Purification and Characterization of a Novel Transforming Growth Factor

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ABSTRACT

Previous studies have indicated that an autostimulatory transforming growth factor was required for the optimal growth of SW-13 adrenal carcinoma cells in soft agar. The production of SW-13 colony-stimulating activity by other human malignant cell lines of both epithelial and mesenchymal origin has been demonstrated. Evidence was presented indicating that the colony-stimulating activity detected in crude acid-ethanol extracts was an acid- and heat-stable polypeptide requiring disulfide bonds for full activity. This activity was detected more frequently in tumors and human cancer cells in culture of epithelial origin than of mesenchymal origin and in a variety of nonneoplastic tissues. In the present study, this activity, termed epithelial transforming growth factor (TGFε) because of its ability to stimulate soft agar growth of certain epithelial cells, was partially purified from bovine kidney. Fourfold purification of the kidney acid-ethanol extract with 50% maximal growth-stimulatory activity of 10 μg was achieved using molecular sieve chromatography where TGFε eluted with an apparent molecular weight of 20,000–25,000. The next purification step, molecular sieve high performance liquid chromatography, yielded a 50% maximal growth-stimulatory activity of 50 μg and an 800-fold purification from the initial acid-ethanol extract. TGFε eluted in the M, 11,000 range. Reverse phase high performance liquid chromatography with a C18 column was then used, yielding a single or double peak of SW-13 colony-stimulating activity at 30–35% acetonitrile. The degree of purification was 11,000-fold with a 50% maximal growth-stimulatory activity of 3.5 ng. Analysis of the peak on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a major and sometimes single band with a molecular weight of 23,000–25,000. Extraction of protein from the polyacrylamide gel demonstrated that only the M, 23,000–25,000 band stimulated soft agar growth of SW-13 cells. The biological activity of the partially purified TGFε was found to differ from other known growth factors with regard to its ability to stimulate soft agar growth of SW-13 cells with the exception of basic fibroblast growth factor (FGF). The acid lability of FGF, the different molecular weights of these two growth factors, the lack of stimulation of soft agar growth of A431 cells, and the lack of binding of TGFε to FGF receptors indicated that TGFε was not related to basic FGF. Partially purified TGFε was also found to stimulate soft agar growth of two squamous cell carcinoma lines, A431 and D562, and the mouse embryo-derived AKR-2B cells. These data suggest that TGFε is a unique factor distinct from those previously described and it is speculated that TGFε may play a role in normal and neoplastic growth of epithelial cells.

INTRODUCTION

TGFs are operationally defined as polypeptides stimulating anchorage-independent growth of nontransformed anchorage-dependent cells through high affinity binding to membrane receptors. TGFα and TGFβ are the two major representatives. TGFα is a M, 5600 single chain polypeptide with 3 intrachain disulfide bonds. It shares 30 to 35% sequence homology with human and rodent EGF (1). TGFα competes with 125I-labeled EGF for binding to the EGF receptor completely and equivalently (2, 3). TGFα activity is similar if not identical to that of EGF. It is a potent mitogen for mesenchymal and epithelial cells in monolayer but only a weak stimulator of anchorage-independent growth in soft agar of mesenchymal cells (4). TGFα acts, like EGF, in synergy with TGFβ in promoting soft agar growth of nontransformed NRK cells (4, 5). Unlike EGF, TGFα has been identified only in embryonic (6) and tumor tissue and transformed cells (1, 4, 5, 7, 8) but not in adult nonneoplastic tissues and organs.

