Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5258–5262, September 1980
Cell Biology

Transforming growth factors produced by certain human tumor cells: Polypeptides that interact with epidermal growth factor receptors

(sarcoma growth factor/cell growth/carcinomas/melanomas)

GEORGE J. TODARO, CHARLOTTE FRYLING, AND JOSEPH E. DE LARCO

Laboratory of Viral Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Communicated by Howard Green, April 21, 1980

ABSTRACT **Three different human tumor lines in culture, a rhabdomyosarcoma, a bronchogenic carcinoma and a metastatic melanoma, release proteins (transforming growth factors, TGFs) into the medium that confer the transformed phenotype on untransformed fibroblasts. These proteins are acid and heat-stable; produce profound morphologic changes in rat and human fibroblasts; and enable normal anchorage-dependent cells to grow in agar. Removal of the transforming protein results in a reversion of cell phenotype. The major activity interacts with epidermal growth factor (EGF) cell membrane receptors. The peptides from these tumor cells are similar in their action to the sarcoma growth factor (SGF) released by murine sarcoma virus-transformed rodent cells. The most anchorage-independent tumor cells released the most TGFs. EGF-related TGFs were not detectable in fluids from cultures of cells with high numbers of free EGF membrane receptors (normal human fibroblasts and human carcinomas).**

Mouse sarcoma virus (MSV)- and rat sarcoma virus-transformed cells release a potent growth-stimulating peptide that interacts with epidermal growth factor (EGF) receptors (1, 2). This ability has been utilized to purify sarcoma growth factor (SGF) produced by MSV-transformed cells (3). It has been noted that certain human sarcoma and carcinoma cells (4) and most melanomas (5) lack EGF receptors and, therefore, may produce endogenous factors related to EGF and SGF. To test this possibility, serum-free media were collected from four human tumors and normal human fibroblasts and partially purified. Cells that lack EGF receptors released a potent growth-stimulating activity that enabled normal fibroblasts and epithelial cells to proliferate in soft agar. Supernates from normal human fibroblasts possessed <2% as much activity.

A human epidermoid carcinoma cell (A431) with an exceptionally high number of EGF receptors (4, 6, 7) released little growth-stimulating activity when compared to the other tumor cells. That activity did not compete with EGF. Tumor cells that lack EGF receptors and form colonies in soft agar released a greater quantity of the transforming growth factors (TGFs) than did normal or tumor cells that grow poorly in agar. We conclude that certain human tumor cells release potent transforming protein(s) that transform normal indicator cells in a manner similar to SGF.

MATERIALS AND METHODS

Cell Cultures. Cell cultures were maintained at 37°C in 75-cm^2 plastic tissue culture flasks (Falcon no. 3024) with Dulbecco's modification of Eagle's medium (DME medium) with 10% calf serum (Colorado Serum). Five human tumor cell cultures were used. The human rhabdomyosarcoma line, A673, and the bronchogenic carcinoma line, 9812, produce progressively growing tumors in immunologically depressed mice and grow readily in soft agar (8). Neither has detectable EGF receptors (4). The human epidermoid carcinoma A431, from a primary vulvar carcinoma in an 85-year-old woman, has an exceptionally high number of EGF receptors (4, 6). The human metastatic melanoma line A2058, from a brain metastasis in a 43-year-old man, has nerve growth factor (NGF) receptors (5) and free NGF on its cell surface (9). TE85 is a human osteosarcoma line from J. Rhim (National Institutes of Health). The human embryonic lung cell HEL 299 is from the American Type Culture Collection, Rockville, MD; the adult skin strain HsF is from a normal female; and 49F is a clone of fibroblastic cells (10) from the normal rat kidney line NRK (11).

Growth Factors. Serum-free conditioned media from the human tissue culture cells were the source of growth factors. In each case, cells were grown to confluency in DME medium with calf serum and washed twice with serum-free Waymouth's medium (GIBCO, MD 705/1), once for 8 hr and once for 16 hr, to eliminate serum proteins. The washes were discarded and subsequent 48-hr collections were taken as "conditioned medium". The medium was centrifuged at 100,000 × *g* for 45 min and concentrated 25-fold using a hollow fiber concentrator (Amicon, Lexington, MA; DC2). The concentrate was dialyzed against four changes (10 vol each) of 1% HOAc, lyophilized, and extracted with 1 M HOAc. This extract was centrifuged (100,000 × *g* for 120 min), and the supernate was subjected to Bio-Gel P-100 chromatography with a column equilibrated and eluted with 1 M HOAc (1).

