Human Transforming Growth Factor

PRODUCTION BY A MELANOMA CELL LINE, PURIFICATION, AND INITIAL CHARACTERIZATION*

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A low molecular weight human transforming growth factor (hTGFs) was isolated from serum-free medium conditioned by a human metastatic melanoma tumor line, A2058. The purification of hTGFs was achieved by gel permeation chromatography on a column of Bio-Gel P-10 in 1 M acetic acid, followed by reverse phase high pressure liquid chromatography on a column of phenylboronapak C8 support using a linear gradient of acetonitrile in 0.045% trifluoroacetic acid, and subsequently by rechromatography of the fractions containing activity on a column of phenylboronapak C8. The estimated molecular weight of hTGFs is 7400. It is a single chain polypeptide with three intrachain disulfide bridges and a molecular weight of 7000. Lacking tyrosine and methionine, but containing three phenylalanine residues, hTGFs is unlike human and mouse epidermal growth factor (EGF). hTGFs competes with 125I-labeled EGF for binding to A431 human carcinoma cells completely and equivalently, and thus is functionally related to EGF. In contrast, hTGFs enabled normal anchorage-dependent rat kidney cells to grow in soft agar, whereas EGF did not stimulate growth of these cells in semisolid medium. The half-maximal growth-stimulating response of hTGFs (50% growth stimulation) was reached with one EGF-competing unit or 1.1 ng of hTGFs.

The anchorage-independent growth of normal and tumor cells in culture is regulated in part by a family of growth factors, designated transforming growth factors. TGFs is functionally related to EGF, confers the transformed phenotype on normal fibroblasts in vitro, and thus can tentatively be considered a proximal effector of cell transformation. The production of TGF appears to be a general expression of the transformed phenotype and is common to tumor cells of different origins. Several polypeptides with the properties of TGF have been partially purified from the conditioned medium of Moloney murine sarcoma virus-infected mouse 3T3 cells, and termed sarcoma growth factors (1), from RNA tumor virus-transformed rat fibroblasts (2), and from certain human tumor cells (3). An intracellular form(s) of TGF has also been detected in tumor cells growing both in culture and in the animal (4). We describe in this report the purification of a small molecular weight human TGF that is produced by the human metastatic melanoma cell line, A2058, and some chemical and biological properties of the homogeneous polypeptide growth factor in order to define the functional and structural relationships that exist among the various TGF polypeptides form different species and different cell types.

EXPERIMENTAL PROCEDURES

Source of hTGFs

hTGFs was purified from the serum-free medium conditioned by a human metastatic melanoma line A2058 (3) derived from a brain metastasis in a 45-year-old male. Cells were grown in serum-free Waymouth's medium (Grand Island Biological Co., 430-2100), supplemented with 10% calf serum (Colorado Serum Co.) at 37 °C. The cells were washed for 1 h with 50 ml of serum-free Waymouth's medium (Grand Island Biological Co., MD 7051/1) (5). This and a second collection of supernatant fluid, 24 h later, were discarded. Subsequent collections were made every other day, or every 3rd day, for a 2-week period.

The medium was collected by decantation, stored for up to 24 h at 4 °C in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (1 µg/ml), and clarified by continuous flow centrifugation at 32,000 rpm at 4 °C. Flow rates of 5 liters/h in the CF-32 continuous flow rotor (Beckman) in the model L5-50 ultracentrifuge (Beckman) were used. The supernatant, after high speed centrifugation, will be referred to as A2058-conditioned medium.

The A2058-conditioned medium was immediately concentrated in the hollow fiber Dialyzer/Concentrator (model DC10) and held at 4 °C. The concentrated hTGF was then purified on a column of Bio-Gel P-10 (10 ml bed volume) and eluted with 1 M acetic acid at 22 °C. Samples of protein (65-115 µg) in 1 M acetic acid (5 ml) were applied to the column. To ensure a constant flow rate, the column effluent was regulated at 12 ml/h with a peristaltic pump. 4.8-ml fractions were collected. Aliquots were lyophilized for subsequent determinations of EGF-competing activity and growth-promoting activity in soft agar. Fractions representing the major portions of a given peak were pooled and concentrated by lyophilization.