TGFβ is a M, 25,000 polypeptide consisting of two identical 112-amino acid chains held together with disulfide bonds (9–12). Platelets are the major source of TGFβ (9, 13). TGFβ is a ubiquitous growth factor. It is produced by transformed and nontransformed cell lines (14–16), solid human neoplasms (17, 18), nonneoplastic tissues (10–13, 17–19), and mouse embryos (20). The ubiquity and high degree of conservation among species of TGFβ (21) suggest an important role in the control of cell growth and proliferation (22). It acts on many cells, both mesenchymal and epithelial, and its effect differs, depending on the cell type tested (22–24). It stimulates anchorage-independent growth of anchorage-dependent mouse embryo AKR-2B cells (14, 16) and confers transformed morphology on them in a reversible manner (13, 25). TGFβ is very similar to the growth inhibitor purified from African green monkey BSC-1 cells by Holley et al. (26, 27). They both inhibit the growth of epithelial cell lines, bind to the same receptor with the same affinity, and have the same molecular weight of 25,000 (23).

Previous studies have indicated the presence of an apparently novel TGF (18) in a variety of tissues, both neoplastic and nonneoplastic. It stimulated soft agar growth of SW-13 cells, derived from a human small cell carcinoma of the adrenal. Evidence was presented suggesting this TGF was responsible for autocrine stimulation exhibited by these cells. This SW-13-stimulating activity appeared to be an acid- and heat-stable polypeptide requiring disulfide bonds for its activity. It could be separated from TGFβ by molecular sieve chromatography and RP-HPLC. In this paper we report partial purification and further characterization of this novel TGF, called TGFε because of its ability to stimulate soft agar growth of certain epithelial cells.

MATERIALS AND METHODS

Cell Culture. SW-13 (28), HT-1080 (29), JEG-3 (30, 31), AN3 CA (32), and BS-C-1 (33) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). A431, A549, A204, A375 (34), and NRK (clone 49F) (35) cell lines were a gift from Drs. G. J. Todaro and J. E. De Larco. The D562 cell line was provided by Dr. R. E. Scott who purchased it from the ATCC (36). SW620 and SW480 cell lines were given by Dr. R. J. Coffey, Jr., who obtained them from C. Heidelberger (40). All cell lines were maintained in McCoy's Medium 5a (Grand Island Biological Co., Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum. This article must therefore be hereby marked advertisement in all its forms and channels of expression. This work was supported by the National Cancer Institute, Department of Health and Human Services.
bovine serum (Reheis Chemical Co., Phoenix, AZ, or KC Biologicals, Lenexa, KS). All cell lines were used for experiments within 10 to 15 passages of the frozen stock from which they were replaced periodically. Cells were regularly examined after Hoechst 33258 staining to ensure that they stay free of mycoplasmas (41). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Tissue Extraction.** Bovine kidneys were purchased from a local slaughterhouse (Rock Dell, MN), trimmed of fat, cut into 100- to 150-g portions, frozen in liquid nitrogen, and stored at −70°C until used for extraction. A modification (18) of the acid-ethanol procedure reported by Roberts et al. (42) was used.

Soft Agar Colony Stimulation Assay. Soft agar assays were carried out as described previously (18). In some experiments HT-1080 cells were seeded at 5 × 10³, A549 and A204 cells at 2.5 × 10³, and SW620 and SW480 cells at 1.5 × 10³ cells/ml to decrease the height of colonies at higher cell densities. SW-13, A431, and DS62 cells were analyzed at 14 to 21 days; all remaining cell lines were analyzed at 7 to 14 days.

Colonies with a diameter greater than 50 or 60 µm were counted by the Cambridge Quantimet 800 image analysis system (Monsey, NY) or the Bausch & Lomb Feature Analysis Stem Model III (Rochester, NY).

**Hormones and Growth Factors.** The following hormones and growth factors were added to the soft agar assay to test for SW-13 colony-stimulating activity. Sigma Chemical Co. was the source of bombesin and heparin. Basic FGF purified to homogeneity was a generous gift from Dr. D. Gospodarowicz (43). EGF was purified to homogeneity by the method of Savage and Cohen (44) and radiolabeled with ¹²⁵I as described previously (45). TGFα was purified to homogeneity and radiolabeled with ¹²⁵I as described (46). Recombinant DNA produced TGFα was obtained from Genentech, Inc. (South San Francisco, CA). Acridic FGF purified to approximately 90% was purchased from Biomedical Technologies, Inc. (Stoughton, MA).