Chromatography on CM-Cellulose. The lyophilized fractions from the biologically active region from the P-100 column were pooled, reconstituted in 5 ml of 1 M HOAc, and dialyzed against 5 mM NH_4OAc (pH 4.5) overnight at 4°C. The sample was centrifuged at 175,000 × *g* for 30 min at 22°C and applied to a 1.5- × 3-cm column of CM-cellulose (CM-52, Whatman). Elution was achieved with a linear gradient pumped from a two-chamber, constant-level device containing 200 ml of starting buffer (5 mM NH_4OAc, pH 4.5) in the first chamber and 200 ml of limit buffer (0.5 M NH_4OAc, pH 6.8) in the second (flow rate 80 ml/hr at 22°C). Aliquots were made up to 1 M HOAc and concentrated by lyophilization.

EGF Binding Assays. EGF was isolated (12) and radiolabeled with ^{125}I (13) as described (3). Binding of ^{125}I-labeled EGF (^{125}I-EGF) was performed on subconfluent monolayers of HCHO-fixed A431 cells in 16-mm tissue culture wells (Linbro no. 76-033-05). The fixed cells were washed twice with binding buffer (DME medium with 1 mg of bovine serum albumin per ml and 50 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid adjusted to pH 6.8). Competitions were ini-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: TGFs, transforming growth factors; EGF, epidermal growth factor; SGF, sarcoma growth factor; NGF, nerve growth factor; DME medium, Dulbecco's modification of Eagle's medium; MSV, mouse sarcoma virus.

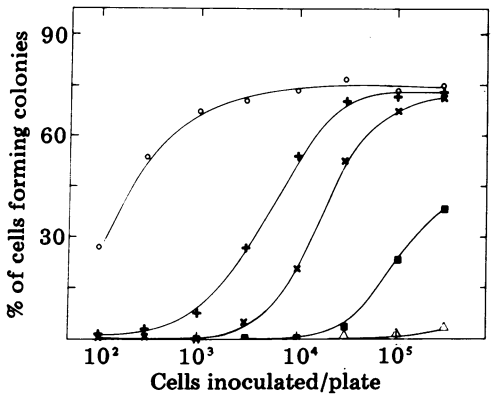

FIG. 1. Soft agar colony formation as a function of cell density. Soft agar assays were set up in 60-mm tissue culture dishes (Falcon no. 3002) by applying a base layer of 0.5% soft agar (Difco, Noble) and a 2-ml layer of 0.3% agar containing the appropriate cell number. △, HEL 299; ■, A431; ○, 9812; +, A673; ×, A2058.

tiated by adding 0.2 ml of binding buffer containing 2 ng of ^{125}I-EGF per ml with or without the potential inhibitor. The mixtures were incubated with the monolayers at 22°C for 1 hr, the ^{125}I-EGF-containing buffer was removed, the cell sheet was washed four times with 1-ml portions of binding buffer, and the quantity of ^{125}I-EGF specifically bound was determined.

Soft Agar Growth Assay. The lyophilized fractions were redissolved in binding buffer at five times the final concentration used in the assay and centrifuged to clarity. Samples containing 3×10^3 cells per ml were suspended in 0.3% agar (Difco, Agar Noble) in DME medium with 10% calf serum; 0.7 ml was pipetted onto a 0.7-ml base layer of 0.5% agar in 35-mm petri dishes (Falcon). Plates were incubated at 37°C for 2 weeks in a humidified 5% CO_2/95% air atmosphere without further feeding. The assay was read unfixed and unstained at 5 days and at 10–14 days.

