Loss of epidermal growth factor receptors and release of transforming growth factors do not correlate with sarcoma virus-transformation in clonally-related NIH/3T3-derived cell lines

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Transformation of NIH/3T3 cells by Kirsten murine sarcoma virus (MSV) caused a dramatic reduction in the number of cell-surface receptors for epidermal growth factor (EGF). However, the number of EGF receptors remained at a very low level in a non-tumorigenic revertant cell line isolated from the virus-transformed cells, indicating that an increase in EGF receptors is not a requirement for the phenotypic reversion of Kirsten MSV-transformed 3T3 cells. Serum-free conditioned medium from normal and virus-transformed cell lines contained similar amounts of cell growth-promoting activity as assayed by the ability to stimulate DNA synthesis in quiescent Swiss 3T3 cell cultures. However, the concentrated conditioned medium from these cell lines showed no evidence of β -transforming growth factor (TGF) activity as assayed by promotion of anchorage-independent growth of untransformed normal rat kidney (NRK) fibroblasts in agarose. The cellular release of α -TGF activity was assayed by measuring the ability of concentrated conditioned medium to inhibit the binding of ^{125}I -EGF to Swiss 3T3 cells. Conditioned medium protein from the virus-transformed cell line inhibited ^{125}I -EGF binding but only to the same extent as conditioned medium protein prepared from the untransformed cell line. The α -TGF secretion by these cell lines was estimated to be 30–45-fold lower than the level of α -TGF released by a well-characterized α -TGF-producing cell line (3B11). These results suggest that the induction of TGF release is not a necessary event in the transformation of NIH/3T3 cells by Kirsten MSV.

Cells transformed by various mammalian sarcoma viruses and by Abelson leukaemia virus display a dramatically reduced level of binding of EGF (DeLarco & Todaro, 1978a; Blomberg *et al.*, 1980). It was originally proposed that the production of an endogenous EGF-related polypeptide might activate and 'block' cellular EGF receptors and that this autocrine stimulation might play an important role in the mechanism of cellular transformation (Todaro & DeLarco, 1978). The subsequent demonstration that these transformed cell

lines produce transforming growth factors (i.e. polypeptides which stimulate the growth of untransformed cells in soft agar), one of which (α -TGF) competes for binding to EGF receptors, has provided further evidence in favour of these possibilities (DeLarco & Todaro, 1978b; Roberts *et al.*, 1980; Twardzik *et al.*, 1982a).

α -TGF has recently been purified from medium conditioned by a rat embryo fibroblast cell line which had been transformed by Snyder–Theilen feline sarcoma virus. Rat α -TGF is a single chain polypeptide of 50 residues (calculated M_r 5616) with a strong sequence homology to mouse EGF and an ability to inhibit EGF binding to cellular receptors (Massagué, 1983; Marquardt *et al.*, 1984). With the possible exception of embryonic tissue (Twardzik *et al.*, 1982b), the production of α -TGF appears to be restricted to transformed cell types. By itself, α -TGF, like EGF, has little ability

Abbreviations used: EGF, epidermal growth factor; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; CCM, concentrated conditioned medium; MSV, murine sarcoma virus; NRK, normal rat kidney.

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to promote the growth of untransformed cells in soft agar. However, both factors potentiate the activity of another polypeptide, β -TGF (Anzano *et al.*, 1982a). This factor, which is present in non-neoplastic adult tissues (Frolik *et al.*, 1983; Roberts *et al.*, 1983) as well as in transformed cells (Anzano *et al.*, 1982a), has an M_r approx. 25000 and does not compete for binding to cellular EGF receptors (Roberts *et al.*, 1983). β -TGF is an EGF-dependent factor in that its ability to stimulate cell growth in soft agar requires the presence of either EGF or α -TGF (Roberts *et al.*, 1983; Anzano *et al.*, 1982b). These results have raised the possibility that the co-ordinated action of TGFs are centrally involved in the mechanisms of cellular transformation. Further support for this hypothesis comes from the finding that α -TGF production and release apparently correlate with the transformed phenotype in cells transformed by a temperature-sensitive murine sarcoma virus (MSV) mutant (DeLarco *et al.*, 1981), and from the finding that transformation-defective mutants of Abelson leukaemia virus do not induce α -TGF production (Twardzik *et al.*, 1982a).