**Physical and Chemical Treatment.** Aliquots of material purified by RP-HPLC were analyzed for heat stability and sensitivity to a disulfide bond-reducing agent as described previously (18).

**Molecular Sieve Chromatography.** Two hundred mg of protein from the crude acid-ethanol extract from bovine kidney suspended in 15 to 20 ml of 1 M acetic acid were clarified at 1000 x g for 30 min. The supernatant was then applied to a 5- x 87-cm Bio-Gel P-60 (Bio-Rad, Hercules, CA) column equilibrated with 0.004 M HC1 with 1 mg of BSA/ml as a carrier, lyophilized, and used. Aliquots of every second fraction were suspended in 40 nL of 0.004 M HC1 and 2 ml fractions were collected. The effluent was monitored at 254 nm. The flow rate was 1 ml/min for the acetonitrile gradient elution. Three-mi fractions were collected and stored at −20°C until used for extraction. A modification (18) of the acid-ethanol procedure reported by Roberts et al. (42) was used.

Soft Agar Colony Stimulation Assay. A modification of the method described previously by Roberts et al. (11) was used. Samples of material purified by RP-HPLC were applied boiled and nonboiled to SDS-polyacrylamide gel and run as described above. Following electrophoresis, one portion of the gel containing a fraction of the tested sample and molecular weight standards was stained with silver nitrate.

The second portion of the gel was transferred on a glass plate, kept frozen on dry ice, and cut with a scalpel into 3-mm slices. Each slice was crushed and transferred onto glass wool packed tightly in a 3-ml plastic syringe with their tips sealed. The crushed gel slices were extracted for 24 h at room temperature in a small volume (0.5 to 1 ml) of 1 M acetic acid containing 200 µg BSA/ml as a carrier. Eluates were dialyzed for 36 h either in a microdialysis unit (Bethesda Research Laboratories) against three 12-h changes of 4 liters of 1 M acetic acid or in dialysis tubing against three 12-h changes of 4 liters of 1% (v/v) acetic acid, lyophilized, and tested for soft agar-stimulating activity.

**RESULTS**

**Acid-Ethanol Extraction.** Data obtained in previous experiments demonstrated the production by a variety of tissues both malignant and nonneoplastic of a polypeptide growth factor involved in stimulation of SW-13 cells (18). Bovine kidney was selected as a source of TGFα because of the high SW-13-stimulating activity present in nonneoplastic kidney, both human and bovine, and because of its ready availability. Freshly obtained kidneys were trimmed free of fat, cut into 100- to 150-g portions, and stored at −70°C until used. The acid-ethanol procedure of Roberts et al. (42) was used as a first purification step. The yield was 15 to 20 mg protein/g of wet tissue as determined by the dye-binding assay. ED₅₀ was defined as the concentration of protein required to give 50% maximal response measured as SW-13 colony-stimulating activity. The ED₅₀ at this stage of purification was 40 µg protein/ml (Table 1).

**Molecular Sieve Chromatography.** A Bio-Gel P-60 molecular
column was eluted with 1 M acetic acid. Aliquots of 1/40 to 1/500 fraction were used to stimulate soft agar growth of SW-13 cells. 

The ED50 was 20 ng/ml therefore approximately 10-fold degree of purification and 75% recovery of initial biological activity was achieved (Table 1). Lyophilization of material starting with aliquots of 1/400 to 1/500 from a SDS-polyacrylamide gel was used to avoid losses frequently associated with this step. Peak fractions from several RP-HPLC runs were pooled and lyophilized to small volume (stock solution). One unit was measured as ED50 which was determined as the volume of stock solution of TGF e required for 50% maximal stimulation of soft agar growth of SW-13 cells (Fig. 4). A preparation contained 0.5 to 5 ng/μl.