Cellular DNA Synthesis Assay. Serum-deprived, subconfluent, NRK cells (clone 49F) (10) were seeded at 5×10^4 cells per 16-mm well (Linbro no. 76-033-05) in DME medium with 10% calf serum. After 4 hr, the medium was removed; the cells were washed with fresh serum-free medium and incubated with 1 ml per well of Waymouth's medium containing 0.1% calf serum. Seven days later, 0.1 ml of binding buffer containing the sample to be tested was added. After 16 hr, the cells were exposed for 5 hr to 4.0 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]thymidine (New England Nuclear; NET-027), washed twice with 1 ml of DME medium containing 100 μg of unlabeled thymidine per ml, incubated for 30 min, washed three times, and disrupted with 0.5% sodium dodecyl sulfate/1 mM EDTA. DNA was precipitated by adding the lysate to 3 vol of cold 10% CCl_3COOH and was removed by filtration (Millipore; HA, 0.45 μm); the filters were dried and added to counting vials with 5 ml of toluene/Liquifluor (New England Nuclear, NEF 903), and the radioactivity was measured in a liquid scintillation counter (Beckman, LS-250).

RESULTS

The three tumor cells tested for production of factors analogous to SGF were chosen for study because they had no apparent EGF receptors and readily form colonies in soft agar. Normal embryonal lung fibroblasts, which are unable to grow in soft agar, and A431 cells, which have a very high number of EGF receptors and grow poorly in soft agar, were used as controls.

The five cultures were compared for their ability to form colonies in soft agar. The cells were grown in monolayer cultures, harvested, and seeded at varying densities into medium with 0.3% agar. Colonies were scored at 5 and 10 days. Colonies with more than 10 cells were counted as positive. The results shown in Fig. 1 were obtained at 5 days; the later reading showed no additional positive cells. The cell line 9812 formed progressively growing colonies even when relatively low numbers of cells were seeded, whereas A431 cells only showed colony growth when high-cell inocula were used. This suggests

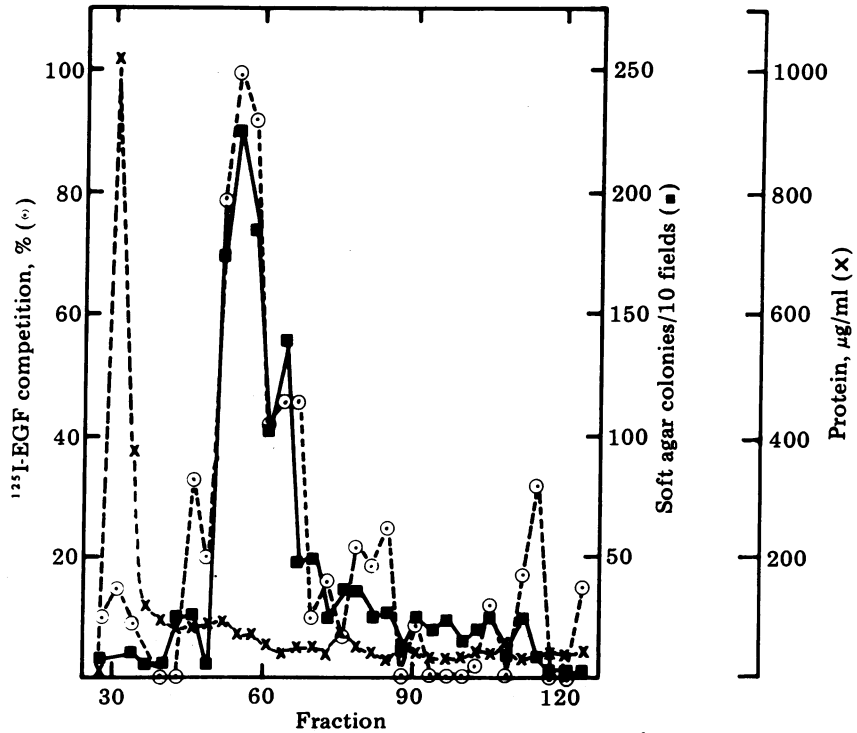

FIG. 2. Biological activity and protein determination of Bio-Gel P-100 column fractions of concentrated conditioned medium from A673 cells. Nonspecific binding, determined by an addition of a 500-fold excess of unlabeled EGF, was approximately 200 cpm. Specific binding was approximately 1200 cpm. Percentage of competition was determined after correcting for nonspecific binding. Protein concentration was determined by the method of Lowry *et al.* (24).