Starting with freshly cloned NIH/3T3 cells we have isolated a series of untransformed, Kirsten MSV-transformed and flat revertant cell lines (Morris *et al.*, 1980). These clonally-related cell lines allow the study of the expression of the virus genome and its effects on the cell against a constant cellular background. We have investigated ^{125}I -EGF binding and TGF production by these cell lines in order to determine whether either, or both, parameters are correlated with the transformed phenotype.

Materials and methods

Culture medium, antibiotics, trypsin and newborn calf serum were from Flow Laboratories. Na^{125}I and $[^3\text{H}]$ thymidine were from Amersham International. EGF was prepared from male mouse submaxillary glands by the method of Savage & Cohen (1972) and labelled with ^{125}I by using lactoperoxidase (Thorell & Johansson, 1971). The labelled protein was separated from unreacted Na^{125}I by passage of the iodination mixture through a Sephadex G-25 column equilibrated and eluted with a buffer containing 0.01 M-phosphate (pH 7.4) and 0.15 M-NaCl. The ^{125}I -EGF was stored frozen in the presence of 0.1% bovine serum albumin. The specific radioactivity of the ^{125}I -EGF, at preparation, was 65000–135000 c.p.m./ng.

Cell lines

The isolation and characterization of clonally-related NIH/3T3 derived cell lines infected with

Kirsten MSV has been reported previously (Morris *et al.*, 1980; Brown & Blakeley, 1984). The cells used in the present work were an untransformed line (NIH.4E), a non-producer Kirsten MSV-transformed clone (CC1), and a revertant clone (R5-5-1) derived from CC1 cells. The transformed and the revertant cells, but not the untransformed cells, release fully functional Kirsten MSV after superinfection with Moloney murine leukaemia virus (Morris *et al.*, 1980). The lines were used at passage numbers (after cloning) of 13–18, 30–35 and 13–25 for the NIH.4E, CC1, and R5-5-1 cells respectively. There were no changes in cellular morphology or cell growth characteristics during these intervals. The cloned cell line ST4 was isolated from spontaneously transformed NIH/3T3 cells (Clewley & Avery, 1982). The indicator cells used in the soft agar cell growth assay were normal rat kidney fibroblasts, clone 49F (DeLarco & Todaro, 1978a,b). The cells used to measure competition for ^{125}I -EGF binding were a line of Swiss 3T3 cells (Todaro & Green, 1963) which have been extensively characterized in their response to polypeptide growth factors and their interaction with ^{125}I -EGF (Brown *et al.*, 1979a, 1980, 1983; Dicker & Rozengurt, 1980; Rozengurt *et al.*, 1982). The Moloney MSV-transformed 3T3 cell line (3B11; DeLarco & Todaro, 1978b), which produces both α - and β -TGF (Anzano *et al.*, 1983), was used for comparative purposes.

Cell culture

Stock cultures of all cell lines were propagated in 9 cm plastic dishes (Nunc) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Dishes were incubated at 37°C in a humidified atmosphere of CO_2 /air (1:9). Stock cultures were routinely subcultured shortly before achieving confluence. For measuring ^{125}I -EGF binding, approx. 2.5×10^4 cells were seeded into 3-cm plastic dishes (Nunc) and used after 4 or 5 days of growth. In assays of the stimulation of DNA synthesis, the Swiss 3T3 indicator cells were used after 6–8 days of growth, by which time the cells were blocked in the G_0/G_1 phase of the cycle as judged by the low (<2%) proportion of labelled nuclei after a 40 h exposure to $[^3\text{H}]$ thymidine.

