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Purification and initial characterization of a type β transforming growth factor from human placenta

(epidermal growth factor/soft agar growth/cell transformation)

CHARLES A. FROLIK, LINDA L. DART, CHESTER A. MEYERS, DIANE M. SMITH, AND MICHAEL B. SPORN

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

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ABSTRACT **A polypeptide transforming growth factor (TGF) that induces anchorage-dependent rat kidney fibroblasts to grow in soft agar has been isolated from human placenta and purified to homogeneity. This polypeptide is classified as a type β TGF because it does not compete with epidermal growth factor (EGF) for membrane receptor sites but does require EGF for induction of anchorage-independent growth of indicator cells. Purification of this peptide was achieved by acid/ethanol extraction of the placenta, followed by gel filtration, cation exchange, and HPLC of the acid-soluble proteins. Homogeneity of the TGF-β from the final column was shown by its constant specific activity and amino acid composition across the peak of soft agar colony-forming activity and by its migration as a single band at M_r 23,000–25,000 on $NaDodSO_4$/polyacrylamide gel electrophoresis. Under reducing conditions, the protein migrated on a gel as a single band at M_r 13,000. The purified placental TGF-β caused half-maximal growth stimulation of indicator cells in soft agar at 64–72 pg/ml (3 pM) in the presence of EGF at 2 ng/ml (0.34 nM).**

Transforming growth factors (TGFs) have been operationally defined as low molecular weight acid-stable polypeptides that induce nonneoplastic anchorage-dependent rat kidney cells to form colonies that grow in soft agar (1, 2). These TGFs have been shown to have important functional interactions with epidermal growth factor (EGF) and can be subdivided into at least two groups based on these interactions (3–5). One group, defined as type α, is able to compete with EGF for membrane receptor binding sites and includes sarcoma growth factor (1), a TGF purified from human melanoma cells (6), and EGF itself (5). The second group, type β, does not bind to the EGF receptor. Type β TGFs were originally isolated from nonneoplastic tissues (3) but have recently been shown to occur in neoplastic cells as well (4, 5, 7). Both TGF-α and TGF-β must be present to induce the growth of nonneoplastic cells in soft agar (8). The normal physiological functions of these potent growth factors are not yet known. It has been suggested that they may be involved in embryological development (9, 10), wound healing (11), and tissue repair (11). In this report, the presence of a TGF-β in human placenta is documented and the total purification to homogeneity of a TGF-β derived from a human organ is described.

MATERIALS AND METHODS

Soft Agar Assay. Soft agar colony-forming activity was determined as described (2) except that the cells were stained (12) at the end of 1 wk in assay and the numbers and sizes of the colonies were determined using a Bausch and Lomb Omnicon image analysis system (3).

Extraction. Normal term human placentas were frozen on dry ice within 30 min after delivery and stored at −60°C until used. Placentas were extracted as described (2) except that the homogenized tissue (600–1,000 g) was stirred in the acid/ethanol solution at room temperature for 2 to 3 hr prior to centrifugation. The resulting supernatant was adjusted to pH 3.0 and protein was precipitated with ether and ethanol (2). The precipitate was collected by filtration and dissolved in 1 M acetic acid (1 ml/g of tissue). Insoluble material was removed by centrifugation, the supernatant was lyophilized, and the residue [27 mg/g (wet weight) of placenta] was stored at −20°C.

Gel Filtration Chromatography. The lyophilized extract (239 g) from 11 placentas (8.8 kg) was dissolved in 1 M acetic acid (50 mg of residue per ml) and applied in two portions (107 g and 132 g of protein) to a column (35.6 × 90 cm) of Bio-Gel P-30 (100–200 mesh, Bio-Rad) equilibrated and eluted (1.6 liters/hr) with 1 M acetic acid at room temperature. Fractions (800 ml) were collected and aliquots of the even-numbered fractions were assayed for protein and for growth-promoting activity in soft agar. The fractions containing TGF activity were combined into three pools (A–C) (see Fig. 1) and lyophilized. Pool B (6 g of residue per column) was dissolved in 1 M acetic acid (60 mg/ml) and applied to a column (10 × 91 cm) of Bio-Gel P-6 equilibrated with 1 M acetic acid. The protein was eluted from the column with 1 M acetic acid (150 ml/hr), collecting 37-ml fractions. Aliquots of even-numbered fractions were assayed for TGF activity. The fractions containing this activity were pooled and lyophilized.