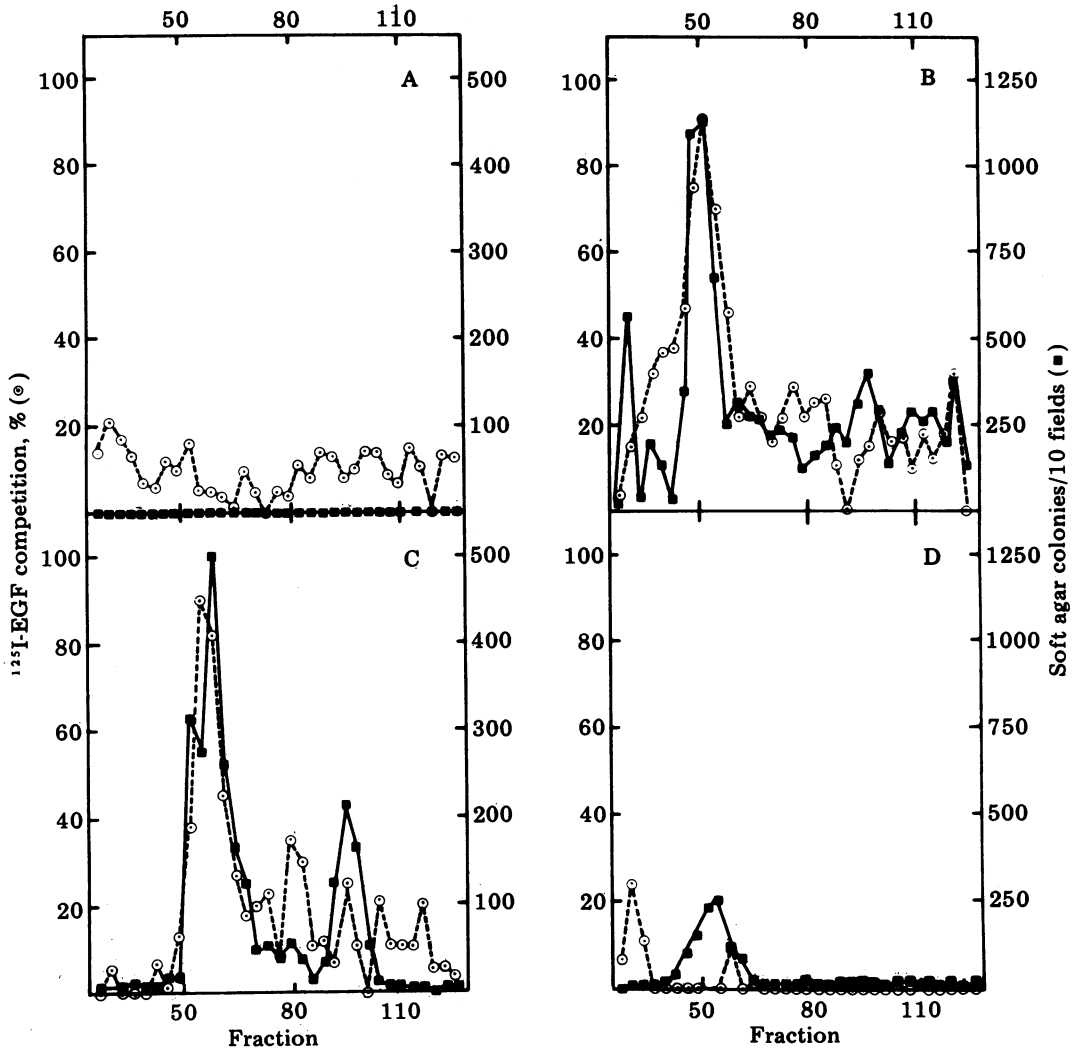

FIG. 3. Biological activity in Bio-Gel P-100 column fractions of serum-free conditioned media from four human cells in culture. *A*, HEL 299; *B*, 9812; *C*, A2058; *D*, A431.

that a critical concentration of diffusible factors from these cells is required for anchorage-independent growth.

