DIFFERENTIAL EFFECTS OF EPIDERMAL GROWTH FACTOR (EGF) ON CELL LOCOMOTION AND CELL PROLIFERATION IN A CLONED HUMAN EMBRYONAL CARCINOMA-DERIVED CELL LINE IN VITRO

WILHELM ENGSTRÖM

Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

SUMMARY

The effects of epidermal growth factor (EGF) on clones from a human embryonal carcinoma-derived cell line (Tera-2) have been studied. Cells were plated at clonal densities, whereafter the effects of serum and EGF on cell locomotion and cell proliferation were examined. The addition of 50 ng EGF ml⁻¹ resulted in increased migration, as judged by increased colony diameter in the presence of EGF. However, the effect of EGF on cell locomotion was rarely accompanied by any effect on cell proliferation. It was concluded that EGF exerts a preferential effect on cell migration in human embryonal carcinoma cells in vitro.

INTRODUCTION

The human embryonal carcinoma-derived cell line Tera-2 expresses approximately 26 000 membrane receptors for epidermal growth factor (EGF) per cell (Engstrom et al. 1985). Similar results have been obtained by Carlin & Andrews (1985), who discovered 20 000 to 30 000 EGF receptors per cell in a related cell line (NTera-2). In both cases the receptors appeared to be functional, in the sense that addition of EGF resulted in an increased intrinsic kinase activity (Engstrom et al. 1985; Carlin & Andrews, 1985). The present study examines the possible biological effects of EGF on this cell line. We report that the addition of EGF results in increased cell migration, whereas we observe only minor effects on cell proliferation. This suggests that Tera-2 cells may have some properties in common with two other human tumour cell lines in which EGF exerts a preferential effect on cell locomotion (Westermark et al. 1982; Gregoriou & Rees, 1984).

MATERIALS AND METHODS

Growth factors, basal media and tissue-culture material

Recombinant epidermal growth factor was a gift from Dr Mary Gregoriou, Laboratory of Molecular Biophysics, University of Oxford. Porcine insulin was purchased from Sigma (UK). Transferrin was obtained from Behring (FRG) and loaded with iron according to Iscove et al. (1980). Multiplication stimulating activity (MSA) was purified from the conditioned medium of Buffalo rat liver cells as described by Marquardt et al. (1981). High-density lipoprotein (HDL)
and low-density lipoprotein (LDL) were prepared by centrifugation of fresh human plasma in potassium bromide (Brown et al. 1974). MSA and the lipoproteins were generously provided by Dr John K. Heath, Department of Zoology, University of Oxford. Alpha-modified Eagle's Medium (α-MEM), was obtained as dry powder from Flow Laboratories (UK) and made up in accordance with the manufacturer's instructions. All tissue-culture plastic was obtained from NUNC (through GIBCO, UK) with the exception of the 6 mm × 35 mm cluster dishes that were purchased from Sterilin (UK). Heat-inactivated foetal calf serum was obtained from Flow Laboratories (UK) and trypsin was purchased from Difco (UK). Tissue-culture dishes were precoated for more than 24 h at 4°C with gelatin (swine skin type I gelatin (Sigma, UK), 0.1% solution (w/v) in calcium/magnesium-free phosphate-buffered saline (PBS)). All dishes were thoroughly rinsed in ice-cold PBS before use.

Cell culture
The human embryonal carcinoma cell line Tera-2 was originally established in 1971 by Dr Jørgen Fågh (Fågh & Trempe, 1975), who also kindly provided us with the cells. The cell line was cloned at the 41st passage (Thompson et al. 1984). The Tera-2 cell line was established from a lung metastasis from a primary testicular embryonal carcinoma (Fågh & Trempe, 1973). Tera-2 cells have been shown to give rise to embryonal carcinoma-like tumours in nude mice (Jewett, 1978; Andrews, 1983; Thompson et al. 1984). Tera-2 cells possess an aneuploid karyotype with a near triploid number of chromosomes, and one such clone (clone 13) was used in this study (Thompson et al. 1984). All experiments were performed on cells that had undergone fewer than 20 passages after cloning.

