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Characterization and Partial Purification of Human Epithelial Transforming Growth Factor

Damien J. Dunnington, Robert G. Scott, Mario A. Anzano, and Russell Greig

Department of Cell Sciences, L-109, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-2799

A polypeptide growth factor has been partially purified from medium conditioned by the human adrenocortical carcinoma cell line SW13. This factor, designated h-TGFe, stimulates anchorage-independent growth of the SW13 cells. Similar activity was observed in human milk, and in conditioned media from seven of 14 epithelial cell lines. The SW13-derived activity is stable to low pH and 8M urea but labile to dithiothreitol and 2% sodium dodecyl sulfate. Human TGFe does not bind to heparin and fails to stimulate growth of endothelial cells in monolayer culture. The apparent molecular weight of h-TGFe is 59k by size exclusion chromatography in the presence of 8M urea and the activity binds strongly to cation exchangers. The activity elutes at 15–30% acetonitrile from a C18 reverse-phase column and has been partially purified by using a four-step chromatographic procedure. TGFe appears to be a novel growth factor produced by many epithelial cells and tissues.

Key words: adrenocortical carcinoma cells, protein purification, anchorage independent growth, epithelial cell proliferation

Proliferation of epithelial cells is affected by a number of polypeptide growth factors including transforming growth factors (TGF) type alpha [1] and beta [2], fibroblast growth factor [3], and an apparently novel protein known as TGFe [4]. Production of TGFe appears to be restricted to epithelial cells and tissues [5] and is measured by quantitation of colony formation in soft agar by the adrenocortical carcinoma cell line SW13 [5]. A form of TGFe present in acid ethanol extracts of bovine kidney has been extensively characterized [4,6] but its amino acid sequence has yet to be determined. This material has a molecular weight of 22,000–25,000 and appears to be a single-chain protein requiring intrachain disulfide bonds for full activity [4]. Bovine TGFe has been discriminated from previously described growth factors by molecular weight, receptor binding studies, and physical and biological properties [4].

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In contrast to bovine TGFe, relatively little is known about the activity produced by human cells and tissues. Acid-ethanol extracts of human carcinoma tissues contained acid- and heat-stable polypeptides with molecular weights of 20,000–22,000 that were active in the SW13 soft agar assay [5]. The same report described growth-stimulating activity in medium conditioned by SW13 cells, implying the presence of an autocrine loop in these cells [5]. However, the properties of the SW13-secreted factor were not examined. In the present report, we describe partial purification of the SW13-derived material and its relationship to bovine TGFe.

MATERIALS AND METHODS

Cell Cultures and Growth Factors

All cell lines were obtained from the American Type Culture Collection and were free from mycoplasma contamination as determined by the Gen Probe assay (Gen Probe, San Diego, CA 92123) and broth culture (Microbiological Associates). Isoenzyme analysis demonstrated the human origin of SW13 cells (Corning Authenti-kit). Bovine basic fibroblast growth factor (bFGF) was supplied by Collaborative Research.

Collection of Conditioned Media

Cells were grown to confluence in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, in roller bottles. The cells were washed with phosphate-buffered saline (PBS), incubated in serum-free DMEM for 4–5 h, and again washed with PBS. Each roller bottle received 200 ml of serum-free, phenol red-free RPMI 1640 medium (Gibco) supplemented with 10 mg/L freshly prepared ferrous sulfate and 5 ng/ml sodium selenite (Collaborative Research). Cells were incubated with serum-free medium for 2 days and medium was acidified with 5 ml/L trifluoroacetic acid (TFA) and stored at 4°C. The acidified conditioned medium was centrifuged at 1,500*g* for 15 min and filtered through a 0.45 μm membrane (Gelman), and biological activity was determined after concentration by ultrafiltration (Amicon YM5 membrane, 5,000 mw cutoff) and dialysis against 0.05% TFA. The following cell lines were surveyed for secretion of activity: SW48, SKC01, LOVO, CACO 2, HT-29, COLO 320DM, COLO 320HSR, SW948, DLD-1, HCT-15, WIDR, SW403, and SW742 (all derived from colon carcinomas) and NRK-52E (rat kidney epithelial cells) and SW13.

Biological Assays

TGFe activity was measured by using growth of SW13 cells in soft agar as described [5] except that the base and upper layers contained 0.5% and 0.3% agarose respectively in DMEM, and 10% fetal bovine serum, and 5,000 cells were plated per 3 cm dish.