Cells that are potential producers of factors that stimulate growth in soft agar (e.g., human tumor cells) were seeded in one layer of agar at 1×10^6 cells per plate and overlaid with indicator cells (e.g., rat fibroblasts) at 1×10^4 cells per plate. The indicator cells formed colonies when certain human tumor cells were seeded in the other layer. A673, 9812, and A2058 cells elicited the greatest response and released as much growth-stimulating activity in agar as did a comparable number of MSV-transformed mouse 3T3 cells.

Fig. 2 shows the results of experiments in which serum-free supernates from A673 cells were collected, concentrated, and passed over a Bio-Gel P-100 column in 1 M HOAc. Individual fractions were tested for protein concentration, ability to stimulate cells to form colonies in soft agar, and ability to compete with ^{125}I-EGF (3). The majority of the protein is in the void volume of the column. A major peak of growth-stimulating activity in soft agar was found in the included volume, with maximal activity in fraction 54. When the same fractions were tested for competition with ^{125}I-EGF binding, one major peak was again found, with maximal activity also in fraction 54. Aliquots were tested for stimulation of cell division in serum-depleted cultures of mouse 3T3 cells, rat NRK cells, and human skin fibroblasts; in all cases, the major growth-stimulating activity was found in fraction 54. Fractions 51–57 were pooled, concentrated by lyophilization, and used for further studies.

The identical procedure was used to test for growth-stimulating factors and EGF-competing peptides from the supernates of four other human cell cultures. Fig. 3 shows that the two highly transformed tumor cell lines, 9812 and A2058, release a growth-stimulating and EGF-competing material with an apparent molecular weight of 20,000–23,000 (Fig. 3 *B* and *C*). A2058 cells release a second factor with an apparent molecular weight of 6000–7000. Fig. 3*A* shows that the supernate from normal human fibroblast cells did not release a detectable growth-stimulating activity and had no significant EGF-competing activity. A431 cells showed a smaller peak of growth-stimulating activity with an apparent molecular weight of 21,000; no EGF-competing activity was found.

Fig. 4*A* shows a dose–response curve measuring growth in soft agar as a function of protein concentration. The pooled, peak fractions from A673 cells are compared with those from normal human fibroblasts. There was a 50- to 100-fold difference in growth-stimulating activity in soft agar.

The relative sensitivities of three different assays for growth-stimulating activity are compared in Fig. 4*B*. The data is presented as the percentage of the maximal response. Induction of DNA synthesis as tested with serum-depleted rat fibroblast monolayer cultures was slightly more sensitive than the soft agar growth assay; EGF-competing ability was the least sensitive. The latter two assays were used in further studies because they have greater specificity. Each of the TGF activities

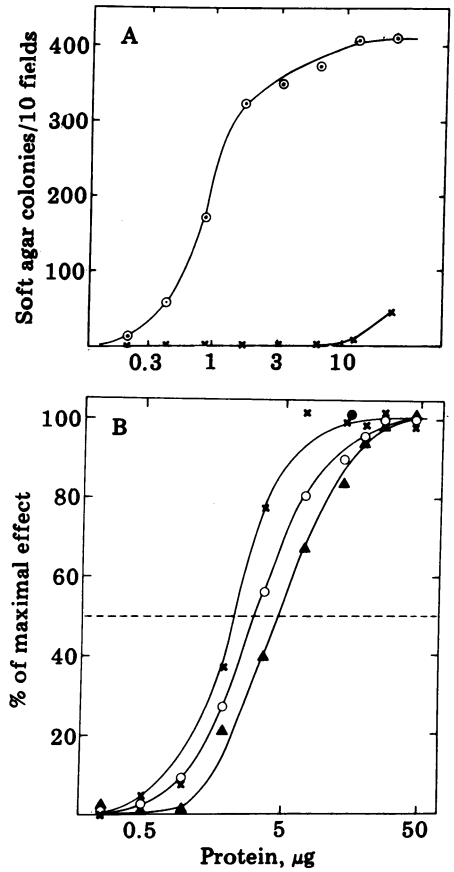

FIG. 4. (*A*) Soft agar colony (>10 cells) formation as a function of protein concentration. Bio-Gel P-100 column fractions from the 20,000–23,000 dalton region were pooled and lyophilized. Aliquots in 0.1 M HOAc were added with the cells in the soft agar overlay. O, A673; X, HEL 299. (*B*) Plot of the percentage of the maximal effect as a function of protein. [^{3}H]Thymidine incorporation, EGF competition, and soft agar assays were performed as described. Maximal response was seen at 25–50 μg of protein; 73,500 cpm and 1,800 cpm, respectively, were incorporated for the [^{3}H]thymidine assays and control plates; 430 colonies per 10 fields (control plates with none) for the soft agar growth assay; 96% inhibition of ^{125}I-EGF binding.