Analysis on and Elution from SDS-Polyacrylamide Gels. When analyzed on 12.5% SDS-polyacrylamide gels under non-reducing conditions, a major protein band with an apparent molecular weight of 23,000 to 25,000 was noted only in fractions containing SW-13 colony-stimulating activity (Fig. 3). To prove that this M, 23,000 to 25,000 polypeptide is responsible for soft agar growth of SW-13 cells, and not one of the other bands observed in some preparations usually in the lower molecular weight range or around 43,000, the activity was eluted from a SDS-polyacrylamide gel. RP-HPLC fractions containing SW-13 colony-stimulating activity were pooled, lyophilized, resuspended in Laemmli sample buffer, and run in two lanes of a 12.5% SDS-polyacrylamide gel. Absorbance (O.D.) K, molecular weight in thousands; C4, carb />vice column was used as the second purification step. Two hundred-mg aliquots of protein from acid-ethanol extracts of bovine kidney were solubilized in 1 M acetic acid and loaded on a Bio-Gel P-60 column equilibrated with 1 M acetic acid. The ED50 was 10 ng/ml therefore approximately 4-fold purification was achieved (Table 1).

To achieve further purification of TGF e, active P-60 fractions were pooled, lyophilized to a volume of 2 ml, applied to a molecular sieve Spherogel TSK SW3000 HPLC column, and eluted with 1 M acetic acid. A single peak of SW-13 soft agar activity emerged with an apparent molecular weight of 11,000 appeared after the elution of majority of protein. This difference in apparent molecular weight between Bio-Gel P-60 and Spherogel column was attributed to the adherence of TGF e to silica-based Spherogel. Aliquots of 1/400 to 1/500 fraction were sufficient to elicit marked soft agar growth activity which was dose dependent (Fig. 2). The protein recovery in the active fractions was somewhere between 5 and 20% of the biological activity were recovered in this peak. The ED50 was 10 μg/ml; therefore approximately 4-fold purification was achieved (Table 1).

Reverse Phase HPLC. RP-HPLC was used as the next purification step. Ten to 16 active fractions from the Spherogel TSK SW3000 column were pooled and further purified by HPLC on a reverse phase C18 (ODS) column using a linear gradient of 20 to 50% acetonitrile in 0.1% TFA. Aliquots of 1/500 of fractions were used in the soft agar growth assay. A single or double peak of SW-13 soft agar activity eluted at approximately 30 to 35% acetonitrile corresponding to a M, 23,000 to 25,000 band when 1/50 to 1/50 of fractions was run on a 12.5% SDS-polyacrylamide gel (Fig. 3). The protein recovery was 5% and the ED50 was 3.5 ng. This represents an 11,000-fold degree of purification and 40% recovery of initial biological activity (Table 1). Lyophilization of material starting with fractions from the Spherogel TSK SW3000 column was omitted to avoid losses frequently associated with this step. Peak fractions from several RP-HPLC runs were pooled and lyophilized to small volume (stock solution). One unit was measured as ED50 which was determined as the volume of stock solution of TGF e required for 50% maximal stimulation of soft agar growth of SW-13 cells (Fig. 4). A preparation contained 0.5 to 5 ng/μl.

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Fig. 3. RP-HPLC of pooled peak fractions from Spherogel TSK SW3000 column. In the final purification step peak fractions from the molecular sieve Spherogel TSK SW3000 column were pooled and loaded directly on an Altex Ultrasphere ODS column (1 x 25 cm). A linear gradient of 20 to 50% acetonitrile containing 0.1% TFA over 75 min at a flow rate of 1 ml/min was used. SW-13 colony-stimulating activity eluted as a single or double peak at 35% acetonitrile. This activity from fraction 20 corresponded to a M, 23,000-25,000 band on a 12.5% SDS-polyacrylamide gel (bottom). Top, typical protein profile for a RP-HPLC run.