Growth of fetal bovine heart endothelial (FBHE) cells in monolayer culture [7] was used to measure FGF activity. Samples were added to 3 cm dishes containing 2 ml DMEM, 10% fetal bovine serum. Each dish then received 100 μl of a suspension of 5×10^4 FBHE cells in DMEM, 10% serum. Cultures were incubated for 5 days, fixed with 10% buffered formalin, and stained with 0.5% crystal violet. Cell growth was quantitated by measurement of the total colony areas for each dish by image analysis.

Analysis of TGFe Activity in Human Milk

Human milk pooled from ten donors was delipidated by three successive extractions with an equal volume of diethyl ether at 4°C followed by centrifugation at 12,000*g* for 20 min. The supernatant was acidified with TFA (0.5% final concentration) and centrifuged at 12,000*g* for 20 min before being filtered through a 0.45 μm membrane and concentrated five-fold by using an Amicon YM5 membrane. The concentrate was dialyzed against 0.05% TFA, lyophilized, reconstituted at 10 × the original concentration, and bioassayed on SW13 cells.

Characterization of SW13-Derived TGFe

The affinity of TGFe activity for heparin was determined by passage of crude conditioned medium reconstituted in 50 mM NaH_2PO_4, 0.25 M NaCl pH 7.0 through a 0.5 × 3 cm column packed with 1 gm of heparin-Sepharose conjugate (Pharmacia). Samples of the starting material and the column effluent and material eluted with 2M NaCl, 50 mM NaH_2PO_4 were bioassayed on SW13 cells. As a control, 50 ng/ml bFGF in the same buffer was passed through the same column and samples were bioassayed by using FBHE cells. Conditioned medium was bioassayed against FBHE cells and on NIH-3T3 cells by using the thymidine uptake assay [8] with platelet-derived growth factor as positive control.

The sensitivity of h-TGFe activity to denaturing agents was tested by incubating crude conditioned medium concentrate with either 2% sodium dodecyl sulfate, 0.065 M dithiothreitol, or 8M urea for 1 h. The treated samples along with an untreated control were exhaustively dialyzed against 0.05% TFA to remove the denaturing agent and bioassayed on SW13 cells.

The molecular weight of h-TGFe was estimated by gel filtration chromatography of conditioned medium concentrate on a BioGel P60 column in the presence of 8M urea. Lyophilized concentrate was dissolved in 20 ml 8M urea in 0.05% TFA and applied to a 5 × 86 cm column (Pharmacia) containing BioGel P60 resin (BioRad). The flow rate was 36 ml/h, 20 ml fractions were collected, and 100 μl aliquots were dialyzed against 0.05% TFA to remove urea and bioassayed. Molecular weight standards were ovalbumin (43k), chymotrypsinogen (25k), and ribonuclease A (13.7k).

Purification of TGFe

TGFe was purified from SW13-conditioned medium by preparative reverse-phase, size exclusion, and cation exchange HPLC. A 25 L batch of acidified SW13-conditioned medium was loaded at 75 ml/min onto a 5 × 30 cm preparative column containing Vydac 218TP C18 packing, particle size 15–20 μm, by using a Waters Delta-Prep system. The column was washed with 0.05% TFA until the absorbance at 214 nm reached baseline and a gradient of 0.05% TFA in acetonitrile was run at 0–30% over 50 min, 30–50% over 20 min, and 50–100% over 10 min at a flow rate of 70 ml/min. Fractions were collected every 2.5 min, acetonitrile was removed by vacuum evaporation, and aliquots were bioassayed on SW13 cells.

Active fractions from the reverse-phase step were concentrated 40× by using Amicon YM5 membranes and concentrates were loaded onto two series connected TSK 3000 SW size exclusion columns (each 2.5 × 60 cm). Material was eluted from the columns with 0.05% TFA at a flow rate of 2 ml/min and 4 ml fractions were collected.

Sodium phosphate was added to the active material from the size exclusion step to a final concentration of 20 mM. The pH was adjusted to 6.0 with sodium hydroxide and the samples were centrifuged at 90,000*g* for 30 min. Supernatants were loaded onto a CM2SW cation exchange column (Beckman) and washed with 20 mM phosphate buffer pH 6.0. A linear gradient of 0–0.6M sodium chloride in phosphate buffer pH 6.0 was run at 0–95% over 90 min and 2 ml fractions were collected and bioassayed.