was destroyed by trypsin or dithiothreitol but was stable at 100°C for 2 min and to repeated lyophilization from 1 M HOAc.

Table 1 shows that the growth-stimulatory factor(s) released by the human tumor cells induce anchorage-independent growth of normal human fibroblasts. Two cell strains were tested; passage 8 of HEL 299 and passage 14 of HsF. A673 cells were tested at concentrations of 10 μg/ml and 1 μg/ml. Cells were seeded (1×10^4 per plate) and 1000 single cells were followed for 2 weeks. Those that grew to colonies containing 10 cells were scored as positive. The percentages of HEL 299 and HsF single cells that gave rise to colonies were 4.2% and 3.1%, respectively, using 10 μg of P-100-purified TGF per ml. In contrast, 23.6% of the rat fibroblast cells showed a pronounced response, even at a concentration of 1 μg/ml. TGF also induced soft agar growth of a mouse epithelial cell line, MMC-1 (14) (data not shown).

In order to test whether human tumor cell lines could also respond to TGFs, cells such as A431, which untreated could not form colonies in agar unless inoculated at high density, were used. Carcinoma cell growth in agar also depends on "conditioning" factors, such as TGFs, which partially replace the requirement for high-cell density. The results were more striking when the human osteosarcoma line TE85, which can be further transformed by MSV and certain chemical carcinogens (15), was used as an indicator cell. These results demonstrate that

Table 1. Stimulation of growth in agar of human diploid fibroblasts and human tumor cells by TGF

Cell	Type	Colonies >10 cells/1000 cells: Control	With TGF, 1 μg/ml	With TGF, 10 μg/ml
HEL 299	Embryonic lung fibroblast	1	3	42
HsF	Adult skin fibroblast	2	2	31
A431	Epidermoid carcinoma	3	8	31
TE85	Osteosarcoma	1	14	75
NRK (clone 49F)	Rat kidney fibroblasts	0	37	236

normal and tumor cells respond to TGFs in the same manner as rat fibroblasts. The results, then, are not dependent on an unusual property of a particular indicator cell.

The active fractions from Bio-Gel P-100 columns of A673 and A431 cells were pooled, concentrated, and applied to CM-cellulose columns. Two peaks of agar growth-stimulating activity were obtained from A673 cells; only the major activity was associated with the peak of EGF-competing activity. Dose–response curves from each peak show an activity detectable when concentrations of 10 to 20 ng/ml are added to soft agar. The comparable fraction from supernates of cultures of the normal human fibroblast showed no activity. Fractions derived from A431 cells showed (Fig. 5) only the less active, earlier eluting peak that is not associated with EGF-competing activity. We conclude that A431 cells that grow poorly in agar and have a high level of EGF receptors produce a factor capable of stimulating anchorage-independent growth of cells through a mechanism independent of the EGF receptor system. The highly transformed A673 cells, however, make at least two different factors. One interacts with the EGF receptor system and accounts for >90% of the total activity in the fraction. The other is independent of the EGF receptor system and may be analogous to the factor produced by the A431 cells.

