Transforming Growth Factors from a Human Tumor Cell: Characterization of Transforming Growth Factor β and Identification of High Molecular Weight Transforming Growth Factor α

Linda L. Dart,* Diane M. Smith, Chester A. Meyers,‡ Michael B. Sporn, and Charles A. Frolik§

Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT: Intracellular transforming growth factors (TGFs) were extracted from a human rhabdomyosarcoma cell line and purified to apparent homogeneity by using gel filtration, cation-exchange, and high-performance liquid chromatography. Two types of transforming growth factor activities, TGF-α and TGF-β, were detected. The intracellular polypeptides which belonged to the TGF-α family required TGF-β for full activity in inducing nonneoplastic normal rat kidney fibroblasts to grow in soft agar. These peptides also bound to the membrane receptor for epidermal growth factor. As determined by sodium dodecyl sulfate–polyacrylamide gels, the apparent molecular weight of these intracellular TGF-α's was 18 000. Intracellular TGF-β required either epidermal growth factor or TGF-α for stimulation of soft agar growth. The intracellular TGF-β was purified to homogeneity as judged by a single peak after reverse-phase high-performance liquid chromatography and a single band on a sodium dodecyl sulfate–polyacrylamide gel. The intracellular TGF-β from the human tumor cell line was similar in all respects tested (migration on sodium dodecyl sulfate–polyacrylamide gels, stimulation of soft agar growth, binding to the membrane receptor for TGF-β, and amino acid composition) to intracellular TGF-β from normal human placenta.

Transforming growth factors (TGFs),[1] low molecular weight acid-stable polypeptides that are able to confer a transformed phenotype to normal rat kidney (NRK) fibroblasts, were originally identified in the conditioned medium from murine sarcoma virus transformed cells (De Larco & Todaro, 1978). Later these growth factors were also shown to be present in certain human tumor cells (Todaro et al., 1980), in chemically transformed cells (Roberts et al., 1980; Moses et al., 1981), and also in nonneoplastic tissues (Roberts et al., 1981, 1982a). TGFs have been operationally classified as type α or β depending on their relationship with epidermal growth factor (EGF) in NRK cells (Frolik et al., 1982a; Anzano et al., 1982; Roberts et al., 1982b, 1983c). Type α TGFs compete with EGF for binding to the EGF receptor while type β TGFs do not compete. Type β TGFs require EGF or a TGF-α for the induction of growth in soft agar.

Recently, TGF-α's have been purified to homogeneity from the conditioned medium of a human metastatic melanoma cell (Marquardt & Todaro, 1982) and from the medium of several virus-transformed rat and mouse cell lines (Twardzik et al., 1982a; Marquardt et al., 1983, 1984; Massague, 1983). These growth factors are single-chain polypeptides with molecular weights of 5600, are structurally and functionally highly conserved among different species and cell types (Marquardt et al., 1983), and have significant sequence homology with EGF (Marquardt et al., 1983, 1984). TGF-β has been purified from normal human placenta (Frolik et al., 1983), from platelets (Assoian et al., 1983), from bovine kidney (Roberts et al., 1983a), and from feline sarcoma virus transformed rat cells (Massague, 1984). These TGFs are composed of two polypeptide chains held together by disulfide bridges. The TGF-β's from the three different nonneoplastic tissues are structurally very closely related as shown by chromatographic behavior on HPLC, migration rates on $NaDodSO_4$–polyacrylamide gels, and amino acid composition (Sporn et al., 1984). In this paper, the purification to homogeneity of a type β TGF from human tumor cells is described. In addition, several intracellular TGF-α's were detected with apparent molecular weights on $NaDodSO_4$–polyacrylamide gels approximately 2.5 times larger than that reported for previously characterized extracellular TGF-α's.

Experimental Procedures

Extraction. Human rhabdomyosarcoma cells (A673) were grown at the Frederick Cancer Research Facility. In a typical preparation, 1 kg of cells was extracted as previously described (Roberts et al., 1980) except that frozen cells were thawed in 4 mL/g of a solution consisting of 3189 mL of 95% ethanol, 770 mL of distilled water, and 66 mL of concentrated HCl plus 210 mg of phenylmethanesulfonyl fluoride and 12 mg of pepstatin A. The mixture was homogenized in a Waring blender, stirred overnight at 4 °C, and then centrifuged. The pH of the supernatant was adjusted to 5.3 with NH_4OH, protein was precipitated by the addition of 8 L of cold ethanol and 16 L of cold ether, and the mixture was allowed to stand overnight at 4 °C. The precipitate was collected by filtration, dissolved in 1 M acetic acid (1 mL/g of cells), and stirred overnight at 4 °C. Insoluble material was removed by centrifugation, and the supernatant was frozen and lyophilized to dryness. The residue [7 mg/g (wet weight) of cells] was stored at −20 °C.

Gel Filtration Chromatography. The lyophilized extract (45.7 g) from 6.5 kg of A673 cells was dissolved in 1 M acetic

* Correspondence should be addressed to this author.

‡ Present address: Smith Kline & French Laboratories, Philadelphia, PA 19101.

§ Present address: Eli Lilly Research Laboratories, Indianapolis, IN 46285.