Purification of hTGFs

Dialysis and Centrifugation—The combined retentate and cartridge wash after ultrafiltration of A2058-conditioned medium was dialyzed for 60 h against 0.1 M acetic acid in Spectrapor 3 dialysis tubing (Spectrum Medical Industries). The retentate was centrifuged at 100,000 × at 1 h at 4 °C. The pellet was discarded. The supernatant was concentrated by lyophilization and reconstituted in 0.5 ml of 1 M acetic acid/liter of original A2058-conditioned medium.

Chromatography on Bio-Gel P-10—Following concentration, dialysis, and centrifugation, the supernatant containing hTGF activity was further purified by gel permeation chromatography on a column (25.5 × 85 cm) (420 ml bed volume) of Bio-Gel P-10 (200-400 mesh, Bio-Rad Laboratories). The column was equilibrated with 1 M acetic acid at 22 °C. Samples of protein (65-115 µg) in 1 M acetic acid (5 ml) were applied to the column. To ensure a constant flow rate, the column effluent was regulated at 12 ml/h with a peristaltic pump. 4.8-ml fractions were collected. Aliquots were lyophilized for subsequent determinations of EGF-competing activity and growth-promoting activity in soft agar. Fractions representing the major portions of a given peak were pooled and concentrated by lyophilization.

Reverse Phase High Pressure Liquid Chromatography—The final purification of hTGF was achieved by reverse phase HPLC. As previously published (6). All separations were performed on a column.
C, column (10-μm particle size, 0.39 × 30 cm, Waters Associates) at a flow rate of 1 ml/min at 40 °C. Lyophilized samples were reconstituted in 0.05% (v/v) trifluoroacetic acid in water, adjusted to pH 2 with 10% (v/v) trifluoroacetic acid, and applied through the sample injector to the column which was equilibrated with 0.05% trifluoroacetic acid. The column effluent was collected in 1.5-ml fractions. Aliquots were lyophilized for subsequent EGF competition and growth stimulation assays. Pools of fractions comprising the major hTGFs activity were concentrated by lyophilization.

hTGFs-containing pools were reconstituted in 0.05% trifluoroacetic acid and rechromatographed on the same column, previously equilibrated with 0.05% trifluoroacetic acid in water. The column was then eluted with a linear acetonitrile gradient in 0.05% trifluoroacetic acid. The column effluent was collected in 1.5-ml fractions. Aliquots were lyophilized for EGF competition and growth stimulation assays.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described (7). A 15-30% acrylamide gradient slab (140 × 120 × 0.75 mm) was prepared with a 4% stacking gel. The gels were run at 30 V with an electrode buffer containing Teks (0.05 M), glycine (0.38 M), and SDS (0.1%, w/v) until the tracking dye (bromphenol blue) had run off the end of the gel. After electrophoresis, gels were fixed in 50% methanol, 10% acetic acid for 2 h, washed in 5% methanol, 7% acetic acid overnight, and stained with silver (8).

Protein Determination—Total protein was determined (9) using bovine serum albumin as a standard. Prior to protein determination, the starting material was dialyzed against phosphate-buffered saline to remove components of the culture medium interfering with the color reaction or lyophilized if samples had been dissolved in volatile acids. Protein was also determined by amino acid analysis. Lyophilized samples were hydrolyzed at 110 °C for 24 h in evacuated Pyrex tubes with 0.1 ml of 6 n HCl containing 0.1% liquid phenol, and analyzed with a Durrum D-500 analyzer equipped with a PDP 8/A computing integrator using o-phthalaldehyde for the fluorogenic detection of primary amines (10).

Radioreceptor Assay

Purified EGF (11) was labeled with Na125I by a modification of the chloramine-T method (12) as described (1). The 125I-EGF binding assay was performed on confluent monolayers of formalin-fixed A431 (13) human carcinoma cells as previously described (14). The fixed cells were washed twice with 0.5 ml of binding buffer (Dulbecco’s modified Eagle’s medium containing 1 mg/ml of bovine serum albumin and 50 mm 2-(bis(2-hydroxyethyl)amino)ethanesulfonic acid, pH 6.8). Competitions were initiated by the addition of 0.2 ml of binding buffer containing 0.4 ng of 125I-EGF with or without potential inhibitor. After incubation for 1 h at 22 °C, the specifically bound 125I-EGF was determined. The TGF content was expressed by its degree of inhibition of the binding of 125I-EGF to the EGF receptor. One EGF-competing activity unit is defined as the amount of protein that inhibits the binding of 125I-EGF to its receptor by 50%.