An active sample of TGFe from the cation exchange step was analyzed by reverse-phase HPLC. The sample was acidified to pH 2.5 with 1% TFA and loaded onto a 0.46 × 30 cm column containing Vydac C18 218TP 10 μm packing. A gradient of 0–50% 0.05% TFA in acetonitrile was run at 0.25%/min and 2 ml fractions were collected and bioassayed after removal of the acetonitrile by vacuum evaporation. Aliquots of fractions 30–40 were run on a 15% polyacrylamide gel in the presence of 2-mercaptoethanol and examined by silver staining (BioRad). Recovery of activity during purification was monitored by constructing dose-response curves using the purified material. One unit of activity was defined as the dose of test sample needed to give a response equal to an ED_{50} of a standard preparation of concentrated SW13-conditioned medium run in the same assay. Protein concentration was measured by the BCA method (Pierce), or by electronic integration of absorbance peaks at 214 nm by using ovalbumin as a standard.

RESULTS

Presence of TGFe Activity in Human Tissues, Human Milk, and Cell Supernatants

TGFe-like activity was detected in human milk and SW13, SW403, LOVO, NRK-52E, WIDR, and HCT-15 cells released activity into their respective media (Table I) Smaller but detectable amounts were produced by SKC01 and SW48 cells, while DLD-1, HT-29, SW948, COLO 320 (DM or HSR), CACO 2, and SW742 failed to release observable amounts of TGFe. Activity was also detected in unacidified conditioned media from SW13 and LOVO cultures, suggesting that acid activation is not essential for the release of TGFe activity in contrast to TGFβ [9].

TABLE I. TGFe Activity in Media Conditioned by Epithelial Cell Lines*

Cell line	Colonies > 20 μm	Cell line	Colonies > 20 μm
SW48	95	NRK-52E	598
SKC01	70	HCT15	280
LOVO	840	WIDR	360
CACO2	20	SW403	650
HT-29	18	COLO320 HSR	20
COLO320 DM	12	SW742	35
SW948	15	Control[a]	15
DLD-1	18	SW13	370

*Conditioned media were collected from each cell line as described (Materials and Methods), dialyzed against 0.05% TFA, reconstituted at 10× original concentration, and assayed at 100 μl/dish on SW13 cells in soft agar. Human milk extract gave 392 colonies in the same assay.

[a]Control was medium incubated in the absence of cells. Background colony formation in this assay ranged from 0 to 20 colonies (mean of 20 observations = 6 colonies).

Discrimination of SW13-Derived h-TGFe From Fibroblast Growth Factor

When SW13-conditioned medium was concentrated 50-fold by ultrafiltration and dialyzed against 0.05% TFA, the resulting material produced a dose-related increase in colony formation by SW13 cells in the soft agar assay (Fig. 1). The ED_{50} of this crude concentrate was 29 μg protein/ml, giving a specific activity of 34.5 half-maximal units per mg protein. Since basic fibroblast growth factor (bFGF) is also known to stimulate colony formation in soft agar by SW13 cells [4,5], we examined the heparin affinity of h-TGFe and its ability to stimulate growth of endothelial cells and 3T3 fibroblasts, both of which respond to bFGF.

A sample of SW13-conditioned medium concentrate was passed through a heparin-Sepharose column. As shown in Figure 2A, most of the activity was recovered in the column breakthrough fraction, indicating that it had little affinity for heparin. In contrast, bFGF applied to the same column was completely retained, as expected from the known affinity of FGF-like growth factors for heparin [9]. The biological activity of h-TGFe could also be separated from that of bFGF. When an aliquot of h-TGFe from the reverse-phase purification step (see below) was assayed on FBHE cells, no stimulation of proliferation was observed (Fig. 2B), in contrast to an eight- to nine-fold increase in cell growth produced by bFGF. When bFGF was assayed on FBHE cells in the presence of TGFe this proliferative response was not diminished, indicating that the absence of effect of h-TGFe alone was not due to contamination of the samples with inhibitors or toxins. Furthermore, partially purified h-TGFe did not stimulate thymidine uptake in quiescent 3T3 cells in contrast to bFGF (not shown).