[1] Abbreviations: EGF, epidermal growth factor; NRK, normal rat kidney; $NaDodSO_4$, sodium dodecyl sulfate; TGF, transforming growth factor; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

acid (1 mL/50 mg of residue). The entire sample was applied to a column (35.6 × 90 cm) of Bio-Gel P-30 (100–200 mesh, Bio-Rad) and chromatographed as previously described (Frolik et al., 1983). Fractions (850 mL) were collected, and aliquots of the even-numbered fractions were assayed for their ability to stimulate growth of NRK cells in soft agar with and without the addition of EGF. The assays indicated two areas of interest against a background of small colony formation (data not shown). One area (pool 1) contained the fractions showing inhibition of colony formation in soft agar. Pool 2 contained the fractions which eluted after the inhibitory peak and which displayed TGF activity in the presence of EGF.

After lyophilization, both pool 1 (7.2 g) and pool 2 (4.0 g) were separately chromatographed on Bio-Gel P-6 columns (10 × 91 cm). Protein was eluted from each column with 1 M acetic acid (95 mL/h), and 24-mL fractions were collected. Again, aliquots of even-numbered fractions were assayed for TGF activity with and without the addition of EGF. Fractions from both P-6 columns which showed activity in soft agar without the addition of EGF were combined into pool A and lyophilized. Fractions which showed activity in soft agar only with the addition of EGF were combined into pool B and lyophilized.

Cation-Exchange Chromatography. Pool A (2.76 g of protein) and pool B (1.1 g of protein) were dissolved separately in 70 mL of 0.01 M acetic acid. The pH was adjusted to 4.5 with 1 N NaOH and the conductivity to 1.2–1.5 mS/cm. Each pool was then applied to a cation-exchange column (CM-Trisacryl M) and run as previously described (Frolik et al., 1983). Aliquots from every other fraction were removed for determination of TGF-α and TGF-β activity. The fractions from pool A, which showed TGF-α activity, and the fractions from pool B, which showed TGF-β activity, were combined separately for further analysis.

Reverse-Phase HPLC. The sample from the ion-exchange column which contained TGF-β activity was adjusted to 10% (v/v) acetonitrile/0.1% trifluoroacetic acid (pH 2) and then pumped onto a μBondapak C_{18} HPLC column (10-μm particle size, 0.78 × 30 cm; Waters Associates) equilibrated with acetonitrile/water/trifluoroacetic acid, 10:90:0.1 (pH 2). After the sample had been washed onto the column with 50 mL of the initial solvent, the column was eluted (1.2 mL/min) with a 70-min linear gradient from 30:70:0.1 to 37:63:0.1 acetonitrile/water/trifluoroacetic acid (pH 2). A total of 72 fractions (1.2 mL/fraction) was collected. Aliquots were removed for assay of TGF-β activity. The peak fractions were combined and partially evaporated under nitrogen and then directly applied to a μBondapak CN column (10-μm particle size, 0.38 × 30 cm; Waters Associates) equilibrated with 30:70:0.1 1-propanol/water/trifluoroacetic acid. The column was eluted (1.1 mL/min) with a 153-min linear gradient from 30:70:0.1 to 45:55:0.1 1-propanol/water/trifluoroacetic acid (pH 2). A total of 65 fractions was collected, and aliquots were removed for bioassay, amino acid analysis, and gel electrophoresis.

The pool from the ion-exchange column which contained the TGF-α activity was adjusted to 10% (v/v) acetonitrile/0.05% trifluoroacetic acid, and the pH was adjusted to 2 with HCl. This was then pumped onto a μBondapak C_{18} HPLC column (10-μm particle size, 0.78 × 30 cm; Waters Associates) equilibrated with acetonitrile/water/trifluoroacetic acid, 10:90:0.05 (pH 2). The sample was washed onto the column with 90 mL of initial solvent and then eluted (1.2 mL/min) with a 110-min linear gradient from 18:82:0.05 to 28:72:0.05 acetonitrile/water/trifluoroacetic acid (pH 2). A total of 87 fractions (1.8 mL/fraction) was collected, and aliquots were removed for assay of TGF-α activity. The peak fractions were combined and partially evaporated under nitrogen. An equal volume of 0.035% trifluoroacetic acid (pH 2) was added to the sample and applied to the same C_{18} column equilibrated with 0.035% trifluoroacetic acid (pH 2). The column was eluted (1.1 mL/min) by using a 180-min linear gradient from 0.035% trifluoroacetic acid to 20:80:0.035 1-propanol/water/trifluoroacetic acid (pH 2). A total of 80 fractions (1.7 mL/fraction) was collected, and aliquots were removed for receptor binding assay. The fractions which showed activity were combined and partially evaporated under nitrogen. An equal volume of initial solvent, 10:90:0.035 1-propanol/water/trifluoroacetic acid (pH 2), was added to the sample before being loaded onto a μBondapak CN column (10-μm particle size, 0.38 × 30 cm; Waters Associates). The column was eluted (1.1 mL/min) by using a 200-min linear gradient from 10:90:0.035 to 50:50:0.035 1-propanol/water/trifluoroacetic acid (pH 2). A total of 130 fractions (1.7 mL/fraction) was collected and assayed for activity. Fractions showing the peak of TGF-α activity were combined and partially evaporated under nitrogen. An equal volume of initial solvent, 10:90:0.035 1-propanol/water/trifluoroacetic acid (pH 2), was added to the sample. The sample was then applied to the same CN column. The column was eluted (1.1 mL/min) by using a 24-min linear gradient from 10:90:0.035 to 22:78:0.035 1-propanol/water/trifluoroacetic acid (pH 2) followed by a 180-min linear gradient from 22:78:0.035 to 40:60:0.035 1-propanol/water/trifluoroacetic acid (pH 2). A total of 120 fractions (1.7 mL/fraction) was collected, and aliquots were assayed for activity. Fractions containing the peak of activity were combined and partially evaporated under nitrogen. An equal volume of initial solvent, 10:90:0.035 acetonitrile/water/trifluoroacetic acid (pH 2), was added to the sample prior to application to a Synchropak RP-P column (10 mm × 25 cm; SynChrom, Inc.). The column was eluted by using a 5-min linear gradient from 10:90:0.035 to 15:85:0.035 acetonitrile/water/trifluoroacetic acid (pH 2) followed by a 400-min linear gradient from 15:85:0.035 to 55:45:0.035 acetonitrile/water/trifluoroacetic acid (pH 2). A total of 100 fractions (1.7 mL/fraction) were collected and aliquots removed for bioassay and gel electrophoresis.