Preparation of concentrated conditioned medium (CCM)

A procedure similar to that described by DeLarco & Todaro (1978b) was used to concentrate cell-conditioned medium. Briefly, cells were grown in either roller bottles or on plastic dishes (Nunc; 23 cm $\times$ 23 cm) containing DMEM with 10% (v/v) newborn calf serum. The cultures were

washed with 50ml of serum-free medium for 1 h followed by a further wash for 12 h. These washes were discarded. Two successive 48 h collections of serum-free conditioned medium were pooled, centrifuged at 100000g for 45 min and the supernatant was stored at -20°C until used. The viability of the cells at the end of the whole collection period was greater than 85% as measured by Trypan Blue exclusion. After thawing, the conditioned medium was dialysed against 1% acetic acid using Spectrapor 3 dialysis tubing (M_r cutoff approx. 3500). The dialysis residue was lyophilized and dissolved in either 1 M-acetic acid or phosphate-buffered saline (pH 7.4). The extract was clarified by centrifuging at 100000g, divided into portions and stored at -20°C . The protein concentration of the CCM was determined as described by Bradford (1976); 500 ml of conditioned medium gave 12.4, 14.5 and 17.8 mg of acetic acid-soluble protein from the NIH.4E, CC1 and 3B11 cells respectively.

Comparing TGF release by different cell lines on the basis of the amount of CCM protein added to an assay could be misleading, since no account would be taken of possible differences in the relative amounts of non-TGF proteins secreted by the cell lines. We have, therefore, chosen to compare cellular TGF production on the basis of the volume (ml) of original conditioned medium from which a given amount of CCM was derived.

¹²⁵I-EGF binding assay

Cells, grown on 3 cm dishes, were washed with 1–2 ml of binding medium which consisted of DMEM containing 0.1% (w/v) crystalline bovine serum albumin, 0.1 μM -KI, and 50 mM-Bes adjusted to pH 7.0. The cells were incubated at 37°C with 1 ml of binding medium containing ¹²⁵I-EGF at the required concentration. After incubation, unbound radioactivity was removed by washing the cells four times with cold (4°C) phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and 0.1 μM -KI. The washed cells were solubilized (60 min at 37°C) into 1 ml of 0.5 M-NaOH, and cell-associated radioactivity was determined in a γ -radioactivity counter. Cell numbers were determined by trypsin-treating replicate dishes of cells, suspending the cells in Isoton, and counting in a Coulter Counter.

Assay for the stimulation of DNA synthesis in quiescent Swiss 3T3 cells

Aliquots of CCM were added directly to the depleted growth medium of quiescent cells. [³H]Thymidine (20 μl) was added per dish to give a final concentration of 1 $\mu\text{Ci/ml}$ (1×10^{-6} M). After incubation for 40 h at 37°C , the medium was removed from the dishes and the cells were

washed twice with cold phosphate-buffered saline (pH 7.4). The cells were extracted for 30 min with 1–2 ml of cold 5% trichloroacetic acid, rinsed with ethanol, and air dried. The cells were solubilized into 1 ml of 0.1 M-NaOH and 0.5 ml of this solution were mixed with 10 ml of acidified scintillation fluid for ³H counting.

Soft agarose growth assay

This test was carried out as described (DeLarco & Todaro, 1978b) except that agarose (Neugut & Weinstein, 1979) was substituted for agar. Briefly, NRK fibroblasts (2×10^3 cells) suspended in 0.7 ml of DMEM containing 10% (v/v) calf serum and 0.25% agarose, were pipetted onto a base agarose layer (0.5% in the same medium) in 3 cm plastic dishes. CCM, with or without EGF (2 ng/ml), was added to both the base and upper layers at the indicated concentrations. The dishes were incubated at 37°C and fed 140 μl of complete DMEM (with or without CCM and EGF) at weekly intervals. After 3 weeks, cell colonies $> 30 \mu\text{m}$ diameter were counted over a total area of 1.29 cm^2 .

Results

Cell growth characteristics

The untransformed NIH.4E cells grew as a flattened polygonal monolayer in DMEM containing 10% calf serum. In contrast, the virus-transformed CC1 cells were smaller, more spindle-shaped, less adherent to the substratum and formed multilayers in culture. The R5-5-1 revertants were morphologically similar to the untransformed cells, showing a flat, ordered growth pattern.

We have previously shown (Morris *et al.*, 1980) that the growth of the NIH.4E and R5-5-1 cells is strictly anchorage-dependent and that these cells fail to grow in soft agar culture. In contrast, more than 50% of seeded CC1 cells formed colonies in soft agar medium. The CC1 cells were highly tumourigenic when injected into newborn mice whereas neither untransformed nor revertant cell lines formed tumours (Table 1).