Effects of Denaturing Agents on h-TGFe Activity

Crude conditioned medium was treated with 0.065M dithiothreitol, 2% sodium dodecyl sulfate, or 8M urea. After removal of the denaturing agent by dialysis, bioassay

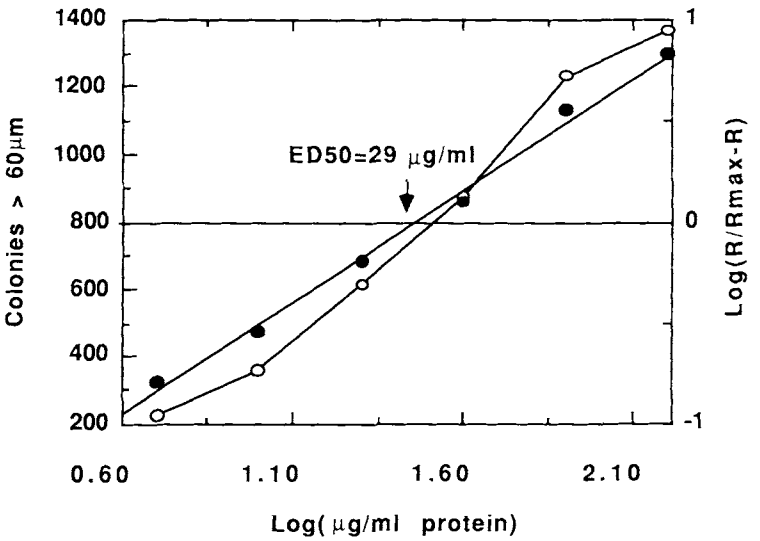

Fig. 1. Dose-response of SW13-conditioned medium concentrated 50-fold, on SW13 cells. The number of colonies >60 μm (○—○) was measured by image analysis. Hill transformation (●—●) of the data gave an ED_{50} of 29 μg protein/ml (one unit).