Stock cultures were maintained in 25 cm² plastic tissue-culture bottles. The cells were grown under humidified 5% (v/v) CO₂ in air at 37°C in alpha medium supplemented with 10% (v/v) foetal calf serum, 50 units ml⁻¹ of penicillin and 50 μg ml⁻¹ of streptomycin sulphate (Engström et al. 1985, 1986). The stock cultures were subcultured every 5 or 7 days at a routine split ratio of 1 in 4. The cells were removed from the bottle before transfer by treatment with 0.125% (w/v) trypsin in Ca²⁺/Mg²⁺-free PBS supplemented with 50mM-EDTA at low temperature, as described by McKeehan (1977).

Clonal analysis of cell growth and cell locomotion
The cells were inoculated at different concentrations ranging from 10 to 50 cells cm⁻², into 3 ml of equilibrated medium in the 35 mm wells of six-well cluster dishes. After 5 days, each culture was fixed in 3:1 (v/v) methanol/acetic acid for at least 24 h, hydrated in Analar water, and finally stained in 1% (w/v) aqueous Crystal Violet. Each dish was examined in a Leitz inverted microscope equipped with an eyepiece graticule inscribed with 10 concentric circles. The distance between two circles was taken as 1 relative unit.

Each dish was scanned in the microscope, and the location of each colony was marked. The diameter of each colony was determined by the use of the graticule, whereafter the number of cells in each colony was counted. In several instances, the colonies were photographed in a Leitz inverted microscope with an attached camera system.

Cell growth determinations
The cells were seeded at a concentration of 100 000 cells ml⁻¹ in 3 ml pre-equilibrated α-MEM supplemented with 10 % foetal calf serum on 35 mm wells of six-well cluster dishes. After 16 h the medium was discarded, the cells thoroughly rinsed in α-MEM, and the experimental media added. At times indicated in the figure legends, the cells were rinsed at least three times in prewarmed PBS and dispersed in 2 ml of 0.125% trypsin (w/v) in Ca²⁺/Mg²⁺-free PBS containing 50mM-EDTA for 10 min at 37°C. The cell suspension was thereafter counted in triplicate in a Coulter counter (Coulter Electronics, USA).

Statistics
Student's t-test was used to analyse the statistical significance of the differences between the variables obtained in this study. The level of significance was set at P < 0.1.
RESULTS

Effects of EGF on Tera-2 cell locomotion

The starting point for this study was the finding by Westermark et al. (1982) that cloned human glioma cells maintained in 10% serum increase their motility after addition of epidermal growth factor (EGF). It was assumed that the effects of EGF on cell locomotion and cell proliferation could be studied in single-cell-derived colonies by seeding cloned Tera-2 cells at very low density. Fig. 1 shows the morphology of four different single-cell-derived colonies of Tera-2 cells at 5 days after seeding. Fig. 1A shows cells that have maintained a round shape and grow in a tightly packed colony. Fig. 1B shows a colony where the cells have begun to flatten but still grow adjacent to one another. Fig. 1C illustrates a colony where some of the cells have migrated from the centre of the colony, whereas Fig. 1D shows elongated cells that are well spread over the surface area.

Fig. 2 shows the effect of EGF addition on Tera-2 cell migration. In this set of experiments the number of cells as well as the diameter was scored for each colony. In order to exclude the possibility that any observed effects on cell locomotion were a consequence of an increased rate of cell proliferation, the diameters of colonies that consisted of comparable numbers of cells were examined in the presence and absence of EGF.

