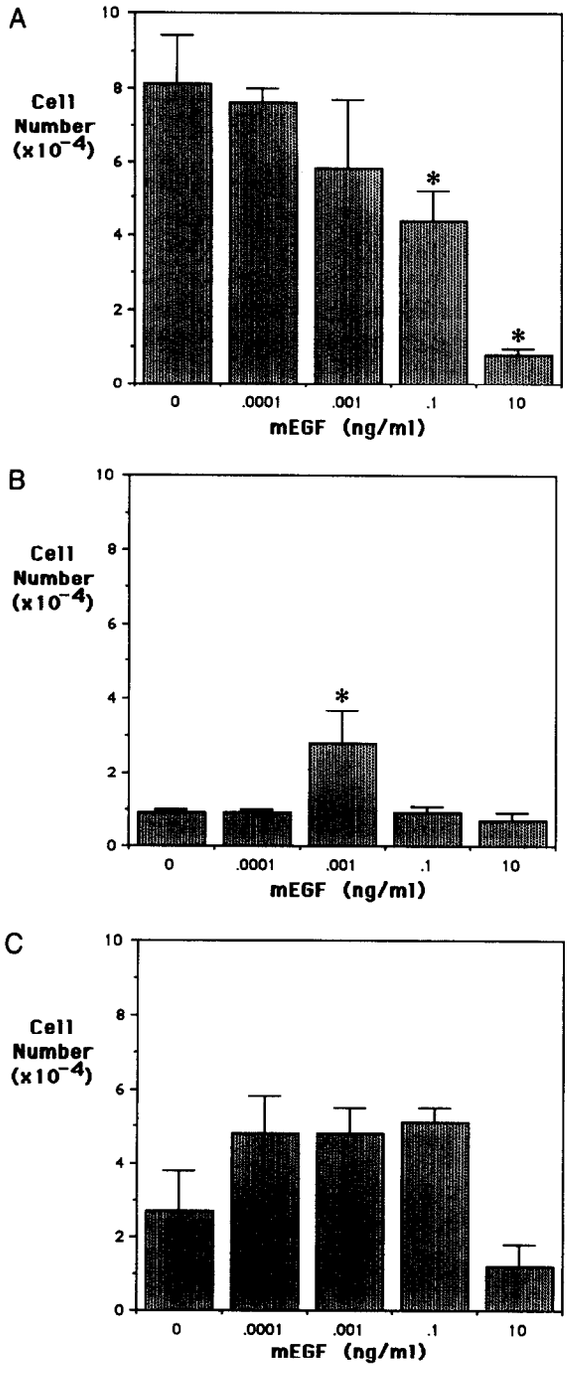

*Fig. 2. Effect of EGF on MDA.MB.436 cell proliferation in serum-free medium. (A) Control and treatment media were not changed during course of experiment. (B) Control and treatment media were changed daily. (C) Control and treatment media removed after 1 day (day 0–1) and replaced with control medium in each case. Results are mean cell numbers on day 3 ± S.D. (bars) of three or four wells. Significant differences between treatment and control are indicated (*P < 0.01, Student's t test).*

0.001 or 0.1 ng/ml had no effect (results not shown). 0.001–10 ng/ml EGF caused significant reduction of final cell numbers if a single dose was left on the cells for the course (6 days) of the experiment (Fig. 1A).

When repeated daily doses were given, concentrations of 0.0001, 0.001 and 0.1 ng/ml EGF caused stimulation of growth above that of the relevant control population by day 6 (Fig. 1B). Daily replacement of medium caused control cells to grow more slowly compared to control cells grown in the absence of medium changes (Fig. 1A and B). The final cell numbers produced by daily doses of 0.001 and 0.1 ng/ml EGF, however, were greater ($P < 0.01$) than in untreated cells given no medium changes (control, Fig. 1A).

In the presence of FCS a one day exposure to EGF (day 0–1) followed by 5 days growth in control medium had no effect on subsequent growth except for significant inhibition at 10 ng/ml (Fig. 1C).

In serum-free conditions, cells did not grow beyond day 3 except when given daily doses of 0.1–10 ng/ml EGF, in which case they grew up to day 6 (not shown). In the absence of FCS continuous exposure to a single dose of 0.1 or 10 ng/ml EGF caused significant inhibition of growth (Fig. 2A). Repeated daily doses of 0.001 ng/ml EGF caused significant stimulation of growth compared to the relevant control on day 3 (Fig. 2B). In experiments where medium was changed daily, floating cells were not detected in the used medium. In serum-free conditions a 24 h exposure to EGF followed by 2 days growth in control medium did not significantly affect final cell numbers (Fig. 2C).

EGF receptor-binding activity in the serum used in these experiments was below the limit of detection (<0.2 ng/ml); the final concentration of EGF-like material in 10% FCS-containing L-15 being <0.02 ng/ml. EGF-like material was undetectable in both conditioned medium (limit of detection = 0.02 ng/ml) and extracts from routinely cultured MDA.MB.436 cells (limit of detection = 0.09 pg/10^6 cells). A dose of 10 ng EGF was reduced to 1.58 ng after a 24 h incubation in the presence of 10% FCS and cells; 1.4 ng being found in the medium and 0.18 ng associated with the cells. After 2 and 6 days incubation the same dose was undetectable.

DISCUSSION

The results show that growth of the MDA.MB.436 human breast cancer cell line may be either inhibited or stimulated by EGF depending on cultural conditions. Continuous exposure to a single dose of EGF for 3 days or longer causes growth inhibition of MDA.MB.436 cells but daily replacement of EGF stimulates growth above control. Treated cells cultured in the presence or absence of serum respond similarly to the frequency of medium changes, suggesting that divergence of response to EGF is not governed by serum-borne factors. The control cells grow more slowly when medium is changed daily, suggesting that in the absence of EGF, MDA.MB.436 cells depend on autocrine secreted growth promoters, which would

be removed with each medium change, and that daily replacement doses of EGF can substitute for this secreted acitivity. As noted in results this putative factor does not interact with EGF receptors nor does the FCS contribute detectable EGF-like material. The inhibitory effects of a single dose of EGF persist long after the peptide has been degraded to undetectable levels suggesting that either inhibitory degradation products (which either do not displace native EGF from its receptor or interact with a site other than the EGF receptor) are produced or that EGF stimulates delayed cellular secretion of a growth inhibitory factor which builds up to effective levels only in the absence of medium changes. It should be noted that not all breast cancer lines respond in this way; untreated ZR-75-1 cells, for example, grow more quickly when medium is changed frequently [15].

Decreased growth of control cells given daily medium changes is not due to floating cells being removed. In experiments where medium was changed daily, floating cells were not detected in the used medium. The decrease in cell numbers in serum-free controls given daily medium changes (Fig. 2A) is therefore due to cell lysis. The decrease in growth resulting from daily medium changes also suggests that nutrients or serum-borne growth factors are not growth-limiting for EGF treated cells.

These results are the first demonstration of a breast cancer cell line which can show both growth-stimulatory and -inhibitory responses to EGF. Early passages of rat intestinal epithelial cells, however, are growth inhibited by nanomolar EGF when sparsely seeded but are growth stimulated when densely seeded; this effect of population density is also independent of the presence or absence of foetal calf serum [16]. The divergence of response in MDA.MB.436 cells is not dependent on seeding density (unpublished results) but is determined solely by the frequency of medium changes. The clinical relevance of these observations is unclear but blood flow rates in breast tumours are variable [17] and this may modulate growth responses to EGF in some cases.

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