Ion-Exchange Chromatography. Twenty-four percent of pool B from the P-6 column (2.1 g of protein) and pool C from the P-30 column (1.9 g of protein) were dissolved separately in 60 ml of 0.01 M acetic acid. The pH was adjusted to 4.5 and the conductivity, to 1.2–1.5 mS/cm. Each sample was then applied to a cation-exchange column (CM-Trisacryl M; LKB; 5 × 10 cm) equilibrated with 0.05 M sodium acetate (pH 4.5) (buffer A). The column was eluted with 300 ml of buffer A (145 ml/hr) and then with a linear sodium chloride gradient to 0.70 M sodium chloride in buffer A at 0.8 mM/min. After 70 fractions (29 ml each), the column was washed with 1 M sodium chloride/0.05 M sodium acetate, pH 2.5, and then reequilibrated with buffer A. Aliquots from the even-numbered fractions were removed for determination of protein and TGF activity. The peak of activity was combined for further analysis.

Reversed-Phase HPLC. The sample from the ion-exchange column was adjusted to 10% (vol/vol) acetonitrile/0.1% trifluoroacetic acid and the pH was adjusted to 2.0. It was then pumped onto a μBondapak C_{18} HPLC column (10-μm particle size, 0.78 × 30 cm; Waters Associates) equilibrated with ace-

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TGF-α and TGF-β, types α and β TGF, respectively.

tonitrile/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 60-min linear gradient from 25:75:0.1 to 45:55:0.1 of acetonitrile/water/trifluoroacetic acid, pH 2. Seventy-five fractions (1.2 ml per fraction) were collected, and then the column was stripped with acetonitrile/water/trifluoroacetic acid, 80:20:0.1 (pH 2), collecting 2.4-ml fractions. Aliquots (5 μl) were removed for assay of TGF activity.

The peak of TGF activity from the C_{18} HPLC column was combined, lyophilized, dissolved in *n*-propanol/water/trifluoroacetic acid, 30:70:0.1 (pH 2) and applied to a μBondapak CN column (10-μm particle size, 0.38 × 30 cm; Waters Associates) equilibrated with the sample solvent. This column was eluted (1.1 ml/min) with a 153-min linear gradient from 30:70:0.1 to 45:55:0.1 of *n*-propanol/water/trifluoroacetic acid, pH 2. Forty-five fractions (2.2 ml per fraction) were collected and aliquots were removed for bioassay, amino acid analysis, and gel electrophoresis.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Samples were analyzed on 1.5-mm slab gels using either a polyacrylamide gradient of 15–28% or a 15% polyacrylamide gel and a discontinuous buffer system (13). Proteins were fixed with formaldehyde (14) and stained using a silver staining technique (Gelcode, Upjohn). Protein standards were obtained from Bethesda Research Laboratory. In some cases, samples were boiled with 5% 2-mercaptoethanol for 3 min prior to application to the gel.

Other Procedures. Total protein was determined by either the dye-binding method (15) or fluorescamine assay (16) using bovine serum albumin as standard or by amino acid analysis. Quantitative amino acid analyses were done with a modified Beckman 121MB amino acid analyzer (17) 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 (7–40 pmol) were hydrolyzed in 100 μl of constant-boiling HCl (Pierce) containing 0.1% liquid phenol at 150°C for 2 hr in sealed evacuated tubes. These modified conditions of decreased hydrolysis time at elevated temperature (18) yielded results for several test proteins that were identical to or better than those obtained after conventional acid hydrolysis (110°C, 24–48 hr) (data not shown). Assays for EGF-competing activity were carried out as described (1, 19).