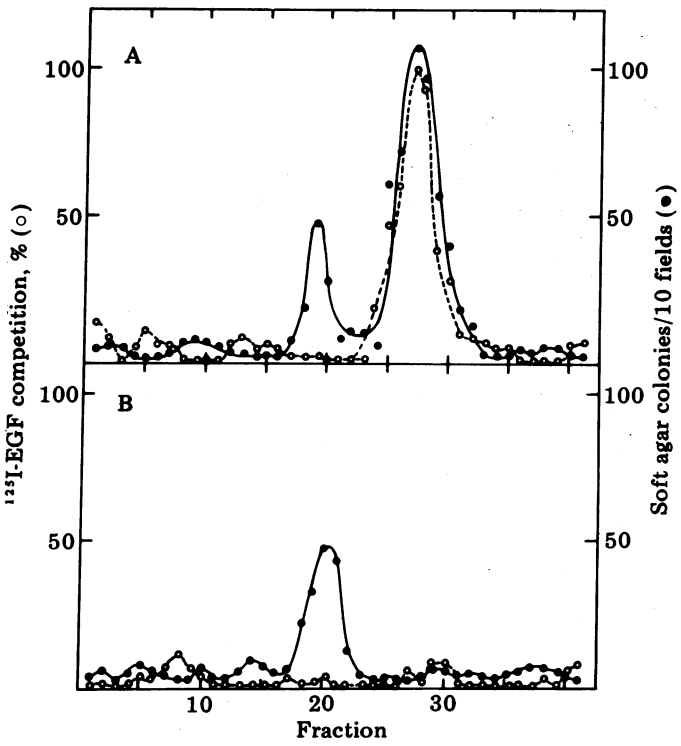

FIG. 5. Chromatography of biological activities in the peak region of Bio-Gel P-100 columns rechromatographed on a CM-cellulose column. *A*, A673; *B*, A431.

DISCUSSION

The results demonstrate that human tumor cells produce a growth factor(s) capable of inducing transformation in normal indicator cells. It has many properties in common with the factor from mouse and rat sarcoma virus-transformed cells. The major activity, although considerably larger than SGF, is closely associated with EGF-competing activity. We have found that a chemically transformed mouse 3T3 cell line produces growth-stimulating factor(s) active in the soft agar growth assay (unpublished data). Production of these factors then, is not restricted to RNA tumor virus-transformed cells, sarcoma cells, or rodent cells, but, rather, may be a more general expression of the transformed phenotype. In assays comparing growth stimulation of mouse, rat, and human fibroblasts in monolayer cultures, there is no evidence for species specificity of the factors produced by human cells. Conclusions as to whether the carcinoma, sarcoma, and melanoma cells are producing an identical factor(s) await further chemical purification. The present experiments show that anchorage-independent growth of tumor and normal cells is stimulated by these growth factors. Their production by transformed cells and the responses of their normal counterparts raise the possibility that cells "auto-stimulate" their growth by releasing factors that rebind at the cell surface (4). Experiments demonstrating that growth in soft agar of tumor cells depends on the number of cells seeded per unit area argue that diffusible substances released by cells stimulate neighboring cells. Those cells that grow best in soft agar are the most efficient producers of transforming peptides. Additional cell lines will have to be tested under different conditions before conclusions can be drawn as to the significance of this association.

Roberts *et al.* (16) described a procedure for purifying TGFs. The peptides are stable in acidic 70% (vol/vol) alcohol. Intracellular growth factors have been extracted from cultured MSV-transformed mouse cells and from tumor cells in athymic mice. The major peptide with growth-stimulating activity in soft agar has an apparent molecular weight of 6700. The peak of EGF-competing activity is in the same fraction. A transplantable, transitional cell, mouse bladder carcinoma had growth-stimulating activity in agar for rat fibroblasts. Ozanne *et al.* (17) described a transforming factor from Kirsten sarcoma virus-transformed rat fibroblasts with properties like SGF and TGFs and reported a similar activity in a spontaneously transformed rat cell line. The effect of the transforming factor on morphologic transformation can be blocked by actinomycin D early after treatment, suggesting that new RNA is produced prior to the change in phenotype of the indicator cells. Inhibitors of protein synthesis also produce a rapid reversion in the phenotype of the treated cells (17).