Fig. 2A,B shows that, irrespective of colony size (as defined by number of cells/colony), EGF addition resulted in increased colony diameter. This difference was in most cases found to be statistically significant. Furthermore, the EGF-induced effect on cell migration was observed at three different seeding densities, namely 10 cells cm\(^{-2}\) (data not shown), 20 cells cm\(^{-2}\) (Fig. 2A) and 50 cells cm\(^{-2}\) (Fig. 2B). Fig. 2 also shows that the presence of EGF resulted in colonies with a larger colony diameter irrespective of the serum concentration. This indicates that the addition of EGF stimulates Tera-2 cell locomotion.

Effects of EGF on Tera-2 cell proliferation

In many cell lines the effects of EGF on cell locomotion are followed by stimulatory effects on cell proliferation (Blay & Brown, 1985; Collins et al. 1979; Westermark & Blomqvist, 1980). It therefore became of interest to estimate the effects on clonal cell proliferation by scoring the number of cells/colony in the presence and absence of EGF. It was found that EGF exerted only negligible effects on cell proliferation (data not shown). Since it is possible, however, that the presence of 1–10% serum masked any effects of EGF on Tera-2 cell proliferation, we extended the analysis to the effect of EGF on cell number in defined serum-free media. Since Tera-2 cells require a high plating density to survive under serum-free conditions (Engström et al. 1985), the effects on cell proliferation had to be determined in bulk cultures.

Fig. 3 shows the effect of EGF on Tera-2 cell proliferation; in these experiments EGF is the sole supplement to a basal medium. Cells were seeded in 10% serum in order to achieve an adequate plating efficiency, and after 16 h the dishes were rinsed...
Fig. 1. Morphological appearance of four different single-cell-derived Tera-2 cell colonies after 5 days maintenance in vitro. Cloned (C13) Tera-2 cells were seeded at low density (10 cells cm$^{-2}$) onto gelatinized dishes in 10% serum. After 5 days the cultures were fixed, stained and photographed in our inverted microscope. ×280.
in the serum-free medium, and the test medium was added. The cells were maintained in the experimental media for 5 days and then counted. Fig. 3 shows that 50 μg EGF ml⁻¹ exerted only a minor effect on Tera-2 cell proliferation. We observed no significant effect on Tera-2 cell proliferation in the presence of any other EGF concentration tested. In some cell lines, EGF exerts little effect on cell proliferation unless it is added together with other growth factors or hormones (Zetterberg et al. 1984). In order to test if EGF depends on the presence of some other factor to sustain cell proliferation in Tera-2 cultures, we seeded Tera-2 cells in a serum-free medium that was designed for this cell line, which contains insulin, MSA, HDL, LDL and transferrin (Engström et al. 1985), and then tested the effects of different concentrations of EGF. Fig. 4 shows that EGF did not increase population numbers, when added over a wide range of concentrations, even though other growth factors and hormones were present. We therefore concluded that EGF exerts a significant effect on Tera-2 cell migration but exerts only minor effects on cell growth and cell multiplication.

DISCUSSION

It has previously been shown that the human embryonal carcinoma-derived cell line Tera-2 expresses low numbers of membrane receptors for epidermal growth
factor (Engström et al. 1985; Carlin & Andrews, 1985). The significance of these findings remained unclear, since we were unable to observe any major stimulatory effects of EGF on Tera-2 cell proliferation, either in combination with different concentrations of serum, or in a defined serum-free medium. It therefore became of

Fig. 2. The effect of EGF and serum on Tera-2 locomotion. CI13 Tera-2 cells were seeded onto 35 mm dishes at a concentration of 20 cells cm$^{-2}$ (A) or 50 cells cm$^{-2}$ (B) in 1–10 % serum with (●) or without (■) addition of 50 μg EGF ml$^{-1}$. After 5 days the dishes were fixed and the diameter and the cell number of each colony were determined in a light microscope. The mean diameters were calculated on colonies with comparable cell numbers. For this purpose the colonies were pooled into clusters that ranged over five cell numbers (abscissa), i.e. 6–10, 11–15, 16–20 cells/colony etc. The curves represent means ± standard deviation for three different experiments.
interest to examine the possibility that EGF exerts other biological effects on this particular cell type. By seeding cells at clonal densities, we were able to examine the effects of EGF on Tera-2 cells within single-cell-derived colonies. The main finding of this study was that EGF stimulates cell locomotion.