RESULTS

The acid/ethanol extract of human placenta displayed activity that stimulated anchorage-dependent normal rat kidney cells to form colonies in soft agar (4). EGF markedly enhanced (150-fold) the activity of this placental TGF. As has been shown for other TGFs (1, 2, 7), the activity of a partially purified placental preparation is destroyed by treatment with either trypsin or dithiothreitol (data not shown).

Chromatography of the undialyzed crude residue from the combined acid/ethanol extractions of 11 placentas on a Bio-Gel P-30 column in 1 M acetic acid gave two peaks of activity when assayed in the presence of EGF (pool A, apparent M_r 5,000–9,000, and pool C, apparent M_r <3,500) (Fig. 1). No colony-stimulating activity was detected when equivalent aliquots were assayed in the absence of EGF. Therefore, all subsequent soft agar assays were carried out in the presence of EGF at 2 ng/ml. None of the three pools shown in Fig. 1 competed with ^{125}I-labeled EGF for EGF membrane receptor sites on CCL-64 cells (data not shown). This TGF is therefore a member of the TGF-β family. Pool A, which contained 31% of the recovered protein, had 17% of the recovered TGF activity (Table 1) while pool C, with only 0.8% of the protein, contained 18% of the recovered activity. Pool B did not give a valid assay for TGF activity because of the presence of a growth inhibitory substance. This inhibitor could be separated from the soft agar colony-forming activity by chromatography on a Bio-Gel P-6 column (data not shown). As shown in Table 1, 69% of the TGF activity found in the crude residue was present in the pool B fraction that eluted from the P-6 column. Pools B and C were therefore further purified.

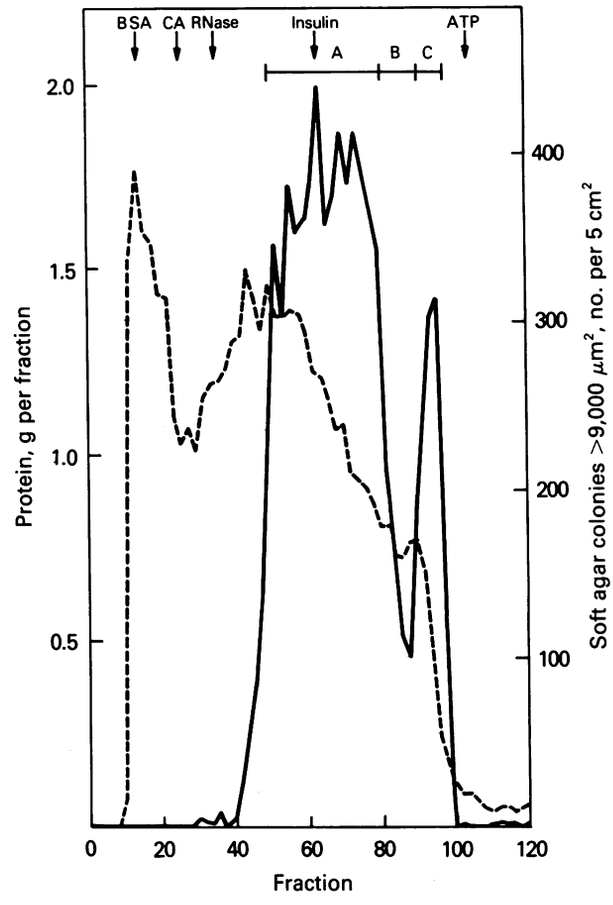

FIG. 1. Bio-Gel P-30 chromatography of the crude acid/ethanol extract of placenta. The residue (132 g) from an undialyzed acid/ethanol extract of 4.4 kg of placenta was applied in 2.6 liters of 1 M acetic acid to a Bio-Gel P-30 column. The column was eluted with 1 M acetic acid. Protein (---) was determined using 10-μl aliquots of the even-numbered fractions and soft agar assays (——) were carried out on lyophilized 150-μl aliquots. Fractions within the horizontal bars were pooled and lyophilized for further analysis. Markers used were BSA, bovine serum albumin (M_r, 68,000); CA, chymotrypsinogen A (M_r, 25,000); RNase (M_r, 13,800); insulin (M_r, 6,000); and Na_2ATP (M_r, 551).