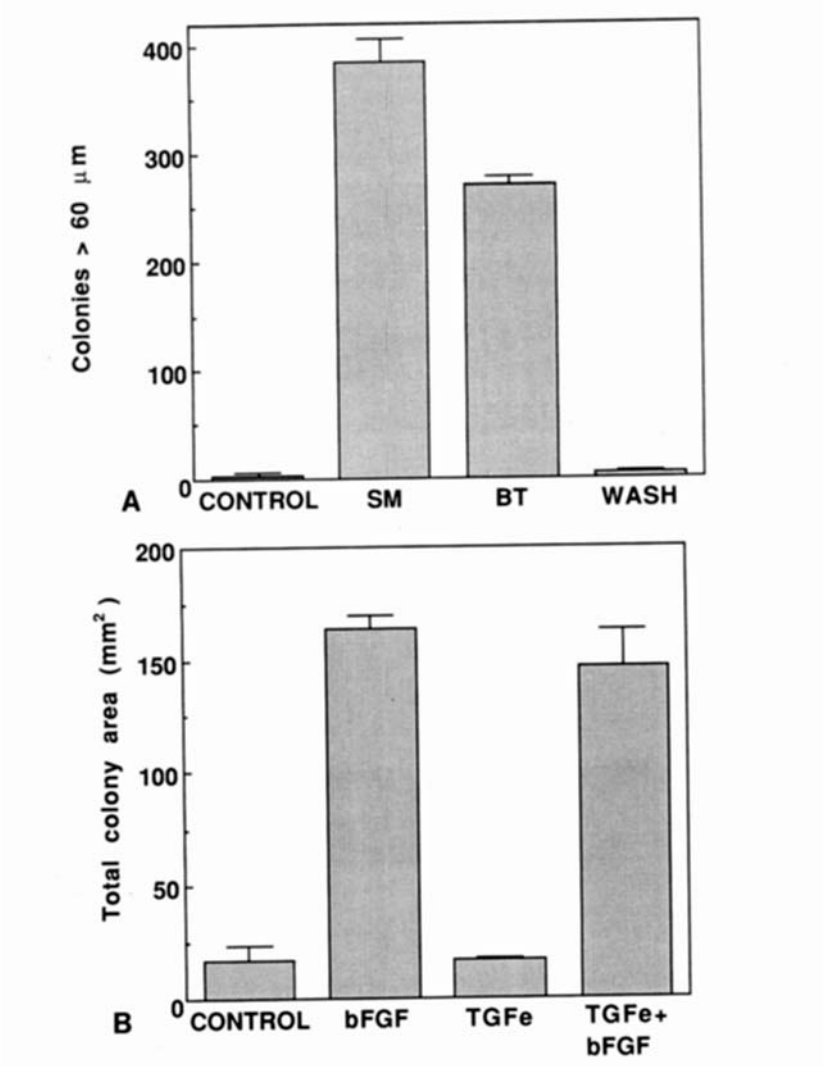

Fig. 2. Discrimination of h-TGFe from bFGF. **A:** Conditioned medium from SW13 cells was concentrated by ultrafiltration, dialyzed, and lyophilized. After reconstitution in 0.1M NaH_2PO_4, 0.25M NaCl pH 7 the sample was loaded onto a heparin-Sepharose column. Samples of the buffer (control), starting material (SM), column breakthrough (BT), and 2M NaCl eluate (wash) were assayed on SW13 cells. **B:** Assay of TGFe on FBHE cells. Cultures of FBHE cells were incubated with phosphate-buffered saline (control), 50 ng/ml bFGF, 2 units/ml TGFe, or 50 ng/ml FGF and 2 units/ml TGFe. After 5 days, colonies were stained with crystal violet and their areas measured by image analysis. Bars represent the range of duplicates.

indicated that the activity was stable to 8M urea but labile to dithiothreitol or sodium dodecyl sulfate (Table II). In addition, the activity was labile to quaternary ammonium salts such as tetrabutylammonium phosphate (not shown).

Purification of h-TGFe

A 25 L batch of SW13-conditioned medium was loaded onto a preparative C18 column and eluted with an acetonitrile gradient. Two peaks of activity were observed

TABLE II. h-TGFe Activity in the Presence of Denaturing Agents*

Dose (μl/dish)	Control[a]	2% $NaDodSO_4$[b]	0.065M DTT	8M urea
50	18	5	4	16
100	71	5	4	39
200	150	3	21	166

*SW13 conditioned medium was concentrated 10-fold and treated with the indicated concentrations of denaturing agents for 1 h at room temperature. Samples were exhaustively dialyzed against 0.05% TFA and assayed at the indicated dose. Figures are the number of colonies > 40 μm in diameter.
[a]Untreated conditioned medium concentrate.
[b]Detergent was removed by dialysis against 8M urea followed by 0.05% TFA.

in the resulting fractions: Peak I eluted at 25–30% acetonitrile and Peak II at 35–40% (Fig. 3). Fractions 17–20 (Peak I) were pooled, concentrated, and applied to a size exclusion column. Activity eluted at an apparent molecular weight of 59k (Fig. 4). Further purification was accomplished by HPLC on a cation exchange column. Activity eluted at 0.3M NaCl (Fig. 5) and was subsequently run on an analytical reverse-phase column. TGFe eluted as a single activity peak at approximately 15% acetonitrile (Fig. 6). This peak was well separated from the majority of contaminating protein as judged by the UV absorbance profile, and analysis by gel electrophoresis and silver staining (Fig. 7).

Fractionation of the Peak II material (fractions 23–26 in Fig. 3) on a Bio Gel P60 column in the presence of 8M urea yielded a peak of activity at an apparent molecular weight of 59k as seen with the Peak I material.

The purification statistics (Table III) show excellent recovery of activity from SW13-conditioned medium by preparative reverse phase HPLC. Over 70% of the total activity was distributed in roughly equal amounts between Peaks I and II. Recovery was 45% of total Peak I activity after the size exclusion step and 29% after cation exchange HPLC. This latter step resulted in greater than 100-fold purification of the material from the size exclusion column. Insufficient material was present after the analytical reverse phase step to determine recovery or degree of purification at this stage.

DISCUSSION

Previous studies by Halper and Moses [5] showed that SW13 cells secrete an autostimulatory growth factor and that similar activity was present in extracts of human colon carcinoma tissue. Our findings confirm the production of active material by SW13 cells and demonstrate similar activity in media conditioned by six of 13 colon carcinoma cell lines, a rat kidney epithelial cell line, and in human milk. We have also detected activity in extracts of human colon carcinoma tissue (not shown). Thus production of TGFe-like activity is not restricted to the SW13 cell line. These findings suggest an association of TGFe activity with epithelial cells and tissues, but we do not yet know whether production of, or response to, TGFe is epithelial-specific.

