

Umbilical Cord Transforming Growth Factor- β 3: Isolation, Comparison with Recombinant TGF- β 3 and Cellular Localization

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The transforming growth factor beta (TGF- β) family of growth modulators play critical roles in tissue development and maintenance. Recent data suggest that individual TGF- β isoforms (TGF- β 1, - β 2 and - β 3) have overlapping yet distinct biological actions and target cell specificities, both in developing and adult tissues. The TGF- β 3 isoform was purified to homogeneity from both natural and recombinant sources and characterized by laser desorption mass spectrometry, by protein sequencing, by amino acid analysis and by biological activity. TGF- β 3 was the major TGF- β isoform in umbilical cord (230 ng/g), and was physically and biologically indistinguishable from recombinant TGF- β 3 and from the tumor growth inhibitory (TGI) protein found in umbilical cord. Immunohistochemistry using antipeptide TGF- β 3 specific antibody showed TGF- β 3 localization in perivascular smooth muscle.

KEYWORDS: transforming growth factor beta-3, TGF- β 3, growth factor purification, umbilical cord, mass spectrometry, TGI

INTRODUCTION

The transforming growth factor-beta (TGF- β) family comprise dimeric proteins which regulate the growth of cells derived from all three germ cell layers. Three structurally and functionally related proteins are found in humans, TGF- β 1, TGF- β 2 and TGF- β 3 (Roberts and Sporn, 1990). TGF- β s arrest or prolong the cell cycling of hematopoietic stem cells and epithelial cells in G1 phase (Hampson et al., 1989; Strife et al., 1991, Moses et al., 1992), at least in part through inhibition of cdk/cyclin E kinase complex (Koff et al., 1993) and transgenic models sug-

gest the TGF- β s act on these cell types to control inflammation and epithelial cell homeostasis. In contrast, TGF- β s serve as indirect mitogens for several mesenchymal cell types, and can regulate inflammatory cell activation and chemotaxis. In addition, TGF- β s regulate the synthesis and degradation of extracellular matrix (Ignatz and Massague, 1986) and play important roles in tissue development and repair. TGF- β 1, β 2 and β 3 are encoded by distinct genes located on chromosomes 19q13.1-13.3, 1q41 and 14q23-24 respectively. TGF- β gene expression can be spatially and temporally controlled in an isoform specific manner during development and in the adult. The individual TGF- β protein isoforms also show clear differences in specific activity, receptor binding, clearance and target cell response, strongly suggesting the TGF- β s have overlapping but distinct roles in the development and maintenance of adult tissue. Here we focus on the most recently identified TGF- β isoform, TGF- β 3.

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TGF- β 3 is synthesized initially as a preprotein in which the signal peptide residues are removed concomitant with translocation across the endoplasmic reticulum, likely by cleavage of a nonconsensus (Walter and Blobel, 1982) Leu-Ser peptide bond. By homology with TGF- β 1 and TGF- β 2, pro TGF- β 3 is predicted to comprise disulfide-linked monomers of 389 amino acids; a 277 amino acid prosegment and the 112 amino acid mature TGF- β 3 protein (ten Dijke *et al.*, 1988; Derynck *et al.*, 1988). Pro TGF- β 3 forms a dimeric polypeptide, which is likely glycosylated at four potential N-linked carbohydrate addition sites per monomer. Proteolytic release of mature TGF- β from the pro domain occurs at the basic sequence motif (Arg-Lys-Lys-Arg) between the pro and mature segments (Madisen *et al.*, 1989, 1990; Sha *et al.*, 1989). Attempts to express biologically active mature TGF- β 1 or TGF- β 3, in eukaryotic cells, in the absence of the Pro segment, suggest the Pro-region is required for correct folding of TGF- β (Gray and Mason, 1990; Haley, unpublished). The Pro-regions of individual TGF- β s are highly conserved between species but not between individual isoforms. TGF- β s are secreted as latent complexes, in which the Pro-polypeptide is physically associated with mature TGF- β via an interaction with a dissociation constant in the nanomolar range (reviewed Miyazono *et al.*, 1990; Gentry and Nash, 1990; Miyazono *et al.*, 1991). TGF- β s can then be converted to a biologically active form by acid activation or by treatment with a chaotropic agent (for TGF- β 3: Graycar *et al.*, 1989; ten Dijke *et al.*, 1990).