¹²⁵I-EGF binding to cell lines

The time course of ¹²⁵I-EGF binding to NIH.4E cells at 37°C is shown in Fig. 1. Maximal binding was achieved after 60–120 min exposure to ¹²⁵I-EGF and continued incubation with ¹²⁵I-EGF led to a decrease in the cell-associated radioactivity. This time-course is very similar to that previously reported for various cell types including human fibroblasts (Carpenter *et al.*, 1975) and BALB 3T3 cells (Brown *et al.*, 1979b). In contrast, no significant ¹²⁵I-EGF binding was detected in either the

Table 1. *Tumourigenicity of cell lines*

Tests were carried out as previously described (Morris *et al.* (1980). The numbers in parentheses indicate passage number.

Cell line	Tumour incidence at 10 ⁶ cells/mouse	TD ₅₀
NIH.4E	0/29 (13)	> 5 × 10 ⁶ (14)
CC1	21/28 (33)	< 1 × 10 ⁵ (35)
R5-5-1	0/27 (13)	> 5 × 10 ⁶ (15)

Fig. 1. Time-course of ¹²⁵I-EGF binding to normal, transformed and revertant cells

Cells were washed with binding medium and 1 ml of binding medium containing 1.2 ng of ¹²⁵I-EGF (43 000 c.p.m./ng) was added per dish. The cultures were incubated at 37°C for the indicated time intervals, washed, and cell-associated radioactivity was determined as described under Materials and methods. Each point represents the mean of four determinations of ¹²⁵I-EGF binding to separate dishes of cells. Error bars show ± S.E.M. Error bars which fall within the symbol have not been marked. Cell numbers were as follows: NIH.4E (●), 0.52 × 10⁶ cells/dish; CC1 (■), 1.70 × 10⁶ cells/dish; R5-5-1 (▲), 0.34 × 10⁶ cells/dish.

CC1 or R5-5-1 cells after exposure to ¹²⁵I-EGF at 37°C for as long as 240 min (Fig. 1).

In order to determine whether the apparent absence of binding to the CC1 and R5-5-1 cells was due to a lower affinity of EGF receptors in these cells, the concentration of ¹²⁵I-EGF in the binding medium was varied in the range 0.05–20 ng/ml and maximal ¹²⁵I-EGF binding (90 min) was measured. The binding of ¹²⁵I-EGF to the NIH.4E cells and to a spontaneously transformed cell line (ST4) was concentration-dependent and saturable (Fig. 2). Scatchard analysis of the data presented in Fig. 2 showed that the maximal amount of ¹²⁵I-EGF bound (B_{max}) was approx. 2-fold higher in

Fig. 2. Binding of ¹²⁵I-EGF as a function of ¹²⁵I-EGF concentration

Cells were washed with binding medium and 1 ml of binding medium containing the indicated concentrations of ¹²⁵I-EGF (41 000 c.p.m./ng) was added per dish. Cell-associated radioactivity was determined after a 90 min incubation at 37°C as described under Materials and methods. Each point represents the mean of two or four determinations of ¹²⁵I-EGF binding to separate dishes of cells. Error bars show ± S.E.M. Cell numbers were as follows: NIH/4E (●), 0.34 × 10⁶ cells/dish; CC1 (■), 1.06 × 10⁶ cells/dish; R5-5-1 (▲), 0.56 × 10⁶ cells/dish; ST4 (▼), 0.16 × 10⁶ cells/dish.

the ST4 cells than in the NIH.4E cells. However, the apparent dissociation constants (K_D) for the two cell lines were similar at approx. 4×10^{-10} M. These values for B_{max} and K_D are similar to those previously reported for human and mouse fibroblasts (Carpenter *et al.*, 1975; Brown *et al.*, 1979b). The Kirsten MSV-transformed and the revertant R5-5-1 cells, on the other hand, showed no evidence of saturable ¹²⁵I-EGF binding (Fig. 2). At high concentrations of ¹²⁵I-EGF (20 ng/ml) there were significant levels of cell-associated radioactivity (Fig. 2). However, this binding is probably non-specific since, in separate experiments (not shown), only 10–40% of cell-associated radioactivity was displaced by 20 µg of unlabelled EGF/ml. In contrast, in the NIH.4E and ST4 cell lines, more than 90% of total cell-associated radioactivity is displaceable by competition with unlabelled EGF. Taken together, these results suggest that both the CC1 and R5-5-1 cells are devoid of cell-surface EGF receptors, though the presence of a small number of relatively low-affinity binding sites cannot be ruled out.