Soft Agar Assay. Soft agar colony-forming activity was determined as described (Roberts et al., 1980) except that the cells were stained at the end of 1 week in assay and the numbers and sizes of the colonies were determined by using a Bausch and Lomb Omnicon image analysis system. Assays were run either with the addition of murine EGF (2 ng/mL) to measure TGF-β activity or with the addition of human placental TGF-β (0.2 ng/mL) to measure TGF-α activity.

^{125}I-EGF and ^{125}I-TGF-β Binding Assays. Column fractions were assayed for their ability to compete with murine ^{125}I-EGF or placental ^{125}I-TGF-β for binding to NRK cells in a radioreceptor assay. Human placental TGF-β was obtained as described previously (Frolik et al., 1983) and iodinated by using a modified chloramine-T method (Frolik et al., 1984). NRK cells were seeded in 24-well plates at 1×10^5 cells mL^{-1} well^{-1} in Dulbecco's modified Eagle's medium containing 10% calf serum (Gibco) and 50 μg/mL gentamycin (Gibco) and incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were washed 2 times with binding buffer (Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and 25 mM Hepes, pH 7.4) prior to the addition of 200 μL of binding buffer containing either ^{125}I-EGF or ^{125}I-TGF-β and an aliquot of the column fraction

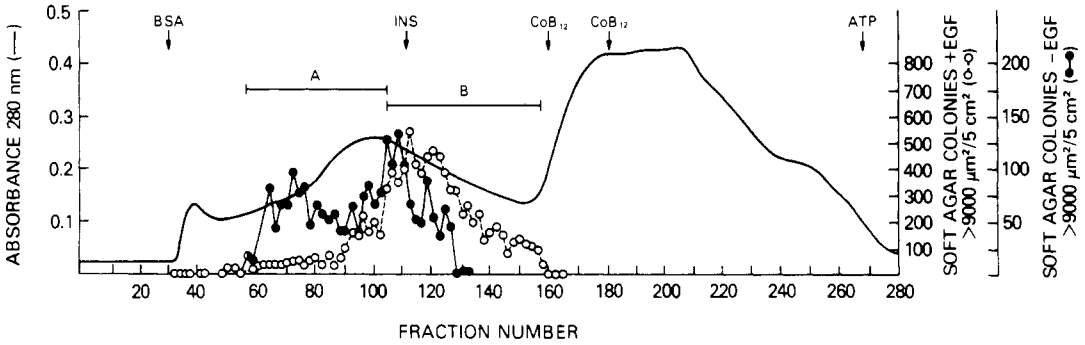

FIGURE 1: Bio-Gel P-6 chromatography of pool 1 from a P-30 gel filtration column effluent. The lyophilized residue (7.2 g) from pool 1 of a P-30 column effluent was applied in 130 mL of 1 M acetic acid to a Bio-Gel P-6 column (10 × 91 cm). The column was eluted with 1 M acetic acid, and soft agar assays were carried out on lyophilized 75-μL aliquots in the presence (○) or absence (●) of EGF (2 ng/mL). Fractions within the horizontal bars were pooled and lyophilized for further analysis. Markers used were bovine serum albumin (BSA) (M_r 68 000), insulin (M_r 6000); CoB_{12}, coenzyme B_{12} (M_r 1580), and Na_2ATP (M_r 551). CoB_{12} eluted as two distinct peaks.

to be assayed. The cells were then incubated for 2 h at room temperature and 5% CO_2 in air. After incubation, the cells were washed 4 times with cold Hanks solution (Gibco) containing 0.1% bovine serum albumin. The cells were solubilized with 0.6 mL of Triton solution [20 mM Hepes, 1% Triton X-100, 10% glycerol, and 0.01% bovine serum albumin, pH 7.4 (Heldin et al., 1981)] by a 20-min incubation at room temperature. Aliquots (500 μL) were counted on a γ spectrometer to determine the amount of ligand bound.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Samples were analyzed on a 1.5-mm slab gel using a 12.5% polyacrylamide gel with a discontinuous buffer system (Laemmli, 1970). Proteins were fixed with formaldehyde (Steck et al., 1980) and stained by using a silver staining technique (Gelcode, Upjohn). Protein standards were obtained from Bethesda Research Laboratory. In some cases, samples were allowed to stand with 5% 2-mercaptoethanol for 30 min prior to application to the gel.