Soft Agar Growth Assay

The assay for colony growth in soft agar, using normal rat kidney fibroblasts, clone 49F (15), was performed as reported (3). Lyophilized samples to be tested were reconstituted in 0.5 ml of Dulbecco’s modified Eagle’s medium, supplemented with 10% calf serum. 1.5 ml of 0.5% (w/v) agar (Difco) in the supplemented medium and 0.5 ml of supplemented medium containing 2.3 × 10² cells were added. 2.3 ml of the resultant mixture were pipetted on a 2-ml base layer (0.5% agar in supplemented medium) in 60-mm Petri dishes (Falcon). The cells were incubated at 37 °C in a humidified 5% CO₂/95% air atmosphere. The assay was read unfixed and unstained at 5 days and at 10–14 days.

RESULTS

Source, Concentration, and Initial Fractionation of hTGFs—hTGFs was isolated from serum-free conditioned medium of the highly transformed human metastatic melanoma cell line, A2058. The quantitation of hTGFs was based on two of its properties: the capacity to induce anchorage-independent growth of normal rat kidney fibroblasts in soft agar, and the ability to compete with 125I-EGF for the EGF receptor sites on A431 human carcinoma cells. A summary of the steps leading to the isolation of hTGFs and its recovery is presented in Table I.

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Gel permeation chromatography of A2058-conditioned medium on Bio-Gel P-10 (420-ml bed volume). Elution pattern of 6.97 mg of protein (acid-soluble protein from 9.2 liters of A2058-conditioned medium). The elution was performed with 1 ml of 0.1 M acetic acid at 22 °C at a flow rate of 12 ml/h. 4.8-ml fractions were collected. Aliquots of the indicated fractions were lyophilized and assayed for: 1) EGF-competing activity with 100-μl aliquots (□—□); 2) soft agar growth with 200-μl aliquots (○—○). The solid line gives the protein absorbance at 280 nm. The following proteins and peptides were used to construct standard plots of log molecular weight versus elution volume: bovine pancreatic ribonuclease A (RNAase A), Mr = 13,700; lima bean trypsin inhibitor (T. Inh.), Mr = 8,400; bovine pancreatic insulin (insulin), Mr = 5,700. The elution volumes of the standard peptides are indicated.

**TABLE I**

| Purification step | Protein recovered mg | EGF-competing activity recovered units | Relative specific activity units/mg | Degree of purification fold | Recovery % |
|------------------|----------------------|----------------------------------------|-------------------------------------|-----------------------------|------------|
| 1. A2058-conditioned medium | 1,020 | 4,525 | 4.4 | 1 | 100 |
| 2. Acid-soluble supernatant | 837 | 4,299 | 5.1 | 1 | 95 |
| 3. Bio-Gel P-10 | | | | | |
| Pool P-10-A | 28.7 | 2,077 | 70 | 16 (1) | 45.9 (100) |
| Pool P-10-B | 14.5 | 2,033 | 140 | 32 (1) | 44.9 (100) |
| 4. Bondapak C₆ (acetonitrile) | 0.202 | 1,698 | 8,059 | 1,832 (E7) | 56.0 (80.1) |
| 5. Bondapak C₆ (1-propanol) | 0.0015 | 1,476 | 984,000 | 223,636 (6,988) | 32.6 (72.6) |

* Total protein was determined using bovine serum albumin as a standard. The quantitation of step 5 hTGFs was based on amino acid analysis. The absolute specific activity of a companion aliquot was found to be 1-1.5 × 10⁶ units/mg (see text).