Application of the protein from the gel filtration column to a cation-exchange column and subsequent elution of the applied material with a linear sodium chloride gradient gave a single peak of soft agar colony-forming activity (Fig. 2). Although 85–96% of the applied protein was recovered from the column, only 10–45% of the applied TGF activity was detected. Whether this loss of activity is due to specific loss of the TGF protein, to denaturation of the TGF, or to the separation of the TGF from an activator is, at this time, not known. Fractions indicated in Fig. 2 were pooled and chromatographed on a μBondapak C_{18} HPLC column using an acetonitrile/0.1% trifluoroacetic acid gradient (data not shown). The TGF activity for both pools eluted from the column as a single peak at an acetonitrile concentration of 35%. Chromatography of this ma-

Table 1. Purification of TGF-β from human placenta

Step	Procedure	Protein recovered,* mg	ED_{50},† ng/ml	Specific activity,‡ units/μg	Total activity, units $\times 10^3$	Degree of purification, fold	Recovery of activity, %
1	Crude extract	239,000	7,600	0.09	21,510	1.0	100
2	Bio-Gel P-30						
	Pool A	73,900	15,000	0.05	3,695	0.6	17
	Pool B	27,720	—	—	—	—	—
	Pool C	1,900	360	2.0	3,800	22	18 (100)
3	Bio-Gel P-6: Pool B	8,700	410	1.7	14,790	19	69
4	Ion-Exchange						
	Pool B§	140	62	11.5	1,610	128	31
	Pool C	46.3	85	8.4	390	93	1.8 (10)
5	HPLC-C_{18}						
	Pool B	0.27	0.10	7,142	1,928	79,000	37
	Pool C	0.26	1.2	595	155	6,610	0.7 (4.1)
6	HPLC-CN						
	Pool B	0.025	0.072	9,920	248	110,000	4.8
	Pool C	0.022	0.064	11,160	245	124,000	1.1 (6.4)

* For steps 1–4, total protein was determined by the dye-binding procedure (15). For steps 5 and 6, total protein was based on amino acid analysis.

† Defined as the concentration (ng/ml) of TGF-β required to give a response of 1 unit in the presence of EGF (1 unit of activity gives 50% maximal response, ≈1,000 colonies >3,000 μm^2 per plate).

‡ Defined as [1 unit/ED_{50} (total ml per Petri dish)] × 1,000.

§ Twenty-four percent of pool B from step 3 was used for further purification.

terial on a μBondapak CN support equilibrated with *n*-propanol/0.1% trifluoroacetic acid yielded a single peak of TGF activity at 35% *n*-propanol that corresponded to a strong absorbance peak (Fig. 3). The homogeneity of the final preparation was indicated by a constant amino acid composition (Table 2) across the peak as well as by gel electrophoresis (Fig. 3 *Inset*). The final degree of purification of TGF-β from the crude extract was 110,000- to 124,000-fold with a 1.1% recovery of activity in pool C and 4.8% in pool B. Placental TGF-β at only 64–72 pg/ml gave a half-maximal growth stimulatory response (ED_{50}) in the presence of EGF at 2 ng/ml.