Extraction of Colony-Forming Activity from NaDodSO$_4$–Polyacrylamide Gels. Following electrophoresis, the gels were cut into 0.5-cm slices, crushed with glass wool in sealed Eppendorf pipet tips, and extracted for 24 h at room temperature in a solution of 1 M acetic acid containing 200 μg of bovine serum albumin per milliliter (Roberts et al., 1983). Eluates were dialyzed for 66 h in a microdialyzer (Bethesda Research Labs) against 8 L of 1 M acetic acid, lyophilized, and assayed for colony-forming activity in the soft agar assay.

Other Procedures. Total protein was determined by the fluorescamine assay (Udenfriend et al., 1972) using bovine serum albumin as a standard or by amino acid analysis. Quantitative amino acid analyses were done with a modified Beckman 121 MB amino acid analyzer (Barbarash & Quarles, 1982) equipped with a Gilson Model 121 fluorometer for detection of primary amines using *o*-phthalaldehyde reagent solution (Fluoraldehyde, Pierce) and an Autolab system 1 computing integrator. Lyophilized samples (30–50 pmol) were prepared for analysis by hydrolysis in 100 μL of constant-boiling HCl (Pierce) containing 0.1% liquid phenol at 150 °C for 2 h in sealed evacuated tubes (Frolik et al., 1983).

Results

Initial Purification. The undialyzed crude acid/ethanol extract from 6.5 kg of A673 cells when chromatographed on a Bio-Gel P-30 column in 1 M acetic acid showed an area (apparent M_r 2600–5000) of decreased soft agar colony-forming activity. This inhibitory activity has been shown to be due to oxidized polyamines which can be separated from TGF activity by chromatography on a Bio-Gel P-6 column (Frolik et al., 1984). The inhibitory region was combined into pool 1. There was also a peak (apparent M_r 850–2600) of soft agar colony-forming activity when assayed in the presence of EGF similar to that observed for an extract from human placenta (Frolik et al., 1983) (data not shown). Fractions from this active region were combined into pool 2. Pool 1 (7.2 g of residue) and pool 2 (4.0 g of residue) were then chromatographed separately on a Bio-Gel P-6 column in 1 M acetic acid (Figure 1). Each column showed similar chromatographic profiles with two broad peaks of activity. The higher molecular weight peak (M_r 9000–32 000) showed soft agar activity in the absence of EGF. The fractions from both P-6 columns which showed this TGF-α activity were combined into pool A and lyophilized. The lower molecular weight peak (M_r 2000–9000) showed soft agar activity in the presence of EGF. The fractions from both P-6 columns which showed this TGF-β activity were combined into pool B and lyophilized.

Pools A and B from the P-6 column were then applied to separate cation-exchange columns, and the proteins were eluted by using a linear sodium chloride gradient (Figure 2). Pool A, in the presence of TGF-β, gave a single peak of soft agar-stimulating activity (Figure 2A). Pool B also gave one peak of soft agar activity but required the presence of EGF (Figure 2B). A peak of EGF-competing activity coeluted with the soft agar-stimulating activity in pool A.

Purification of TGF-β. Fractions from pool B (Figure 2B) were combined and chromatographed on a μBondapak C_{18} HPLC column using an acetonitrile/0.1% trifluoroacetic acid gradient (data not shown). The TGF activity eluted from the column as a single peak at an acetonitrile concentration of 34%. When the peak fractions were assayed for soft agar colony formation, the addition of EGF was necessary for activity. The same fractions also showed inhibition of ^{125}I-TGF-β binding to NRK cells. The TGF-β fractions from the C_{18} HPLC column were combined and run on a μBondapak CN HPLC column using a 1-propanol/0.1% trifluoroacetic acid gradient (Figure 3). Soft agar activity and ^{125}I-TGF-β binding inhibition eluted as a single peak at a 1-propanol concentration of 34%. Approximately 5 μg of pure TGF-β, as determined by fluorescamine assay, was recovered. Aliquots were taken from the peak fraction for characterization studies.