In previous studies, tissue from human umbilical cord, which is fetal in origin, was used to investigate embryonic isoforms of growth factors and growth inhibitors. Here we report the isolation of homogenous TGF- β 3 from human umbilical cord, and its comparison to recombinant TGF- β 3 by laser desorption mass spectrometry, protein sequencing, amino acid analysis and biological activity. TGF- β 3 was the major TGF- β isoform isolated from umbilical cord and was identical to a 'tumor growth inhibitor' protein previously described (TGI; Iwata *et al.*, 1986). TGF- β 3 was localized in umbilical cord by immunohistochemistry to perivascular smooth muscle.

MATERIALS AND METHODS

Bioassay

CCL64 cells, which have been shown to be highly

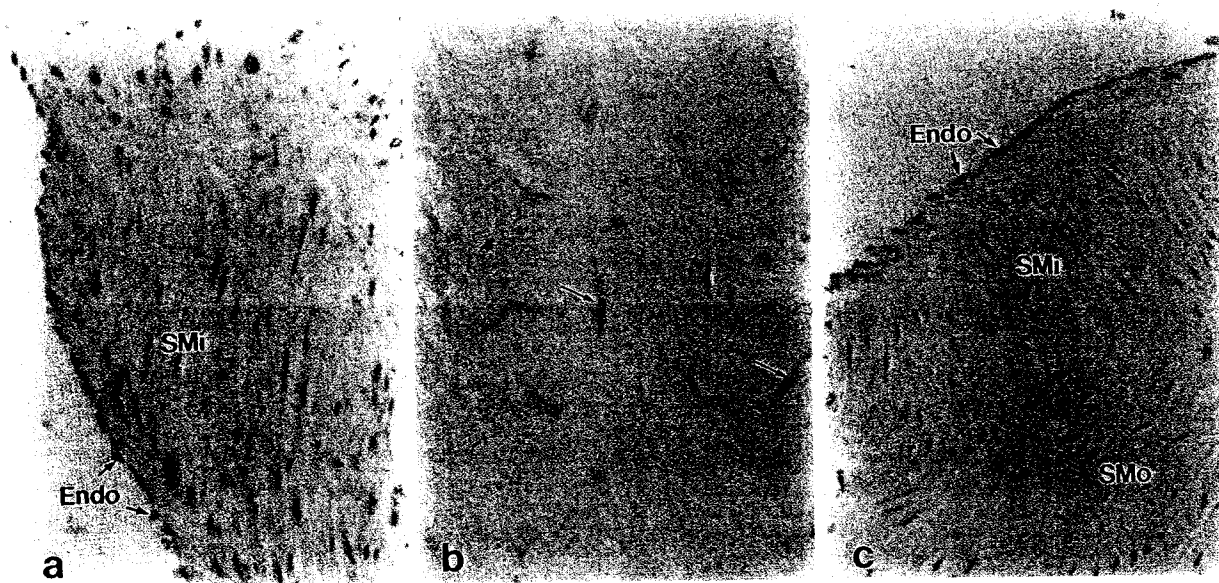
sensitive to growth inhibition by TGF- β s (Ranchalis *et al.*, 1987), were used for measurement of biological activity. Cells were plated on 96 well tissue culture plates (Falcon 3072) at a concentration of 3×10^3 cells per 50 μ l of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Partially purified or pure TGF- β 3 preparations were added directly to cell culture media. Supernatants from transfected cell lines (5 hours after plating) were acid activated prior to addition to tester cells. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was used to measure cell proliferation. Briefly, a filtered 0.2% solution of MTT/phosphate buffered saline (PBS) was prepared; 25 μ l of MTT was added to 100 μ l of CCL64 cells in culture media. Cells were incubated for 4 hours at 37°C, 5% CO₂. Cell media and MTT was aspirated. Metabolized MTT (blue formazan precipitate) was solubilized with 0.04 N HCl in 2-propanol and the optical density (540 nm) measured using a Bio Tek EL320 plate reader. For bioactivity neutralization experiments, TGF- β samples were first incubated in media containing 10 or 25 μ g/ml of the neutralizing antibody for 4 hrs at 37°C, prior to bioassay.