Fig. 4. Determination of ED₅₀ of pooled RP-HPLC fractions. RP-HPLC peak fractions from several runs were pooled and lyophilized to small volume (stock solution). ED₅₀ was determined as the volume of stock solution of TGFe required for 50% maximal stimulation of soft agar growth of SW-13 cells. A typical preparation contained 0.5 to 5 ng of TGFe/ml.

Effects of Growth Factors on SW-13 Colony Formation. In order to determine the relationship, if any, of TGFe to other known growth factors, a variety of growth factors was examined for SW-13 colony-stimulating activity in soft agar cultures. Only the preparation of basic FGF (used at concentrations of 0.001-100 ng/ml) from all of the growth factors and hormones tested stimulated soft agar growth of SW-13 cells (Table 3).

EGF (0.1-1000 ng/ml) and TGFe (0.1-30 ng/ml) had no effect on SW-13 cells, and neither did bombesin (0.1-1000 ng/ml). TGFβ (0.01-30 ng/ml) did not stimulate soft agar growth of SW-13 cells (Table 3); in some experiments it decreased the number of SW-13 colonies as compared to the background. Acidic FGF did not stimulate soft agar growth of SW-13 cells (0.01-100 ng/ml) with or without the addition of heparin (10 µg/ml). Heparin inhibited soft agar growth of SW-13 cells regardless of the presence of acidic FGF. Insulin (1-1000 µg/ml), hydrocortisone (10⁻¹⁰⁻¹⁰⁻⁴ M), dexamethasone (1-1000 µg/ml), concanavalin A (1-100 µg/ml), phytohemagglutinin (1-100 µg/ml), lysine-vasopressin (1-100 µg/ml), and multiplication-stimulating activity (1-100 ng/ml) had no effect on SW-13 colony formation in soft agar as reported previously (18).

Comparison with Basic FGF. Pure basic FGF stimulated soft agar growth of SW-13 at concentrations of 1 ng or more per ml. However, the following data indicate that TGFe is distinct from FGF. The ability of FGF to stimulate soft agar growth of SW-13 cells was abolished upon exposure of FGF to pH 1.5 to 2.0 (data not shown), confirming previous data (51) that FGF is acid labile. Unacidified FGF did not stimulate soft agar colony formation of A431 cells even at concentrations of 30 ng/ml (data not shown), whereas TGFe elicited soft agar growth of A431 cells (Fig. 7A). Both growth factors also differ in their molecular weights as determined by SDS-polyacrylamide gel electrophoresis. The molecular weight of TGFe is 23,000 to 25,000 and that of basic FGF is 16,000 (51). Perhaps most important is the fact that partially purified TGFe did not bind to the FGF receptor as determined in the FGF radioreceptor binding assay using BHK-21 cells (43) kindly performed by Dr.

chemical treatment described under "Materials and Methods" of aliquots of TGFe purified by RP-HPLC was performed to compare the nature of the purified material with the properties of impure preparations as described previously (18). Ninety-one and 103% activity was retained by exposure to 56°C for 30 min or to 100°C for 5 min, respectively (Table 2). Trypsin treatment abolished 87% activity, whereas 65% activity was retained with trypsin preincubated with soybean trypsin inhibitor. Dithiothreitol abolished 17% of the activity. Under reducing conditions, the migration of the M, 23,000 band corresponding to SW-13 colony-stimulating activity on a 12.5% SDS-polyacrylamide gel was only slightly retarded (data not shown), thus suggesting that this protein is a single chain. These results indicate that TGFe is indeed a heat-stable single chain polypeptide only partially sensitive to reducing agents (Table 2).
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Fig. 5. Elution of TGFe from SDS-polyacrylamide gel. To prove that the $M_r$, 23,000-25,000 band represents TGFe, a SDS-polyacrylamide gel was cut into 3-mm slices. Each slice was eluted with 1 M acetic acid and tested in soft agar for SW-13 colony-stimulating activity. A single peak of activity was associated with a $M_r$, 23,000 band in this experiment.