* One EGF-competing activity unit is defined as the amount of protein that inhibits the binding of 125I-EGF to its receptor by 50%.
To remove serum proteins, A2058 cells were extensively washed with Waymouth's medium prior to their culture in serum-free medium. The supernatant fluids were collected every other day for a 2-week period. Culture conditions were such that at the end of the culture period more than 90% of the cells were still viable and attached as monolayers. The initial clarified A2058-conditioned medium of 136 liters, containing 1.02 g of total protein and 4525 units of EGF-competing activity, was concentrated to about 900 ml using a hollow fiber concentrator with cartridges of 5000 molecular weight cutoff. The total EGF-competing activity was retained and a recovery above 95% was obtained.

Dialysis of the concentrated A2058-conditioned medium against acetic acid and subsequent centrifugation resulted in 95% recovery of the initial total EGF-competing activity. 18% of the protein was acid-insoluble and was discarded. The acid-

![Graph](http://www.jbc.org)
soluble, partially purified hTGF was subjected to gel permeation chromatography on Bio-Gel P-10. The column was eluted with 1 M acetic acid. A representative chromatogram is illustrated in Fig. 1. The bulk of the contaminating protein was eluted in the exclusion volume of the column and was well separated from the EGF-competing activity and growth-stimulating activity. Two peaks of activity were found to be well resolved from each other. Fractions with both EGF-competing and growth-stimulating activity (P-10-A and P-10-B) had apparent molecular weights of 10,500 and 6,800, respectively. Fractions having only one of the two activities were not observed. hTGF-containing fractions were pooled as indicated, lyophilized, and further purified. The larger molecular weight TGF eluted from the column in a broad peak (P-10-A) and represented 45% of the initial total EGF-competing activity. The cumulative yield of total input EGF-competing activity from step 1 through the gel permeation chromatography step was 91% (Table I). hTGF was eluted as two distinct major peaks that varied quantitatively from one preparation to another. In some preparations of A2058-conditioned medium essentially all the growth-promoting activity was in the hTGF region.

**Purification of hTGFs—**hTGFs was further purified by reverse phase HPLC. Pool P-10-B, after gel permeation chromatography of the acid-soluble EGF-competing activity of concentrated A2058-conditioned medium on Bio-Gel P-10, was reconstituted in 0.05% trifluoroacetic acid in water, and then chromatographed on a µBondapak C18 column. A typical elution pattern is illustrated in Fig. 2A. EGF-competing and growth-stimulating activities in soft agar of individual fractions were determined. hTGFs was well separated from the bulk of contaminating protein which eluted at higher concentrations of organic solvent (not shown). Fractions indicated with a bar were pooled, lyophilized, and taken for rechromatography. A 57-fold purification of hTGFs after gel permeation chromatography was obtained. 80% of the initial EGF-competing activity in pool P-10-B was recovered (Table I).

Rechromatography of the hTGFs-containing fractions on µBondapak C18 support was chosen for the final purification step since only relatively small losses of EGF-competing activity were observed on these columns. In order to obtain a distinct separation of hTGFs from impurities, it was necessary to use a shallow linear 1-propanol gradient in 0.035% trifluoroacetic acid. Fig. 2B shows that the bulk of contaminating peptide material was separated from a well-defined peak of activity. EGF-competing and growth-stimulating activities copurified with a distinct absorbance peak at 13% 1-propanol. Fractions indicated with a bar were pooled and further analyzed. The purification of hTGFs was approximately 7000-fold after gel permeation chromatography with a yield of 33% of the initial total EGF-competing activity. The overall recovery of hTGFs from step 3 through the final reverse phase HPLC was 25%.

### Table II

**Amino acid compositions of human TGFs and human EGF (20)**

| Amino acid   | hTGFs | hEGF |
|--------------|-------|------|
| Residues/mol |       |      |
| Aspartic acid| 5.1   | 7    |
| Threonine    | 2.9   | 0    |
| Serine       | 5.8   | 3    |
| Glutamic acid| 9.8   | 5    |
| Proline      | ND    | 1    |
| Glycine      | 7.1   | 4    |
| Alanine      | 5.2   | 2    |
| Half-cystine | 5.8   | 6    |
| Valine       | 6.0   | 3    |
| Methionine   | 0     | 1    |
| Isoleucine   | 0.9   | 1    |
| Leucine      | 3.8   | 5    |
| Tyrosine     | 0     | 5    |
| Phenylalanine| 2.8   | 0    |
| Histidine    | 4.1   | 2    |
| Lysine       | 3.9   | 2    |
| Arginine     | 3.9   | 3    |
| Tryptophan   | ND    | 2    |

Values are based on two aliquots (350 ng each) hydrolyzed for 24 h and calculated by assuming Mₙ = 7400.