Characterization of TGF-β. The purified TGF-β from A673 cells was shown to be homogeneous as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis with an apparent molecular weight of 25 000 in its unreduced state and of 13 000 in its reduced state (Figure 4, lanes 3 and 5). In this system, the TGF-β derived from the tumor cell line behaved identically with the TGF-β previously isolated from normal-term human placenta (Figure 4, lanes 2 and 4). Similarly, the amino acid composition of the TGF-β obtained from the two different

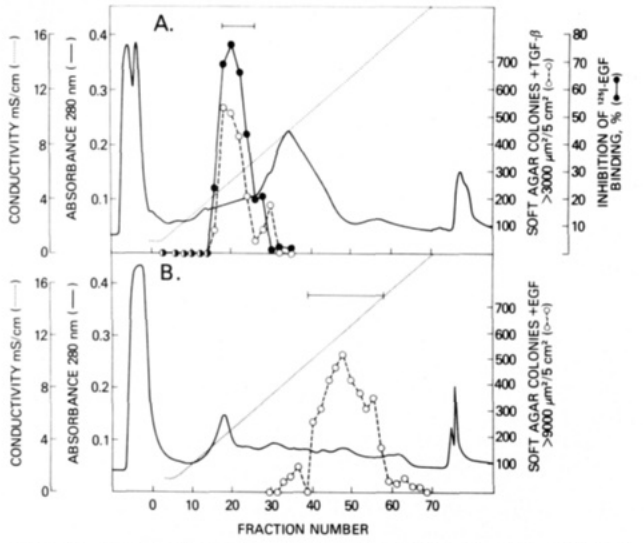

FIGURE 2: Cation-exchange chromatography of A673 TGF. (A) Pool A from a gel filtration column (Figure 1) was applied to a cation-exchange column equilibrated with 0.05 M sodium acetate (pH 4.5). After a 300-mL wash with starting buffer, TGF was eluted by using a linear sodium chloride gradient to 0.70 M, and fractions (29 mL) were collected. Aliquots (40 μL) were removed from even-numbered fractions, lyophilized, and assayed for ^{125}I-EGF binding inhibition (●). In addition, lyophilized aliquots (20 μL) were assayed for soft agar colony formation (○) in the presence of TGF-β (0.2 ng/mL). (B) Pool B was applied to a cation-exchange column and eluted as above. Fractions (27 mL) were collected, and 100-μL aliquots were removed from odd fractions and dialyzed against 1 M acetic acid for 24 h prior to lyophilization and soft agar assay in the presence of EGF (2 ng/mL). Fractions indicated by the horizontal bar were combined for further chromatography. No activity was observed outside of the areas shown in the figure.

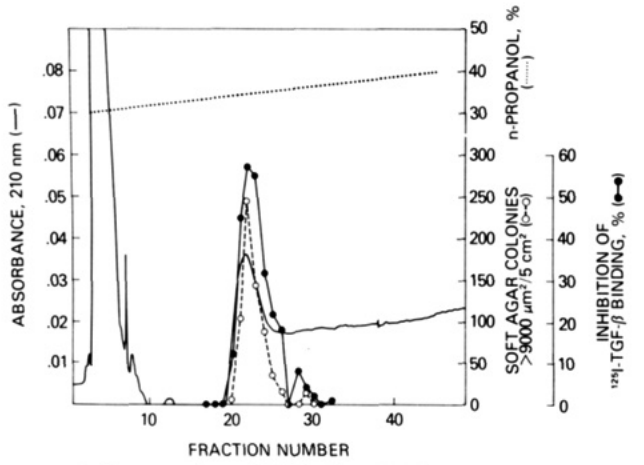

FIGURE 3: Reverse-phase HPLC of A673 TGF-β on a μBondapak CN column. Pooled fractions from a μBondapak C_{18} column were partially evaporated under nitrogen to remove acetonitrile. The sample was then loaded onto a μBondapak CN column equilibrated with 30:70:0.1 1-propanol/water/trifluoroacetic acid (pH 2). After a 3-min delay, TGF-β was eluted by using a linear gradient of 1-propanol (···). A total of 72 fractions (2.4 mL/fraction) were collected. Aliquots of 10 μL were removed for ^{125}I-TGF-β binding inhibition (●), and 1-μL aliquots were taken for soft agar assay (○) in the presence of EGF (2 ng/mL). No activity was observed outside of the area shown in the figure.

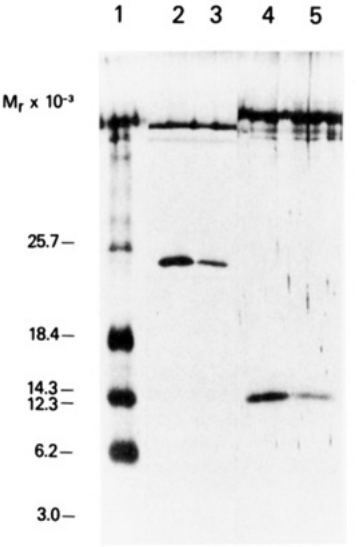

FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of human placental TGF-β and A673 TGF-β. Aliquots of purified TGF-β (20 ng of protein) were dissolved in 40 μL of sample buffer in either the absence (placenta, lane 2; A673, lane 3) or the presence (placenta, lane 4; A673, lane 5) of 5% 2-mercaptoethanol. The samples were applied to a 1.5-mm, 12.5% polyacrylamide gel with a 3% acrylamide stacking gel. After electrophoresis, the gel was fixed with 14% formaldehyde and stained by using a silver staining technique (Gelcode, Upjohn). Molecular weight standards (lane 1) were α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), lysozyme (14 300), cytochrome *c* (12 300), bovine trypsin inhibitor (6200), and insulin A and B chains (3000). The bands at the top of the gel are artifacts which were also observed in the blank lanes.