Preparation of Umbilical Cord Extracts

Human umbilical cord (100 g) was washed extensively to remove residual blood, extracted using a Waring Blender in acid-ethanol (375 ml of 95% (v/v) ethanol, 7.5 ml of concentrated HCl, 33 mg of phenylmethylsulphonyl fluoride (PMSF), 10 μ g/ml aprotinin, and 192 ml of water at 4°C) and stirred overnight at 4°C. The extract mixture was pelleted by centrifugation (3500 \times g), the supernatant was adjusted to pH 5 with concentrated ammonia and 2 M ammonium acetate (pH 5.2) was added to 1% of the total volume. The precipitate was removed by centrifugation (4900 \times g), the supernatant was collected and 4 volumes of diethyl ether and 2 of ethanol (95%, v/v) at 4°C were added. The mixture was allowed to stand for 48 hours at -20°C, allowed to reach ambient temperature, decanted, and residual organic phase allowed to evaporate. The precipitate was dissolved in and dialyzed against 1 M acetic acid.

Chromatography Conditions

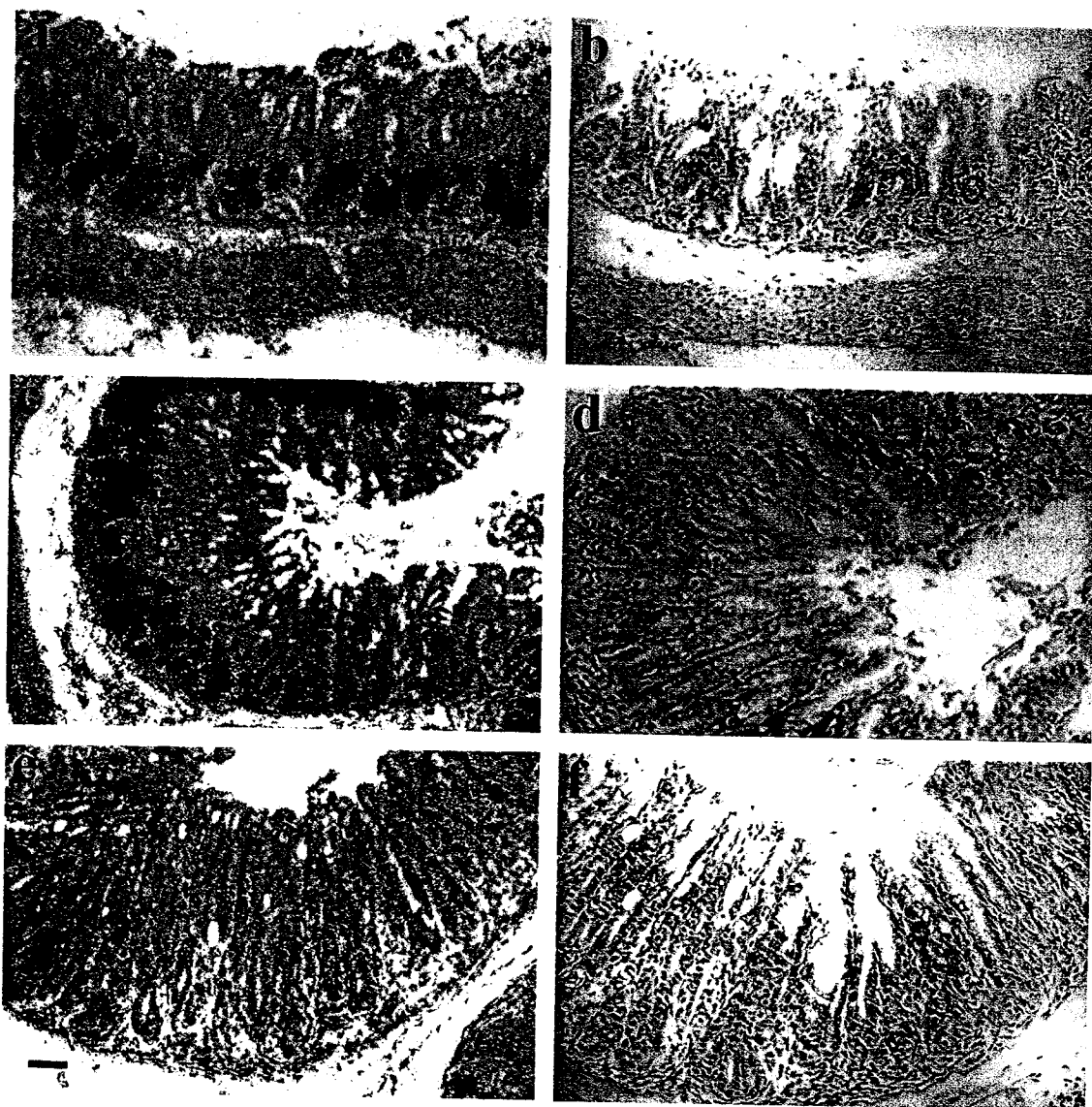
The ether/ethanol precipitate containing 47 mg of total protein was applied to a 5 cm \times 100 cm Biogel