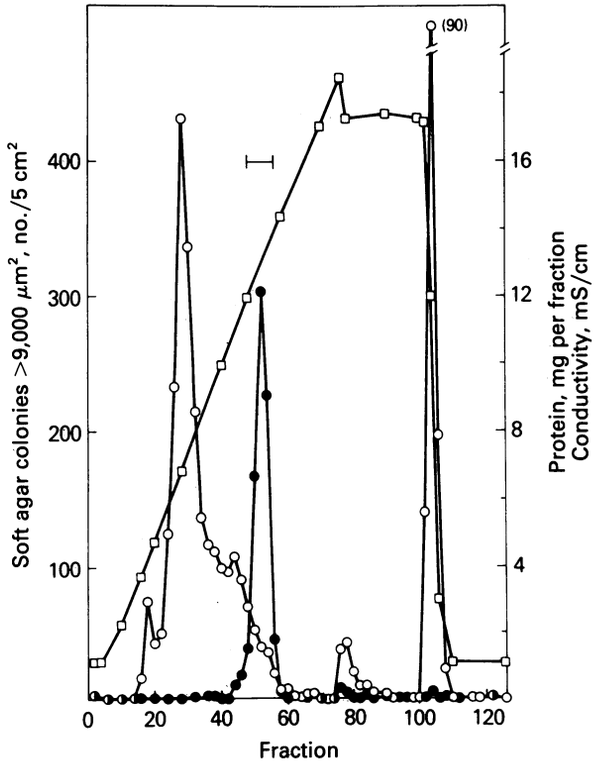

FIG. 2. Cation-exchange chromatography of placental TGF-β. Pool C from a gel-filtration column (Fig. 1) was applied to a cation-exchange column equilibrated with 0.05 M sodium acetate (pH 4.5). TGF was eluted from the column by using a linear sodium chloride gradient. Fractions (29 ml) were collected and 50- and 5-μl aliquots were removed from the even-numbered fractions for protein determination (○) and for soft agar assay (●). Fractions indicated by the horizontal bar were pooled for further chromatography. □, Conductivity.

The purity of the final TGF preparation was also shown by $NaDodSO_4$/polyacrylamide gradient gel electrophoresis (Fig. 4). In the absence of 2-mercaptoethanol, a single polypeptide band with an apparent M_r of 23,000–25,000 was observed for TGF from either pool B or pool C. Reduction of the protein with 2-mercaptoethanol produced a single band at M_r ≈13,000. When the gel was sliced into 0.5-cm strips and the unreduced protein was eluted into 1 M acetic acid, all the TGF activity was found in the slice that corresponded to a M_r of 23,000–25,000 (data not shown), indicating that the TGF activity corresponded to the only detectable protein band.

DISCUSSION

A TGF has been isolated from an acid/ethanol extract of human placenta. It is classified as a type β TGF (4, 5) because it does not compete with EGF for membrane receptor sites but requires EGF for the induction of colony growth in soft agar, with 50% maximal formation of colonies >60-μm diameter occurring at 64–72 pg of TGF/ml (3 pM). The factor has been purified to homogeneity by gel filtration, cation-exchange chromatography, and HPLC. It is a protein of M_r 23,000–25,000 and is composed of two polypeptide chains of M_r ≈13,000 held together by disulfide linkages. Whether these chains are identical or different remains to be determined. Although the protein contains 16 half-cystine residues, it is not yet known whether all of these residues are involved in disulfide linkage. However, the stability of the TGFs to acid treatment and heat denaturation (1, 2, 7, 20) suggests the presence of a large number of such bonds.