Table I: Amino Acid Composition of TGF-β[a]

amino acid	no. of residues/mol[b] (mean ± range) A673	placenta[c]
aspartic acid	23 ± 3	24 ± 2
threonine	8 ± 1	8 ± 1
serine	17 ± 1	17 ± 1
glutamic acid	26 ± 0	25 ± 1
proline	ND[d]	ND
glycine	28 ± 9	17 ± 4
alanine	17 ± 1	18 ± 1
half-cystine[e]	ND	16 ± 2
valine	15 ± 1	15 ± 2
methionine	1 ± 1	2 ± 1
isoleucine	10 ± 1	11 ± 1
leucine	21 ± 5	24 ± 2
tyrosine	12 ± 4	17 ± 1
phenylalanine	8 ± 1	8 ± 1
histidine	7 ± 1	7 ± 1
lysine	18 ± 2	19 ± 2
tryptophan	ND	ND
arginine	12 ± 1	11 ± 1

[a] Amino acid composition was determined after 2-h hydrolysis in 6 N HCl containing 0.1% liquid phenol at 150 °C in sealed, evacuated tubes. Values are based on two separate determinations of two different preparations. [b] The residue number per mole is based on apparent molecular weight of 25 000. [c] Data previously reported (Frolik et al., 1983). [d] ND, not determined. [e] Determined by performic acid oxidation and acid hydrolysis.

sources (Table I) is strikingly similar. Furthermore, the purified tumor cell TGF-β had an ED_{50} for soft agar colony formation of 0.7 pM while placental TGF-β had an ED_{50} of 1.0 pM (Figure 5A); the small difference is within the variability range of the assay. Finally, comparative dose–response curves for inhibition of placental ^{125}I-TGF-β binding to NRK fibroblasts showed an ED_{50} of 2.3 nM for A673 TGF-β and 1.7 nM for placental TGF-β (Figure 5B). Therefore, the data support the conclusion that both TGF-β's, the one derived from tumor cells and the other from normal tissue, are biologically and chemically equivalent in all respects examined.

Purification of TGF-α. The combined TGF-α fractions from the cation-exchange column were run on a Waters μBondapak C_{18} column using an acetonitrile/0.05% trifluoroacetic acid gradient (data not shown). Activity eluted

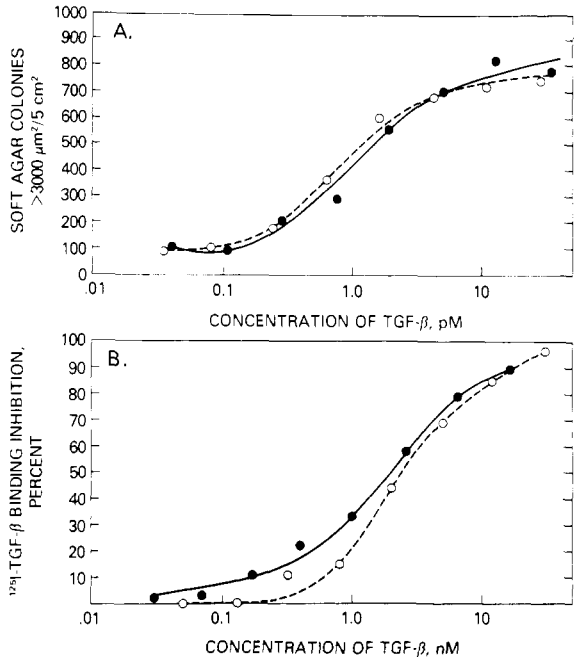

FIGURE 5: Dose–response curves of purified TGF-β derived from human placenta and A673 cells. (A) Soft agar colony formation in the presence of EGF (2 ng/mL) and (B) ^{125}I-TGF-β binding inhibition at various concentrations of TGF-β derived from human placenta (●) and A673 cells (○). Both assays were performed with NRK cells.

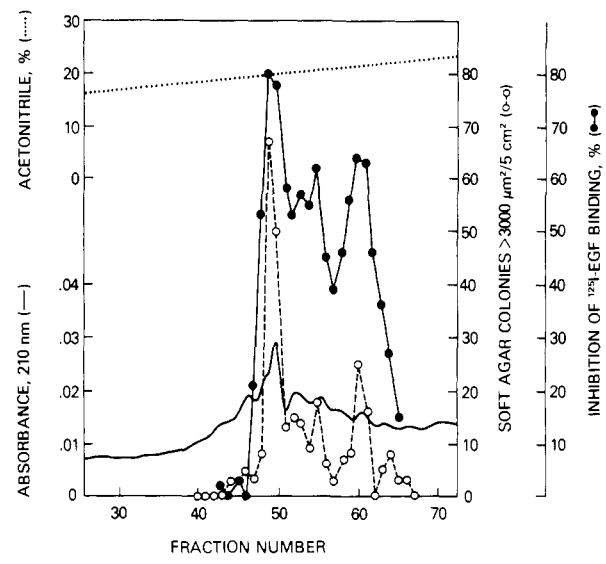

FIGURE 6: Reverse-phase HPLC of A673 TGF-α on a Synchropak RP-P column. Pooled fractions from a CN HPLC column (see Experimental Procedures) were reduced in volume by half by evaporation under nitrogen. An equal volume of initial solvent was added, and the sample was loaded onto a Synchropak RP-P column equilibrated with 10:90:0.035 acetonitrile/water/trifluoroacetic acid (pH 2). After a 5-min linear gradient of 10:90:0.035 to 15:85:0.035 acetonitrile/water/trifluoroacetic acid (pH 2), TGF-α was eluted by using a 400-min linear gradient of 15:85:0.035 to 55:45:0.035 acetonitrile/water/trifluoroacetic acid (···). A total of 100 fractions (1.7 mL/fraction) were collected. Aliquots of 50 μL were removed for ^{125}I-EGF binding competition (●), and aliquots of 30 μL were removed for soft agar assay (○) in the presence of TGF-β (0.2 ng/mL).