Table 2 Effects of physical and chemical treatment of 10-unit aliquots of TGFe on stimulation of SW-13 cell colony formation in soft agar*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF, no dialysis</td>
<td>100</td>
</tr>
<tr>
<td>TGF, dialysis</td>
<td>110</td>
</tr>
<tr>
<td>56°C, 30 min</td>
<td>91</td>
</tr>
<tr>
<td>100°C, 5 min</td>
<td>103</td>
</tr>
<tr>
<td>Trypsin</td>
<td>13</td>
</tr>
<tr>
<td>Trypsin, + soybean trypsin</td>
<td>65</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>83</td>
</tr>
<tr>
<td>No TGF</td>
<td>29</td>
</tr>
</tbody>
</table>

* Mean of 2 assays each performed in duplicate.

Table 3 Stimulation of SW-13 cell colony formation in soft agar by hormones and growth factors

<table>
<thead>
<tr>
<th>Hormone or growth factor</th>
<th>Soft agar colonies (&gt;60 µm diameter)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF (4 units)</td>
<td>599*</td>
</tr>
<tr>
<td>Basic FGF (0.001-10 ng/ml)</td>
<td>869*</td>
</tr>
<tr>
<td>Acidic FGF (0.01-100 ng/ml)</td>
<td>0*</td>
</tr>
<tr>
<td>EGF (0.1-1000 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>TGFB (0.1-30 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>TGFB (0.01-30 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Bombesin (0.1-1000 ng/ml)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Background of 1 to 479 colonies/dish subtracted.
* Positive control run in the same assay with basic FGF.
* At 3.3 ng/ml which gave maximal number of colonies.
* With or without heparin (10 µg/ml).

D. Gospodarowicz. The sensitivity (50 pg of FGF/10 µl) of this assay virtually excludes the presence of significant amounts of basic FGF in preparation of TGFe. These data suggest that TGFe is distinct from FGF and that it binds to a receptor other than the FGF receptor.

125I-labeled TGFB and 125I-labeled EGF Binding Assays. RP-HPLC-purified TGFe was examined in radioreceptor assays for its binding to TGFB and EGF receptors. No competition for the TGFB receptor was obtained when using 0.01 to 30 units of TGFe in several assays, whereas 50% inhibition of specific binding was achieved with 3 ng of pure unlabeled TGFB.

When competition assay for 125I-labeled EGF binding was performed, only weak (33%) binding at high concentrations (30 units) of TGFe was observed and was probably a nonspecific effect. Comparable weak binding to the EGF receptor was obtained with high concentrations of pure TGFB (data not shown). Fifty and 94% competition with 125I-labeled EGF for binding to A431 cells was achieved with 6 and 100 ng of unlabeled EGF, respectively, in the same assay.

The 125I-labeled EGF binding assay was also performed using SW-13 cells rather than A431 cells to determine the level of EGF binding to SW-13 cells. No binding was detected even when using high cell concentrations, such as 2 x 10^6 SW-13 cells/ml. The level of TGFB receptors in SW-13 cells was determined previously to be one-fifth of TGFB receptor level in AKR-2B cells. These results indicate that TGFe is not related to previously described TGFs, α and β or other growth factors and does not bind significantly to either TGFB or EGF receptors and thus raise the possibility that TGFe binds to a yet not described receptor.