If release of the factor and rebinding to EGF receptors is essential for growth stimulation, tumor cell growth could be interrupted by exogenous agents, perhaps analogues that interact with the receptors but do not confer the ability to proliferate under anchorage-independent conditions (18). Anchorage-independent growth is a cell culture property closely associated with the transformed state *in vivo* (19, 20). These peptides, then, are potent proximal effectors of cell transformation. Their continued production appears to play a role in maintaining the transformed phenotype. This can be directly demonstrated in temperature-sensitive mutant transformants of rodent cells (2, 17) but has not yet been shown for factors produced by human tumor cells. The approach described here offers a sensitive assay for growth-stimulatory factors associated with maintaining the transformed state. Purification of such factors may lead to the development of specific immunologic assays for their production by tumor cells and their presence in body fluids. The factors may be analogous to peptide growth factors expressed early in normal embryonic development (2). This is supported by experiments by Nexo *et al.* (21). In the mouse embryo (days 11–18), there is 5–10 times more EGF-like material than mouse EGF. Why the factors produced by transformed cells are so potent in stimulating anchorage-independent growth whereas EGF is not effective is unclear, but that fact suggests the possibility that there may be more "transforming" variants of the normally expressed growth factors produced in adult life. We suggest that these factors, like SGFs, are EGF-related peptides [as insulin and somatomedins are related (22)] and appear to have evolved from common ancestral proteins (23). Further purification of these growth factors from human tumor cells is needed to define their relationship to other biologically active peptides that cells produce.

1. De Larco, J. E. & Todaro, G. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4001–4005.
2. Todaro, G. J. & De Larco, J. E. (1980) in *Control Mechanisms in Animal Cells: Specific Growth Factors*, eds. Jimenez de Asua, L., Levi-Montalcini, R., Shields, R. & Iacobelli, S. (Raven, New York), Vol. 1, pp. 223–243.
3. De Larco, J. E., Reynolds, R., Carlberg, K., Engle, C. & Todaro, G. J. (1980) *J. Biol. Chem.* **255,** 3685–3690.
4. Todaro, G. J. & De Larco, J. E. (1978) *Cancer Res.* **38,** 4147–4154.
5. Fabricant, R. N., De Larco, J. E. & Todaro, G. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 565–569.
6. Haigler, H., Ash, J. F., Singer, S. J. & Cohen, S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3317–3321.
7. Carpenter, G., King, L., Jr. & Cohen, S. (1979) *J. Biol. Chem.* **254,** 4884–4891.
8. Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H. & Parks, W. P. (1973) *J. Natl. Cancer Inst.* **51,** 1417–1423.
9. Sherwin, S. A., Sliski, A. H. & Todaro, G. J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1288–1292.
10. De Larco, J. E. & Todaro, G. J. (1978) *J. Cell. Physiol.* **94,** 335–342.
11. Duc-Nguyen, H., Rosenblum, E. N. & Zeigel, R. F. (1966) *J. Bacteriol.* **92,** 1133–1140.
12. Savage, C. R., Jr. & Cohen, S. (1972) *J. Biol. Chem.* **247,** 7609–7611.
13. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89,** 114–123.
14. Keski-Oja, J., De Larco, J. E., Rapp, U. R. & Todaro, G. J. (1980) *J. Cell. Physiol.* **104,** 41–46.
15. Cho, H. Y. & Rhim, J. S. (1979) *Science* **205,** 691–693.
16. Roberts, A. B., Lamb, L. C., Newton, D. L., Sporn, M. B., De Larco, J. E. & Todaro, G. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3494–3498.
17. Ozanne, B., Fulton, J. & Kaplan, P. L. (1980) *J. Cell. Physiol.*, in press.
18. Sporn, M. B., Newton, D. L., Roberts, A. B., De Larco, J. E. & Todaro, G. J. (1980) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents*, eds. Sartorelli, A. C., Bertino, J. R. & Lazo, J. S. (Academic, New York), in press.
19. Shin, S., Freedman, V. H., Risser, R. & Pollack, R. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4435–4439.
20. Montesano, R., Drevon, C., Kuroki, T., Saint Vincent, L., Handelman, S., Sanford, K. K., DeFeo, D. & Weinsten, I. B. (1977) *J. Natl. Cancer Inst.* **59,** 1651–1656.
21. Nexo, E., Hollenberg, M. D., Figueroa, A. & Pratt, R. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2782–2785.
22. Rinderknecht, E. & Humbel, R. E. (1978) *J. Biol. Chem.* **253,** 2769–2776.
23. Niall, H. D. (1977) in *Peptides: Proceedings of the Fifth American Peptide Symposium*, eds. Goodman, G. & Meienhofer, J. (Halsted, New York), pp. 127–135.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.