in two peak regions with the first peak at 23.5% acetonitrile, containing 43% of the total colony-forming activity, and the second peak at 24.5% acetonitrile, containing 57% of the activity. The fractions forming the first peak were combined and run on a Waters μBondapak C_{18} column using a 1-propanol/0.035% trifluoroacetic acid gradient followed by CN column chromatography; however, no further purification was achieved by these methods (data not shown). Fractions forming the second peak were combined and run on the same C_{18} column under identical conditions (data not shown). Two peak regions of activity were again eluted, one peak at 16% 1-propanol which contained 36% of the colony-forming activity and a second peak at 16.5% 1-propanol which contained 64% of the activity. Of these two peaks, the fractions from the first region were combined and analyzed on CN columns followed by a Synchropak RP-P column which resulted in both poor purification and loss of activity. Chromatography of the material from the second peak on a Waters μBondapak CN column using a 1-propanol/0.035% trifluoroacetic acid gradient at 0.2% 1-propanol/min (data not shown) yielded a single peak of colony-forming activity eluting at 30% 1-propanol which also displayed the ability to inhibit ^{125}I-EGF binding to the NRK cell. Inhibition of ^{125}I-EGF binding was therefore used to monitor this and subsequent columns due to the rapid response of the assay. The peak fractions were combined and rerun on the same column using 1-propanol/0.035% trifluoroacetic acid but with a slower gradient of 0.1%/min. A single broad peak of activity eluted at 28–29% 1-propanol (data not shown). The peak fractions were combined and applied to a Synchropak RP-P column using an acetonitrile/0.035% trifluoroacetic acid gradient (Figure 6). Four peaks of EGF receptor binding activity were eluted between 19.5% and 21% acetonitrile. Two of these peaks (1 and 4) contained the majority of both receptor binding and colony-forming activity and were analyzed in more detail, while peaks 2 and 3 contained only minor binding and colony-forming activity and were not characterized further. Two fractions, one from peak 1 and one from peak 4, were run on polyacrylamide gels and extracted, and soft agar colony-forming activity was measured in the presence of TGF-β. The activity in the first peak migrated on the gel at an apparent molecular weight of 18 000 and appeared to travel with the most densely stained protein band (Figure 7A). (The band at the top of the gel occurred at the interface between the stacking and running gels and was also observed in the blank lanes.) The activity in the last peak also migrated with an apparent molecular weight of 18 000 but corresponded to the lighter staining band of two bands that could be detected on the gel (Figure 7B). There was an insufficient amount of material in either peak to do further purification or characterization. These data therefore suggest the presence of an intracellular TGF-α-like polypeptide that has a higher molecular weight than the previously identified extracellular TGF-α's.

Discussion

In the past, human TGF activity has been detected in a variety of tumor cell lines (Todaro et al., 1980; Frolik et al., 1982b; Halper & Moses, 1983), solid tumors (Halper & Moses, 1983; Nickell et al., 1983), leukemia cell extracts (Nakamura et al., 1983), urine (Twardzik et al., 1982b; Sherwin et al., 1983), platelets (Childs et al., 1982; Assoian et al., 1983), colostrum (Noda et al., 1984), and term placenta (Stromberg et al., 1982; Frolik et al., 1983). Of these factors described, only a small molecular weight extracellular TGF-α from a human metastatic melanoma cell line (Marquardt & Todaro, 1982; Marquardt et al., 1983) and an intracellular TGF-β from human platelets (Assoian et al., 1983) and normal-term placenta (Frolik et al., 1983) have been purified and partially characterized. In this report, an intracellular TGF-β from a human rhabdomyosarcoma cell line has been isolated and compared to normal human TGF-β obtained from placenta. It has been found that the tumor-derived TGF-β is similar in all respects examined to the TGF-β obtained from