Effects of TGFe on SW-13 Cells in Monolayer. Partially purified TGFe was shown to stimulate DNA synthesis in quiescent SW-13 cells. It gave a maximum 2.7- to 3.1-fold increase in [3H]thymidine incorporation at a concentration of 3 to 10 units/ml medium (Fig. 6). A slight increase...
The kinetics showed that the increase was transient, starting to than SO /jm in diameter were counted 7 (for AKR-2B cells) and 14 (for A431 and and stimulated with TGFe at concentrations 0.01 to 10 units/ml. Colonies larger that TGFe may also act as a inilöge n for SW-13 cells grown in

rise at 21 h after the medium was changed, peaking at 24 h,
in [3H]thymidine incorporation was observed at 0.1 unit/ml. The kinetics showed that the increase was transient, starting to rise at 21 h after the medium was changed, peaking at 24 h, and decreasing sharply at 36 h. These preliminary data indicate that TGFe may also act as a mitogen for selected epithelial cell types in addition to SW-13 cells.

DISCUSSION

This paper deals with the purification and initial characteri- zation of a novel transforming growth factor, TGFe. Because TGFe induces anchorage-independent growth of certain cell lines, it fits the operational definition of a transforming growth factor (25). It stimulates soft agar growth of certain epithelial cell lines such as SW-13 cells, derived from a human carcinoma of the adrenal cortex; two human squamous cell carcinoma cell lines, A431 and D562; and mouse embryo AKR-2B cells. Previous studies indicated TGFe to be distributed in a variety of tissues, both neoplastic and nonneoplastic (18). Purification and characterization of TGFe from bovine kidney revealed it to be a single M, 23,000-25,000 polypeptide chain as determined by SDS-polyacrylamide gel electrophoresis. That TGFe is a polypeptide with disulfide bonds can be inferred from its partial sensitivity to reducing agents like dithiothreitol and from its acid and heat stability (10). TGFe probably binds to a membrane receptor distinct from the EGF, TGFB, or basic FGF receptors.

To show that this activity does not represent any of the already characterized growth factors and hormones, purified factors were tested for their SW-13 soft agar-stimulating activity. Of the growth factors tested only basic FGF stimulated soft agar growth of SW-13 cells. However, evidence was gathered indicating that basic FGF and TGFe are two distinct growth factors. The acid lability of basic FGF, the different molecular weights of these two growth factors, the lack of stimulation of soft agar growth of A431 cells by basic FGF, and, perhaps most important of all, the lack of binding of TGFe to the basic FGF receptor strongly indicate that TGFe is a growth factor different from basic FGF.

TGFB, TGFC, EGF, acidic FGF, and bombesin did not have SW-13-stimulating activity. In addition, TGFβ inhibited soft agar growth of SW-13 cells and SW-13 cells did not bind EGF. In view of the lack of detectable EGF receptors and the lack of EGF stimulatory activity in both soft agar and monolayer on SW-13 cells, EGF (and TGFC) is an unlikely candidate for a growth stimulator for these cells. The lack of insulin and multiplication stimulating activity effects on SW-13 cell growth (18) in soft agar strongly indicates that the TGFe is not related to the family of insulin-like growth factors. These findings provide more supporting evidence that SW-13 soft agar activity represents a novel TGF. PDGF was not compared to TGFe for following reasons. Epithelial cells lack PDGF receptors in their membranes (52); one such example is A431 cells (52) which are stimulated by TGFe to grow in soft agar. Under reducing conditions PDGF travels at lower molecular weight in SDS-polyacrylamide gels (52). TGFe is only slightly retarded under

in [3H]thymidine incorporation was observed at 0.1 unit/ml. The kinetics showed that the increase was transient, starting to rise at 21 h after the medium was changed, peaking at 24 h, and decreasing sharply at 36 h. These preliminary data indicate that TGFe may also act as a mitogen for SW-13 cells grown in monolayer.