Fig. 3. The effect of EGF on Tera-2 cell proliferation. The cells were plated in 10% serum on duplicate 35 mm dishes at a concentration of 100,000 cells ml\(^{-1}\) on day 1. After 16 h the serum-containing medium was discarded and fresh α-MEM with 10% serum (●), 50 μg EGF ml\(^{-1}\) (▲) or without any macromolecular supplements (■) were added. After 3 and 6 days the cells were trypsinized and the cell numbers determined in triplicate in a Coulter counter.

Fig. 4. Effects of EGF on the proliferation of cloned Tera-2 cells. Cells were plated in 10% serum on duplicate 35 mm dishes at a concentration of 100,000 cells per ml inoculum on day 1. The top bar (day 1) indicates the number of cells having attached after 16 h. At this point the serum-containing medium was discarded and fresh DMEM–Ham's F12 medium (50:50, v/v) supplemented with 10 μg ml\(^{-1}\) insulin, 10 μg ml\(^{-1}\) transferrin, 50 μg ml\(^{-1}\) HDL, 50 μg ml\(^{-1}\) LDL, 10 ng ml\(^{-1}\) MSA and various concentrations of EGF as indicated. Cells continuously exposed to 10% serum were used as controls. After 5 days the cells were trypsinized and the cell numbers determined in triplicate in a Coulter counter.
This finding is in line with previous studies that have demonstrated an EGF-induced effect on cell migration. Westermark & Blomqvist (1980) showed that EGF significantly stimulates cell migration in human diploid foreskin fibroblast cultures. Similarly, Blay & Brown (1985) have shown that EGF promotes the chemotactic migration of cultured rat intestinal epithelial cells. In the latter cell line, EGF was found to exert a dual stimulatory effect on migration and proliferation. In addition, Brunk et al. (1976) and Collins et al. (1979) showed that EGF also stimulates migration as well as cell multiplication in normal human diploid glial cells. However, it was shown by Westermark et al. (1982) that the stimulatory effects by growth factors on cell migration could be dissociated from those on cell proliferation in a tumour cell line. By exposing proliferating human malignant glioma cells to EGF, the migratory activity could be dramatically stimulated. However, the effects of EGF on membrane motility and cell locomotion occurred in the absence of any effect of EGF on growth rate, since the glioma cells proliferated at the same rate in the absence as in the presence of EGF. More recently, Gregoriou & Rees (1984) have shown that EGF stimulates cell migration in the human cervical carcinoma-derived cell line A431 without any observable effects on cell proliferation. It therefore seems as if EGF exerts a preferential effect on cell locomotion in tumour cells. This is in contrast to normal diploid cells where EGF exerts effects on migration as well as on cell proliferation.

It has been shown that Tera-2 cells, human glioma cells and A431 cells express functional EGF receptors albeit at different numbers (Engström et al. 1985; Westermark et al. 1982; Gregoriou & Rees, 1984b). In these cell lines, the main effect of EGF is to promote cell migration, and it is therefore tempting to speculate whether the stimulatory effect on cell locomotion is a receptor-mediated event. However, it remains to be verified experimentally that the EGF receptor is also involved in the induction of cell migration.

Finally, the present data give rise to the speculation that tumour cells in vivo may respond biologically to EGF or to related growth factors. Malignant cells may require specific growth factors for migration in vivo and consequently for the expression of their metastatic potential. An improved knowledge of the factors and conditions that influence migration and proliferation of human embryonal carcinoma cells in vivo should contribute to an understanding of the cellular origins of human teratocarcinomas as well as of the underlying functional defects that lead to unrestricted proliferation in vivo.

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