Bovine TGFe has been distinguished from bFGF by its biochemical and biological properties and by its inability to bind to bFGF receptors [4]. In the present study, human TGFe did not bind to heparin and was ineffective in promoting growth of endothelial cells or 3T3 fibroblasts in contrast to bFGF which has a high affinity for heparin [10] and readily stimulates proliferation of these cell types. Biochemical

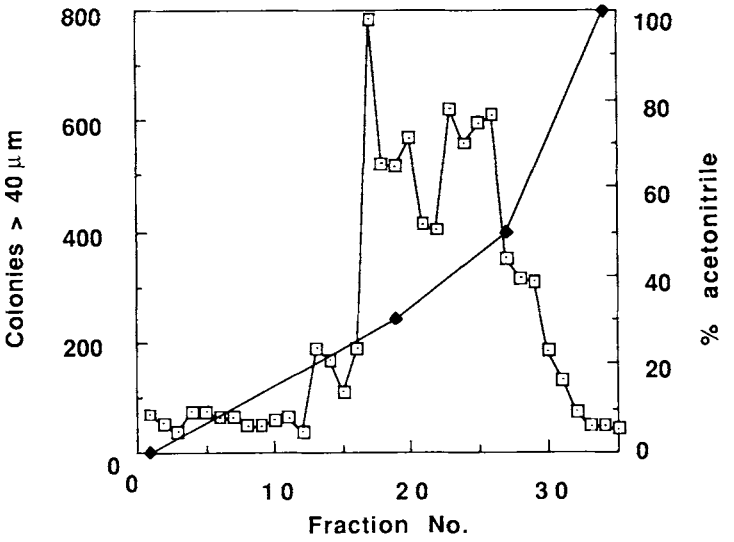

Fig. 3. Preparative reverse-phase HPLC of SW13-conditioned medium. A 25 L batch of conditioned medium was acidified, filtered, and loaded onto a preparative C18 column (see text). The column was washed with 0.05% TFA and material was eluted with a gradient of 0.05% TFA in acetonitrile as shown (◆). Aliquots of each fraction were assayed on SW13 cells (dotted square).

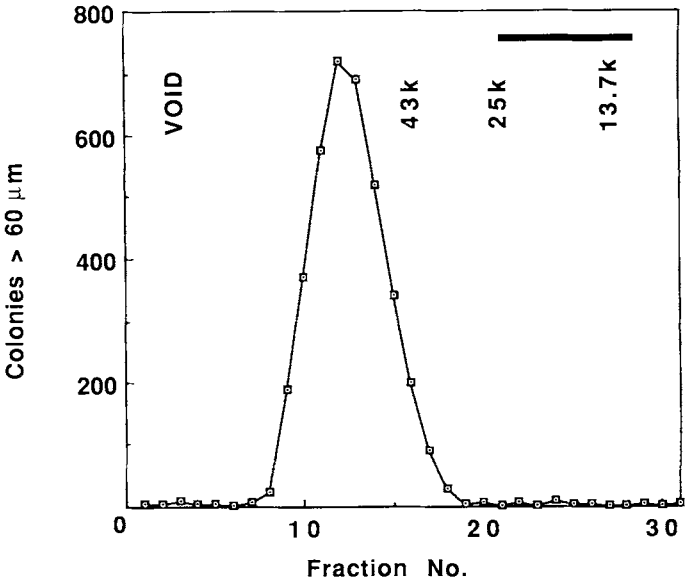

Fig. 4. Chromatography of h-TGFe on BioGel P60 in the presence of 8M urea. Fractions 17–20 from the preparative reverse-phase step were pooled, lyophilized, and reconstituted in 0.05% TFA, 8M urea. The sample was applied to a column of BioGel P60 resin equilibrated and eluted with 0.05% TFA, 8M urea. Aliquots of each fraction were dialyzed against 0.05% TFA and assayed on SW13 cells (dotted square). The bar shows the elution profile of TGFe from bovine kidney purified as in (11) and run under the same conditions. Markers were blue dextran (void), ovalbumin (43k), chymotrypsinogen (25k), and ribonuclease A (13.7k).