Effects of EGFe on Anchorage-independent Growth of Various Cell Lines. Several cell lines of both epithelial and mesenchymal origin were examined for their sensitivity to TGFe in soft agar cultures to determine the tissue and cell type specificity, if any, of action of TGFe. Two human malignant epithelial cell lines A431 and D562, both derived from squamous cell carcinomas, required 10 times higher concentration to achieve the same degree of soft agar growth as SW-13 cells (Fig. 7, A and B). Nontransformed mouse embryo AKR-2B cells were moderately responsive to TGFe and could be stimulated to grow in soft agar with the same sensitivity as A431 and D562 cells (Fig. 7C). It is interesting to note that crude acid-ethanol extracts which stimulated soft agar growth of SW-13 did not stimulate soft agar growth of A431 due to the presence of TGFB which is inhibitory for soft agar growth of A431 cells. Two other human malignant epithelial cell lines, A549 (derived from a bronchioloalveolar carcinoma of the lung) and SW620 (derived from an adenocarcinoma of the colon), showed borderline sensitivity to TGFe with only minimal increase in colony number. However, both lines form soft agar colonies even at cell densities lower than 7.5 x 10⁴ cells/ml. All the remaining cell lines tested were not responsive to this TGFe. These included a human colon adenocarcinoma SW480, an endometrial carcinoma AN3 CA, a choriocarcinoma JEG-3, a rhabdomyosarcoma A204, a fibrosarcoma HT-1080, nontransformed mouse embryo C3H/10T½, their transformed counterpart C3H/58MCA, and African green monkey kidney BSC-1 cells. These findings indicated that TGFe may be a mitogen for selected epithelial cell types in addition to SW-13 cells.

Fig. 7. TGFe stimulation of growth in soft agar of several cell lines. A431 (A), D562 (B), and AKR-2B (C) cells were plated in soft agar at 7.5 x 10³ cells/ml and stimulated with TGFe at concentrations 0.01 to 10 units/ml. Colonies larger than 50 μm in diameter were counted 7 (for AKR-2B cells) and 14 (for A431 and D562 cells) days later. Each experiment was performed twice in duplicate.

J. Halper and H. L. Moses, unpublished observations.
those conditions, indicating that it is a single chain polypeptide, and not a dimer like PDGF.

The group of TGFs which includes well defined TGFβ (12, 22) and the less characterized acid- and heat-labile TGFβ2 (53, 54) and other growth factors, such as PDGF (52, 55) and FGF (56), exert their stimulatory effect on mesenchymal cells. Insulin-like growth factor I (57), EGF (58), and TGFα (4) are nonspecific in their action and stimulate growth in monolayer of both mesenchymal and epithelial cells. Both EGF and TGFα enhance soft agar growth of both mesenchymal and epithelial cells (4, 59) again in a nonspecific way.

Two squamous cell carcinoma cell lines, A431 and D562, were sensitive to TGFα. Squamous cell carcinomas in general possess increased numbers of EGF receptors on their surface as compared to other cell types (60, 61). However, the role of EGF or TGFα (which binds to the EGF receptor) in proliferation of these malignancies is not well understood. Reports on the effect of EGF on A431 cells have been contradictory thus far. Inhibition of growth (61, 62) and stimulation of proliferation with very low concentrations of TGFα63 have been reported. Stimulation of soft agar growth of A431 and D562 cells with TGFα suggests that TGFα may play an important role in growth and proliferation of squamous cell carcinomas. Both A431 and D562 cells were one order of magnitude less sensitive to TGFα than SW-13 cells. The presence of very low concentrations of another growth factor stimulating A431 and D562 copurifying with TGFα on RP-HPLC cannot be excluded. However, this possibility seems unlikely because of relatively low concentrations of TGFα required for their soft agar growth. That different cells exhibit different degrees of sensitivity to various growth factors has been documented in the case of TGFβ (22) and EGF (61–63).

Future studies are necessary to characterize TGFα in more detail. Development of a radioreceptor assay and characterization of its receptor would enable determination of which cells are responsive to this TGF and which cells produce it. The primary amino acid sequence is necessary in order to determine the degree of relatedness or the lack thereof between TGFα and other growth factors. It is likely that other growth factors necessary for the growth of epithelial cells are yet to be discovered.

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