Colour Plate IX

See Stewart *et al.*, Figure 7

Localization of TGF- β 3 in human umbilical cord. Photomicrographs of human umbilical cord sections immunostained for TGF- β 3 protein as described in the Materials and Methods section showing: (a) TGF- β 3 positive vascular smooth muscle layers (SMi) surrounding the umbilical vessel. Endothelial cells lining the blood vessel lumen are also shown, 400X; (b) stromal connective tissue in the umbilical cord did not have detectable TGF- β 3, though some fibroblasts (arrowed) were positively stained, 640X; (c) The specificity of immunohistochemical staining was confirmed by competitive preabsorption of the primary antibody with TGF- β 3 cognate synthetic peptide for both the inner longitudinal (SMi) and outer circular (SMo) layers with vascular smooth muscle around the umbilical vessel, 400X and the fibroblasts in the stromal layer (not shown).



Colour Plate X

See Goldenring *et al.*, Figure 1

TGF α mRNA and protein expression of MT-TGF α mice. *In situ* hybridization for TGF α mRNA (a,c,e) and immunohistochemistry (b,d,f) for TGF α protein were performed on sections of fundic mucosa of a 13 day old MT-TGF α mouse (a,b), a 21 day old mouse treated for 8 days with cadmium (c,d), and a 28 day old mouse treated for 15 days with cadmium (e,f). No expression of TGF α mRNA could be detected in normal littermate mice. Bar = 50 μ m.

P10 column (100–200 mesh; Biorad) equilibrated and eluted in 1 M acetic acid. Bioactive fractions were pooled and injected on an analytical C18 column (0.39 × 30 cm; μ Bondapak, Waters Assoc., Waltham, MA.) and protein elution monitored at 206 nm. TGF- β 3 was chromatographed using an acetonitrile (ACN): 0.05% trifluoroacetic acid (TFA)/H₂O mobile phase and gradient elution (0–15 min: 0–25% ACN; 15–55 min: 25–45% ACN; 55–70 min: 45–100% ACN) with a flow rate of 0.5 ml/min. Fractions were collected and characterized by SDS-PAGE, Coomassie or silver-stained, immunoblot analysis and by the mink cell growth inhibition assay. Material was further purified by reverse phase C 18 chromatography by gradient elution with 2-propanol, 0.05% TFA/H₂O (0–15% 2-propanol in 20 min.; 15–35% in 140 min.; and 35–100% in 160 min.) run at a flow rate of 1 ml/min., monitored at 206 nm.

Material to be characterized by laser desorption mass spectrometry was purified by P10 gel filtration chromatography as described above, followed by cation exchange chromatography (mono-S; Pharmacia) employing a linear gradient of 50 mM MES, pH 6.1, 0.1 M NaCl to 50 mM Hepes pH 7.5, 1 M NaCl. Bioactive material was affinity purified using a β 3-V antibody, Protein-G Sepharose affinity matrix (as described below), with elution of TGF- β 3 in 0.1% TFA.

In later experiments comparing biological activity of recombinant and cord TGF- β 3, umbilical cord extract in 1 M acetic acid (see above) was diluted 5-fold with water and 3 M Tris base added to a final concentration 0.15 M Tris-acetate (pH=7). The extract was centrifuged at 30,000 × g for 30 minutes at 4°C and the supernatant chromatographed by TGF- β 3 specific antibody affinity chromatography followed by reverse phase C4 HPLC (see below).

Preparation of Anti-TGF- β 3 Affinity Matrix

The affinity resin was prepared as follows: β 3-V antibody (ten Dijke et al., 1990) was purified by peptide affinity chromatography to the cognate peptide [NH₂-YLRSDTTHSTVLGLYNTLNPEASASY-COOH], mixed with AffiPrep-10 resin (10 mg protein/ml resin), the complex washed with 0.1 M solution borate pH 9 and dimethylpimelidate added to 20 mM. The resin was incubated for 30 minutes at room temperature and the crosslinking reaction was quenched with an excess of 0.2 M ethanolamine,

pH 8 and finally washed with phosphate buffered saline (PBS).