The presence of TGFs in the crude acid/ethanol extract of

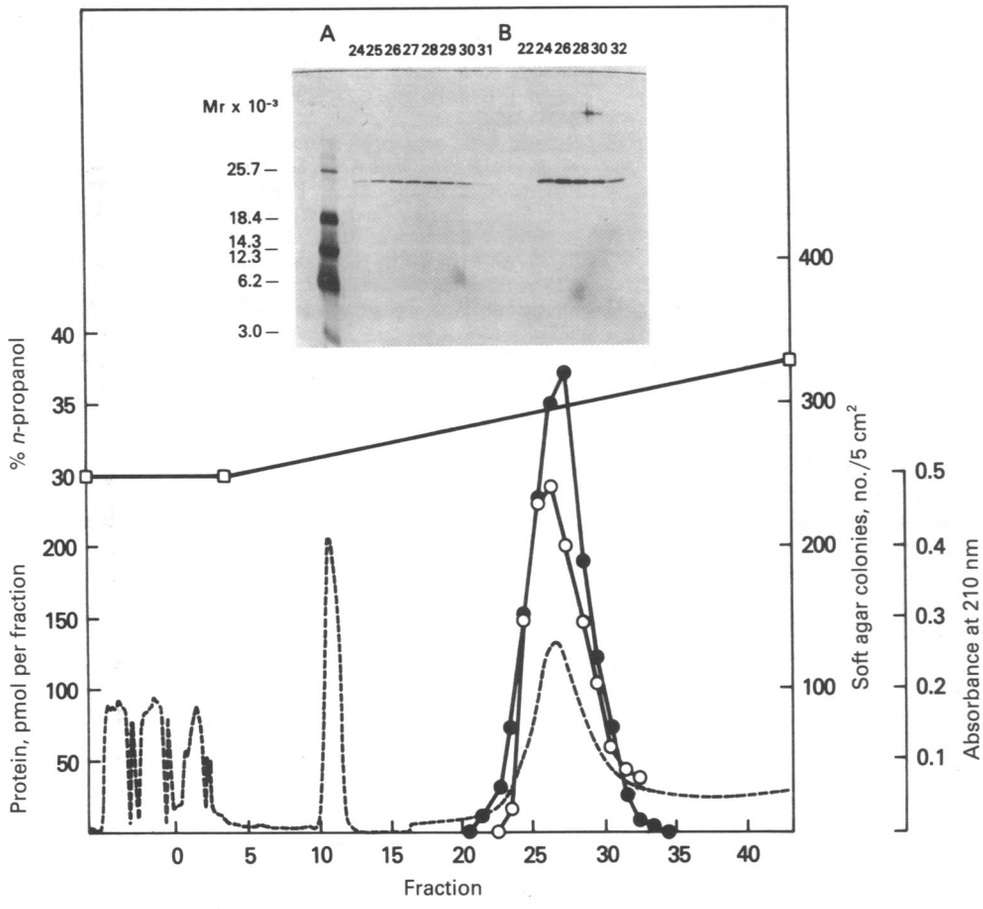

FIG. 3. Reversed-phase HPLC of placental TGF-β on a μBondapak CN column. The pooled fractions from a C_{18} HPLC column were lyophilized, dissolved in *n*-propanol/water/trifluoroacetic acid, 30:70:0.1 (pH 2), and applied to a μBondapak CN column. TGF was eluted from the column with a linear gradient of *n*-propanol (□). Forty-five fractions (2.2 ml) were collected. ○, Protein; ●, soft agar colonies >9,000 μm^2; ---, absorbance at 210 nm. (*Inset*) NaDodSO$_4$/15% polyacrylamide gel electrophoresis of 5-μl (*A*) and 50-μl (*B*) aliquots of selected column fractions. Fraction 26 represents 13 ng of protein in *A* and 130 ng in *B*. Protein was determined by fluorescamine assay with a correction factor based on amino acid analysis.

human placenta that were able to compete with EGF for binding to membrane receptors (TGF-αs) has recently been noted (21). In the present study, a significant amount of TGF-α-like activity was not detected. Although some soft agar colony forming activity was found in the crude residue in the absence of EGF, this activity did not compete with EGF in a receptor binding assay and it was stimulated 150-fold by EGF at 2 ng/ml, indicating that most, if not all, of the TGF present was of the type β class. Also, as the placental TGF was purified, it became totally dependent on exogenous EGF for soft agar col-