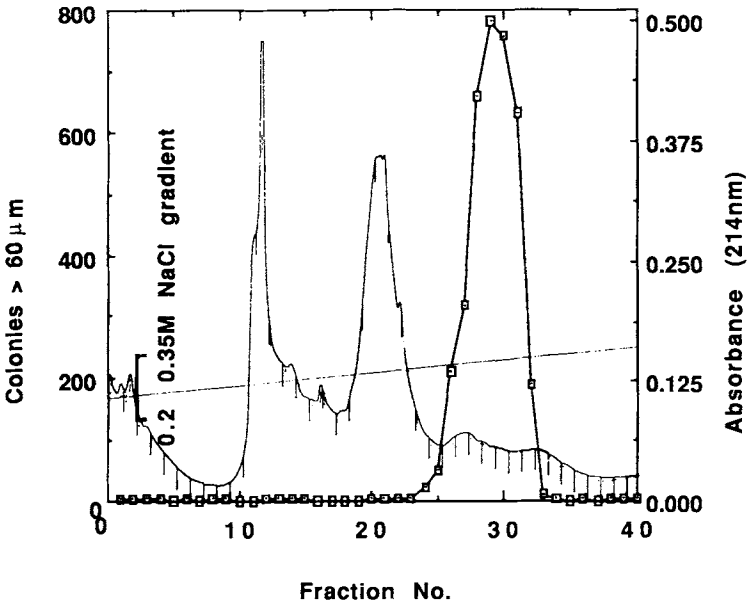

Fig. 5. Cation exchange HPLC of h-TGFe. Fractions 17–20 (Fig. 3) from the preparative C18 column were further fractionated by size exclusion chromatography and the active material from this step was reconstituted in 0.025M NaH_2PO_4 buffer pH 6.0 and loaded onto a CM2SW cation exchange column. Material was eluted with a gradient of 0–0.5M NaCl in 0.025M NaH_2PO_4 pH 6.0. Aliquots of each fraction were bioassayed on SW13 cells (dotted square).

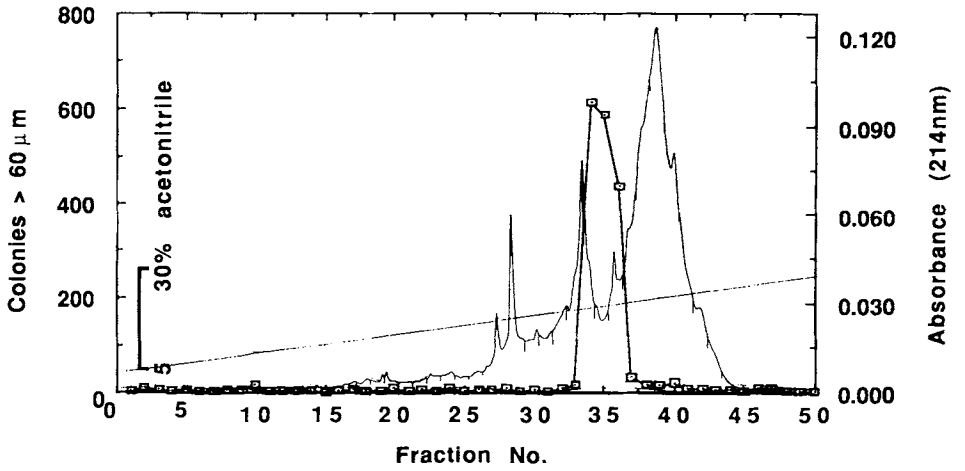

Fig. 6. Analytical reverse phase HPLC of h-TGFe. Fractions 28–31 from the cation exchange step (Fig. 5) were loaded onto a 25 × 0.46 cm C18 column. Material was eluted with a 5–30% acetonitrile gradient and TGFe activity was identified by bioassay on SW13 cells (dotted square).