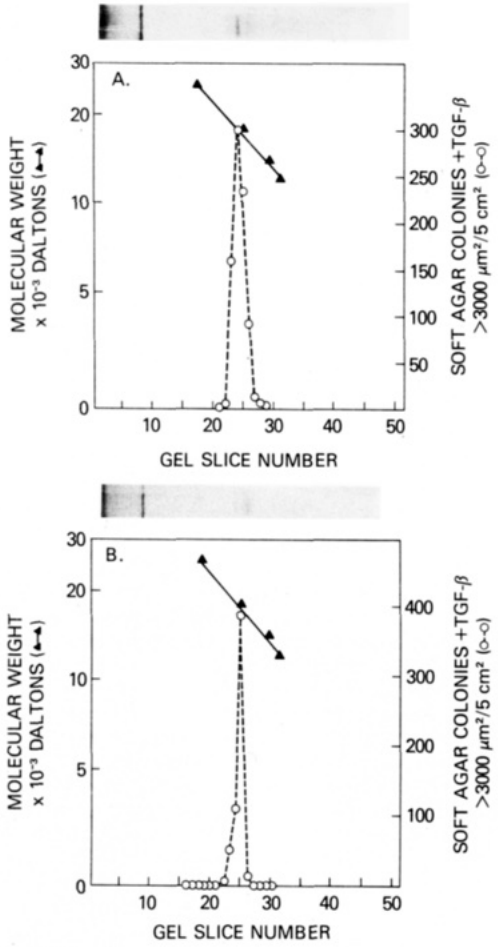

FIGURE 7: $NaDodSO_4$–polyacrylamide gels of A673 TGF-α. Aliquots (100 μL) fraction 50 (A) and fraction 60 (B) from A673 TGF-α chromatographed on a Synchropak RP-P column (Figure 6) were run on a 1.5-mm 12.5% polyacrylamide gel with a 3% acrylamide stacking gel. After electrophoresis, the gel was cut into 0.5-cm slices and extracted as described under Experimental Procedures. The final samples were assayed in soft agar in the presence of TGF-β (○). Standards (▲) containing α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), lysozyme (14 300), and cytochrome *c* (12 300) were fixed in 14% formaldehyde and stained by using a silver staining technique (Gelcode, Upjohn).

normal tissue. This identity includes biological activity in stimulating growth of NRK cells in soft agar (Figure 5A), binding to the TGF-β receptor (Figure 5B), migration on $NaDodSO_4$–polyacrylamide gels (Figure 4), and amino acid composition (Table I). Although subtle sequence differences, as have been observed for various forms of human insulin (Tager et al., 1979; Shoelson et al., 1983), cannot be excluded, it would appear at this level of investigation that the transformed state of the rhabdomyosarcoma cell is not the result of the production of an aberrrant form of a normal TGF-β that displays greater activity in transforming normal rat kidney fibroblasts.

In addition to isolation of tumor cell derived human intracellular TGF-β, we have observed several species of intracellular TGF-α's in the rhabdomyosarcoma cell. These intracellular TGF-α's share common biological properties with extracellular TGF-α (ability to compete with EGF for binding to the EGF receptor and ability, along with TGF-β, to induce NRK fibroblasts to grow under anchorage-independent conditions). However, their apparent molecular weight is approximately 2.5-fold greater than that observed for extracellular TGF-α. Higher molecular weight peptides with TGF-α-like activity have previously been reported in the conditioned medium from both human melanoma cells (Marquardt & Todaro, 1982) and human bronchogenic carcinoma cells (Todaro et al., 1980) and also in human urine (Twardzik et al., 1982; Sherwin et al., 1983). In all cases, the molecular weights of these peptides were determine only by gel filtration chromatography which has been shown not to be a reliable method for determining the molecular weight of transforming growth factors (Roberts et al., 1983b). Marquardt & Todaro (1982) did report that there were several forms of high molecular weight extracellular TGF-α from a human melanoma cell line. As demonstrated in this report, results from the human rhabdomyosarcoma cell line also show a mixture of peptides with TGF-α-like activity (see, e.g., Figure 6). Two of these peptides have an apparent molecular weight of 18 000 on $NaDodSO_4$–polyacrylamide gels (Figure 7). Although one of these factors appeared to be close to homogeneity, there was an insufficient amount of material left for further characterization. Therefore, it cannot be determined at this time whether these higher molecular weight forms of TGF-α-like activity represent precursors to the lower molecular weight TGF-α previously characterized, as has been shown to be the case for several other growth factors including EGF (Frey et al., 1979; Gray et al., 1983; Scott et al., 1983a) and nerve growth factor (Berger & Shooter, 1977; Scott et al., 1983b).

It has previously been suggested that the autocrine secretion of TGFs, especially TGF-α, may be an important mechanism in the malignant transformation of cells (Sporn & Todaro, 1980). In support of this idea, cells transformed by a temperature-sensitive sarcoma virus secrete high levels of sarcoma growth factor (a type α TGF) at permissive temperatures for transformation but negligible amounts at nonpermissive temperatures (De Larco et al., 1981; Ozanne et al., 1981). Similarly, the quantity of type α TGF-like activity secreted into the medium by tumor cells can be correlated with the ability of these cells to grow in soft agar (Todaro et al., 1980). It has also been suggested that the presence of TGF-α-like material in the urine may be a clinically useful marker for certain types of cancer (Twardzik et al., 1982b; Sherwin et al., 1983). Finally, the data in this report show that TGF-α can be detected in human tumor cells by using a procedure that showed no TGF-α activity in normal human placenta (Frolik et al., 1983). These data therefore strengthen the possible correlation between the presence of TGF-α and tumor growth. It should, however, be mentioned that it has recently been demonstrated that TGF-α-like activity can be isolated from normal human platelets (Assoian et al., 1984). It may therefore be that the presence of TGFs may not be the critical factor in tumor growth, but rather the determining event may be their location or concentration or the ability of a given cell to respond to these factors. More work will have to be done before the involvement of the TGFs in tumor formation can be deciphered.

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