Table 2. Amino acid composition of human placental TGF-β

Amino acid	Residues, no./mol
Aspartic acid	24 ± 2
Threonine	8 ± 1
Serine	17 ± 1
Glutamic acid	25 ± 1
Proline	ND
Glycine	17 ± 4
Alanine	18 ± 1
Half-cystine*	16 ± 2
Valine	15 ± 2
Methionine*	2 ± 1
Isoleucine	11 ± 1
Leucine	24 ± 2
Tyrosine	17 ± 1
Phenylalanine	8 ± 1
Histidine	7 ± 1
Lysine	19 ± 2
Tryptophan	ND
Arginine	11 ± 1

Triplicate samples (15 pmol each) were hydrolyzed in constant-boiling HCl/0.1% liquid phenol at 150°C for 2 hr in sealed evacuated tubes. Values are calculated on the basis of an apparent M_r of 25,000. Results represent mean ± range. ND, not determined.
* Determined by performic acid oxidation and acid hydrolysis.

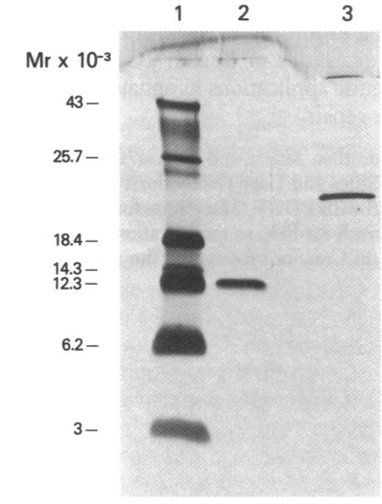

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of placental TGF-β. Aliquots (120 ng of protein) from the TGF-β region of a CN-HPLC column were dissolved in 40 μl of sample buffer and heated for 3 min at 100°C in the presence (lane 2) or absence (lane 3) of 5% 2-mercaptoethanol. The samples were then applied to a 1.5-mm polyacrylamide gradient gel (15–28%) with a 7.5% acrylamide stacking gel. After electrophoresis, the gel was fixed with 14% formaldehyde and stained with silver. M_r standards (lane 1) were ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (14,300), cytochrome *c* (12,300), bovine trypsin inhibitor (6,200), and insulin A and B chains (3,000).

ony-forming activity. Part of this difference may be explained by the fact that in the earlier communication (21) colonies of 6 cells or more were considered to be significant while in the present study only colonies containing at least 60 cells were counted.

A TGF-β at a concentration of 430 ng/g (wet weight) of tissue has recently been purified from human platelets (22). Because placenta contains much blood, it is possible that the placental TGF-β (10 ng/g of tissue) originated from the platelets. However, even assuming that the placenta was 100% blood and that platelets comprised 0.2% of this blood (23), platelet TGF would account for only 8% of the recovered placental TGF. Therefore, if the placental TGF-β did originate from the platelets, it would have to be concentrated by an, as yet, unknown mechanism.

Blood platelets also contain the peptide platelet-derived growth factor (PDGF) (24). However, placental TGF-β is not PDGF, as shown by the amino acid composition of TGF-β (Table 2), which is different from that reported for PDGF (25), and by the results from two different assays. In the first assay, placental TGF-β did not have any chemotactic activity when tested under conditions in which PDGF displayed strong activity (ref. 26; G. Grotendorst, personal communication). Similarly, placental TGF-β did not compete with PDGF in a radioreceptor assay (C. Stiles, personal communication).

Although TGFs were originally found in tumor cells (1, 2, 7, 20) and were postulated to be involved in transformation and neoplastic cell growth (9), their presence in adult cells and tissues (3, 4), in platelets (22, 27), and in embryos (10) implies that TGFs have a normal physiological function as well. The purification of placental TGF-β to homogeneity will facilitate investigation of this function, because it will permit the development of both receptor binding and radioimmunoassays. These assays will not only allow a specific quick procedure for quantitation of TGF-β but will also permit investigation of the mechanism of action and the control of expression of TGF-βs under normal and neoplastic conditions. Finally, structural analysis of purified TGF-β will provide information for initiation of cloning experiments. This will allow eventual production of large quantities of human TGF-β, which might have useful therapeutic applications in enhancement of wound healing and tissue repair.

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