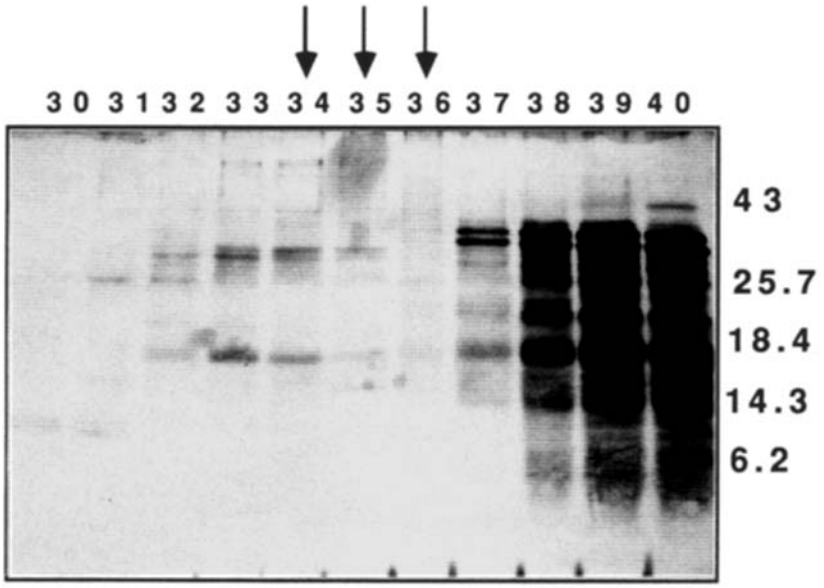

Fig. 7. Gel electrophoresis and silver staining of HPLC fractions. One hundred microliter aliquots of fractions 30–40 (Fig. 6) were run on a 15% polyacrylamide gel under reducing conditions and stained with silver reagents. Arrows indicate the fractions containing biologically active material. Molecular weight markers were ovalbumin (43k), chymotrypsinogen (25.7k), lactoglobulin (18.4k), lysozyme (14.3k), and trypsin inhibitor (6.2k).

TABLE III. TGFe Purification Statistics

Sample	Volume (mL)	Activity (U/mL)	Total activity	mg/ml protein	SP act. (U/mg)	Fold purification	% recovery
Orig CM	25,000	1.4	34,500	0.04	34.5	—	100
Peak I	500	22.2	11,100	0.19	116.8	3.4	32.2
Peak II	400	33.2	13,280	0.71	46.8	1.4	38.5
TSK 3000[a]	205	24.2	43,966	0.14	172.8	5.0	14.4
CM2SW[a]	60	54.0	3,244	0.003	18,018	522	9.4

[a]Peak I material was used for the subsequent purification steps.

characterization of h-TGFe indicates that it is stable to low pH and 8M urea but labile to sodium dodecylsulfate and reducing agents. The molecular mass estimated by gel permeation chromatography under denaturing conditions is 59 kD. This figure differs from the values reported by Halper and Moses both for bovine TGFe [4] and for the activity in acid ethanol extracts of human tissues [5]. In our hands, the bovine form of TGFe, when run under similar conditions, elutes from a BioGel P60 column as a broad peak close to the 25k marker [11]. These findings suggest that h-TGFe differs in size from its bovine counterpart. As expected from the lability of h-TGFe to sodium dodecyl sulfate, activity could not be recovered from slices of polyacrylamide gels, under conditions where bovine TGFe remained active, as seen previously [4]. However, this lability of h-TGFe further suggests that differences exist between bovine and human TGFe's.

We have undertaken preliminary purification of h-TGFe from 25 L of SW13-conditioned medium by using reverse phase, size exclusion, and cation exchange HPLC. Although two peaks of activity were recovered from the preparative C18 column, it is likely that the second (more hydrophobic) peak is an artifact of the HPLC process, since upon treatment with 8M urea and fractionation on a BioGel P60 column, it co-elutes with the Peak I activity. This result suggests that the Peak II material is an aggregate of h-TGFe with unidentified proteins. A similar phenomenon was reported for bovine TGFe [4]. The behavior of h-TGFe on a carboxymethyl cation exchange column indicated that h-TGFe is a highly basic protein. These results suggest that cation exchange and reverse phase methods will be useful in the purification of h-TGFe. Although analysis of reverse phase HPLC fractions by gel electrophoresis and silver staining showed good separation of activity from major contaminating proteins, it did not permit identification of a specific band that could be correlated with SW13 colony formation. In our hands, bovine TGFe does not readily stain with silver reagents and it is possible that h-TGFe may share this property.

In summary, we have characterized and partially purified h-TGFe from SW13 cells. This material differs from bovine kidney-derived TGFe both in behavior on size exclusion columns under denaturing conditions and stability towards ionic detergents. Further studies will be necessary to elucidate the structural basis of these differences.

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