* Residues per peptide determined by amino acid sequence analysis.

**Fig. 3. Dose-response curves of EGF and hTGFs.** A, inhibition of ¹²⁵I-EGF binding to the EGF receptor of formalin-fixed A431 human carcinoma cells by EGF (O—O) and by hTGFs (•—•). Cells, 8 x 10⁶/dish, were incubated with ¹²⁵I-EGF (0.4 ng/dish; 74 µCi/μg) and unlabeled peptides at the indicated concentrations. The ¹²⁵I-EGF bound was determined, and nonspecific binding (binding in the presence of 2 μg of EGF) was 2.9% and has been subtracted. One hundred percent specific binding corresponds to 15.1% of the input radioactivity. B, soft agar growth assay as a function of protein concentration. Normal rat kidney fibroblasts, clone 49F, 1 x 10⁶ cells/dish were incubated with increasing amounts of hTGFs (•—•) and EGF (O—O) in 2.3 ml of 0.3% agar in Dulbecco’s modified Eagle’s medium, supplemented with 10% calf serum, on a 2-ml base layer of 0.5% (w/v) agar in the same medium. The number of colonies (>10 cells) were scored at 2 weeks and are presented as percentage of maximum effect. One hundred per cent maximum response corresponds to 440 colonies/4 fields. No colonies, in the absence of hTGFs, have been seen on control plates.
step was 73%, and the recovery range per step was 80–100% (Table I).

Characterization of hTGFs.—The purity of the final hTGFs preparation was determined by analytical SDS-polyacrylamide gel electrophoresis as shown in Fig. 2C. The gel was stained with silver. One major polypeptide band, with an apparent \( M_r = 7400 \), was observed. The same pattern was obtained when samples were electrophoresed under nonreducing conditions.

The amino acid composition of human melanoma cell-derived hTGFs is shown in Table II. The closest approximation to integral values for all residues was obtained by assuming that hTGFs has a \( M_r = 7400 \). A \( M_r = 7350 \) was calculated for the peptide chain of hTGFs on the basis of its amino acid composition. The contribution of tryptophan and proline to the size of the peptide chain was not taken into account. No free sulfhydryl groups could be found by S-amidomethylation with iod[1-\(^{14}\)C]acetamide in 6 m guanidine-HCl without prior reduction. A comparison of the amino acid compositions of hTGFs, with that of human EGF does not suggest any similarities (Table II).

The receptor reactivity of hTGFs was compared with EGF in the radio-receptor assay. The quantitation of hTGFs was based on amino acid analysis of a companion aliquot. Both hTGFs and EGF competed with \(^{125}\)I-EGF for the EGF receptor sites of A431 human carcinoma cells as shown in Fig. 3A. The specific EGF-competing activity of hTGFs was found to be 1–1.5 \( \times 10^4 \) units/mg; 1.1 ng of hTGFs or EGF were required to inhibit EGF binding by 50%.

hTGFs enabled normal anchorage-dependent rat kidney cells, clone 49F, to grow in soft agar. The half-maximal response of hTGFs in soft agar was reached with 1 EGF-competing unit, or 1.1 ng of hTGFs, whereas EGF does not stimulate growth of these cells in soft agar even when tested with up to 10 \( \mu \)g (Fig. 3B).

DISCUSSION

In the present study, a low molecular weight growth factor produced by the human metastatic melanoma cell line, A2058, and released into serum-free conditioned medium, was purified to apparent homogeneity and partially characterized. The polypeptide has been operationally defined as a member of the TGF family because of its ability to induce transformation of normal fibroblasts, as measured by growth stimulation in soft agar. hTGFs was quantitated by utilizing one of its properties, to compete with \(^{125}\)I-EGF for binding to receptors of normal fibroblasts, as measured by growth stimulation in soft agar. hTGFs was isolated from the acid-soluble fraction of A2058-conditioned medium and purified by gel permeation chromatography and reverse phase HPLC in volatile solvents at acid pH. Gel permeation chromatography produced a broad distribution of hTGFs activity (P-10-A) with an average apparent \( M_r = 10,500 \), and a narrow distribution of hTGFs activity (P-10-B) with an apparent \( M_r = 6,800 \) (Fig. 1), and resulted in excellent recoveries (91%).