Growth factor release by untransformed and virus-transformed cell lines

In order to investigate cellular growth factor release, serum-free conditioned medium was collected from NIH.4E and CC1 cells and concentrated 70–150-fold as described (DeLarco & Todaro, 1978b). CCM prepared from either untransformed or Kirsten MSV-transformed cells stimulated the incorporation of [^3H]thymidine into quiescent Swiss 3T3 cells. CCM from the untransformed cells was a slightly more potent stimulator of DNA synthesis than was CCM from the transformed CC1 cells (Fig. 3). However, CCM from both these cell lines was considerably less active than CCM prepared from the Moloney MSV-transformed cells (3B11) (Fig. 3).

In an attempt to determine whether the growth-promoting activity in CCM was cell-derived, culture bottles containing no cells were incubated with complete serum-containing medium for several days. 'Conditioned' (serum-free) medium from these bottles was harvested and concentrated. Protein was present in this medium but no cell growth-promoting activity was detected (results not shown), suggesting that the stimulatory activity in the CCM was derived from the cells.

Fig. 3. Effect of CCM on DNA synthesis in quiescent Swiss 3T3 cell cultures

Conditioned serum-free medium from NIH.4E cells (○), CC1 cells (■) or 3B11 cells (▼) was harvested, concentrated, and tested for ability to stimulate [^3H]thymidine incorporation in quiescent Swiss 3T3 cells as described under Materials and methods. Each point represents the mean value of three determinations of [^3H]thymidine incorporation. In this experiment the addition of calf serum to the medium (final concentration 10% v/v) produced an incorporation of 348×10^3 c.p.m./dish.

The most specific assay for TGF activity is the ability to stimulate untransformed rat fibroblasts to grow in soft agar culture (Todaro *et al.*, 1981). We have tested the ability of CCM prepared from NIH.4E and CC1 cells in stimulating the growth of NRK fibroblasts in agarose. Cell colonies $> 30 \mu\text{m}$ diameter were not observed at any concentration (up to 20 ml equivalents) of CCM tested (Fig. 4). In contrast, CCM prepared from the Moloney MSV-transformed 3B11 cell line (DeLarco & Todaro, 1978b) produced a marked stimulation of colony formation (Fig. 4). CCM from the NIH.4E and CC1 lines did not stimulate NRK cell growth in agarose even when added together with EGF (Fig. 4). A potentiation of the growth effect by EGF would be expected for CCM which contained β -TGF but lacked α -TGF. As expected, EGF did not potentiate the stimulatory action of CCM from 3B11 cells, which contains both β - and α -TGF (Anzano *et al.*, 1983). These results indicate that, if the CC1 and NIH.4E cell lines release any β -TGF

Fig. 4. Effect of CCM on the growth of NRK fibroblasts in soft-agarose culture

The promotion of NRK fibroblast colony formation by CCM from NIH.4E (○, ●), CC1 (□, ■) or 3B11 (▽, ▼) cells was determined in the presence (closed symbols) or absence (open symbols) of EGF (2 ng/ml) as described under Materials and methods. Each point represents the mean value ($\pm$ S.E.M.) of four determinations.

Fig. 5. Effect of CCM on the binding of ^{125}I -EGF to Swiss 3T3 cells

Conditioned medium from NIH.4E cells (●), CC1 cells (■) or 3B11 cells (▼) was harvested, and concentrated, as described under Materials and methods. Cells were washed with binding medium and 1 ml of binding medium containing 1 ng of ^{125}I -EGF (41000 c.p.m./ng) and the indicated concentration of CCM was added per dish. Cell-associated radioactivity was measured after a 90 min incubation at 37°C . Each point represents the mean value ($n=3$) for ^{125}I -EGF binding expressed as a percentage of binding to untreated control cells.

into their medium, they produce considerably less factor than does the 3B11 cell line.