Preliminary analysis of hTGFs by reverse phase HPLC indicated heterogeneity which was in marked contrast to the results obtained with hTGFs. The increase of its specific EGF-competing activity did not follow that of hTGFs. The slopes of the competition curves were similar, although hTGFs was, on a weight basis, less than 5% as active as EGF. The half-maximal response of hTGFs in soft agar was reached with approximately 120 EGF-competing units (data not shown). Thus, the biological effects of hTGFs are qualitatively similar, although quantitatively different to those described for hTGFs. Despite the existence of multiple molecular weight species, hTGFs represented the major growth-stimulating activity of A2058-conditioned medium and was further purified.

Further purification of hTGFs by reverse phase HPLC on \( \mu \)Bondapak C\(_{18}\) support increased the specific EGF-competing activity about 7000-fold, with little loss of growth-stimulating activity. High recoveries (80–100%) of hTGFs activity were obtained in each chromatographic step. The two-step purification of hTGFs was achieved by using sequentially acetonitrile and 1-propanol as the mobile phase and trifluoroacetic acid as the ionic modifier in the organic solvent for the elution of peptides. Previous studies (16–18), using binary solvent systems consisting of trifluoroacetic acid and a limiting organic solvent, have clearly shown the high resolution of large molecular weight polypeptides by reverse phase HPLC.

The purity of the final hTGFs preparation was established by SDS-polyacrylamide gel electrophoresis. Only one major single band, with an apparent \( M_r = 7400 \), was observed. Reduction with \( \beta \)-mercaptoethanol did not change the mobility of hTGFs, which is evidence for a single chain polypeptide. The lack of free sulfhydryl groups and the presence of six half-cystine residues suggests three disulfide bridges. Reduction of the disulfide bridges completely abolished the ability of hTGFs to compete with EGF for binding to receptors on A431 human carcinoma cells and to stimulate the growth of normal rat kidney cells in soft agar.

The estimated molecular weight of hTGFs, as determined by gel permeation chromatography and SDS-polyacrylamide gel electrophoresis, is in agreement with the calculated molecular weight from amino acid compositional data. The amino acid composition of hTGFs is unique and shows a number of important differences when compared with the amino acid compositions of either human or mouse EGF (19, 20). Both human and mouse EGF have one methionine, whereas hTGFs lacks methionine. Human and mouse EGF have five tyrosine and no phenylalanine residues. In contrast, hTGFs has no tyrosine, but three phenylalanine residues. Although hTGFs has a higher apparent molecular weight than EGF, and thus could represent a pro-EGF (21) or a pre-pro form of EGF, the amino acid compositional data of hTGFs do not support this possibility. hTGFs is a low hydrophobic polypeptide than EGF, in agreement with its lower retention time relative to EGF on \( \mu \)Bondapak C\(_{18}\) columns.  

hTGFs showed remarkable competing activity in a radio-receptor assay developed for EGF, and completely displaced \(^{125}\)I-EGF binding to the EGF receptor of A431 human carcinoma cells. Approximately equimolar concentrations of hTGFs and EGF were required to inhibit EGF binding by 50%, suggesting a close structural relationship between the receptor binding sites on the human TGFs and mouse EGF polypeptides. hTGFs enabled normal anchorage-dependent rat kidney cells to grow in soft agar, whereas EGF did not stimulate growth of these cells in semisolid medium. Polycationic concentrations of hTGFs were required for a measurable effect on specific target cells. Despite the biochemical differences between EGF and hTGFs, both are able to interact specifically with the EGF receptor and bring about its phosphorylation of tyrosine residues (22). Both hTGFs and EGF increase the overall levels of phosphotyrosine-containing proteins, a property they share with a number of different transforming proteins of RNA tumor viruses (23). Primary struc-
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...structure analysis of hTGFs should help to elucidate the receptor binding site common with EGF and to define the structural relationship that exists among functionally indistinguishable TGF polypeptides from different species and different cell types.

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