In order to compare the relative release of α -TGF by the different cell lines we have measured the ability of CCM from NIH.4E, CC1, and 3B11 cells to inhibit cellular ^{125}I -EGF binding. Although CCM from all three cell lines was able to inhibit ^{125}I -EGF binding to Swiss 3T3 cells, the levels of activity in the CCM from NIH.4E and CC1 cells were similar and considerably less (30–45-fold) than the level of activity in CCM from 3B11 cells (Fig. 5). Therefore, transformation of NIH.4E cells by Kirsten sarcoma virus does not necessarily increase α -TGF release.

Discussion

The Kirsten MSV-transformed CC1 cells, like the NIH.4E cells, release little, if any, α - or β -TGF under conditions in which TGF release was clearly demonstrated for the Moloney MSV-transformed

3B11 cells. These differences cannot be explained simply by differences in the number of cells present during medium conditioning, since the CC1 and 3B11 cultures contained similar cell numbers and this was approx. 2-fold higher than the number of NIH.4E cells. (However, it should be noted that the NIH.4E cells are approximately twice as large as the CC1 cells.) These results indicate that induction of the release of TGFs is not involved in maintaining the transformed phenotype of CC1 cells when compared with NIH.4E cells. However, it remains possible that synthesis of TGFs has been induced by Kirsten MSV transformation but the CC1 cells fail to release the synthesized factors. It will be necessary to prepare cell extracts in order to test this possibility.

Blomberg *et al.* (1980) have reported that EGF binding returned to normal levels in a revertant cell line isolated from feline sarcoma virus-transformed cells. Guinavan & Ladda (1979) reported that normal NRK fibroblasts transformed by a temperature-sensitive mutant of Kirsten MSV showed a 10-fold increase in EGF binding when cells were shifted from the permissive to the non-permissive temperature. However, the increased level of binding was still low (approx. 10%) when compared with ^{125}I -EGF binding to the untransformed parent NRK cells. In contrast, other results argue against the generality of restoration of EGF binding after phenotypic reversion of MSV-transformed cells. Retinoic acid has been reported (Mukherjee *et al.*, 1982) to restore anchorage-dependent and density-dependent growth to MSV-transformed cells without increasing cellular binding of ^{125}I -EGF. We have found (results not shown) that neither the retinoid nor interferon, which also produces a partial phenotypic reversion of the Kirsten MSV-transformed cells (Hicks *et al.*, 1981), are able to induce EGF receptors in CC1 cells. Furthermore, the revertant R5-5-1 cells, which are unable to grow in soft agar culture and which are non-tumorigenic, are devoid of detectable EGF receptors. Taken together, these results suggest that an increase in available plasma membrane EGF receptors is not a necessary requirement for the phenotypic reversion of MSV-transformed cells.

The processes involved in the apparent loss of EGF receptors from MSV-transformed cells are not understood. It has been postulated (DeLarco & Todaro, 1978b) that an endogenous EGF-related factor may 'specifically interact with the EGF receptor system in fibroblast membranes'. However, it is not clear how such a mechanism would operate. If the interaction were one of direct competition then 'blocked' surface EGF receptors, if present, should be demonstrable by using high concentrations of ^{125}I -EGF unless the affinity of

the endogenous factor for EGF receptors was exceptionally high. Alternatively, an endogenous EGF-like factor could reduce EGF binding by inducing receptor internalization and degradation. Cooper *et al.* (1983) have provided some evidence in favour of this possibility. Using a monoclonal antibody against the A431 cell EGF receptor it was found that the synthesis of the receptor occurred at the same rate in A431 cells and in A431 cells infected with sarcoma viruses, but virus infection reduced receptor stability by 50% and this increase in receptor turnover was assumed to be due to production of EGF-like factor(s). However, our finding that Kirsten MSV-transformation dramatically reduces cellular ^{125}I -EGF binding without inducing α -TGF release means that alternative mechanisms for receptor depletion must also be considered.

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