High Level Expression of Recombinant TGF- β 3

The TGF- β 3 cDNA was altered by phosphorothioate site-directed mutagenesis (Nakamaye and Eckstein, 1986) to either (1) delete the 5' most of two ATG codons; and (2) convert the Leu-Ser dipeptide within the signal peptide to an Arg-Ala dipeptide. Briefly, the TGF- β 3 cDNA was cloned into mp 18 (KpnI-BamHI; 5' to 3'), single-stranded template was prepared (Messing et al., 1981). The mutation of the 5' most ATG was directed with the oligonucleotide [5'-CCITTCGAAGTGCATGGTGGTGTGTGAGGACCCCTCCG-3']. The signal peptide mutation (Leu-Ser to Arg-Ala) was directed with the oligonucleotide [5'-GGTTCGAAGTGGACAGGGCGCGGCTGACCGTGGCAA-3']. Mutant templates are used to transform E.coli strain JM109 and were screened with 18 base detection oligonucleotides specific for the mutated sequences. Positively hybridizing clones containing the mutation were confirmed by dideoxy DNA sequencing. The expression vector used to transiently express large quantities of wild type and mutant TGF- β 3, was a derivative of plasmid CDM8 originally developed by Brian Seed (Aruffo and Seed, 1987) in which the bacterial origin and SupF gene were replaced by the ampicillin resistance gene and the pUC origin of replication. In addition, the polylinker region, splice site, and polyadenylation signal (polyA) in CDM8 was replaced with another polylinker followed by the very efficient bovine growth hormone splice site and polyA signal. Expression plasmids were purified by two rounds of CsCl gradient centrifugation followed by phenol:chloroform extraction and ethanol precipitation. Mutated and wild type TGF- β 3 expression plasmids were transfected into the COS-1 cell line (SV40 transformed African Green Monkey fibroblast line) by DEAE-dextran transfection. Briefly, 2 × 10⁶ COS-1 cells are placed in DMEM, 10% FCS in a 10 cm dish within 24 hr prior to transfection. DNAs, in 10 mM Tris.HCl, pH 7.5, 1 mM EDTA (TE) was diluted in PBS to 0.1 mg/ml. 250 μ l of diluted DNA was added to 80 μ l of DEAE-Dextran mix (100 mg/ml in PBS; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The mixture was added to COS-1 cells in 4 ml DMEM, 10% FCS, 50 μ g/ml

chloroquine diphosphate (100 mg/ml H₂O; Sigma Chemical Company, St. Louis, MO) and 1X Nutridoma HU serum supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 3 hr at 37°C, 5% CO₂. Cells are washed three times in DMEM, shocked with 2 ml of 10% dimethyl sulfoxide (DMSO)/PBS, washed twice, and replenished with fresh DMEM, 5% FCS. Cell conditioned media was collected 48 hours after transfection, acidified and assayed for biological activity.

The expression plasmids optimized for TGF-β3 production were stably transfected into Chinese hamster ovary (CHO) cells. 1 × 10⁶ dhfr- CHO cells (grown in Ham's F12, 10% FCS) in a 10 cm dish were transfected with 10 μg of DNA by calcium phosphate precipitation in the presence of 100 μg/ml chloroquine diphosphate for 6 hours at 37°C, 5% CO₂. Transfected cells were washed three times in PBS and selected for dhfr+ genotype in alpha MEM 10% dialyzed FCS for 10–14 days (selection medium). Individual clones were isolated using cloning cylinders and expanded. The primary transfectants were selected by increasing concentrations of methotrexate in selection medium (from 0 to 200 ng/ml). TGF-β3 concentration of conditioned media was assessed by electrophoresis and immunoblot with peptide affinity-purified polyclonal antibody β3-V. The CHO-M6 cell line was adapted to suspension cell growth in Iscove's medium (IMDM), 2% dialyzed FCS, 1X HB-CHO serum supplement and 2 mM glutamine.

TGF-β3 containing conditioned media was purified by ion exchange chromatography. Conditioned media was adjusted to 4 mM EDTA, 1 mM EGTA, 10 mM Tris-Cl Ph 7.5, 0.1 mM PMSF. One hundred ml of SP-Sepharose FF (Pharmacia) was added to 4 liters of CHO conditioned medium, pH adjusted to 6.0 using 2 M acetic acid and stirred overnight at 4°C. The resin was washed in a Buchner funnel with 100 mM NaCl, 25 mM MES pH 6.1, 0.1 mM PMSF until no further protein was eluted. Activity was eluted in a 6 cm × 25 cm column (~100 ml/hr) with 1 M NaCl, 50 mM HEPES pH 7.50, 0.1 mM PMSF at 4°C and collected in siliconized glass vials. EDTA was added to 1 mM, leupeptin to 4 μg/ml and soybean trypsin inhibitor to 10 μg/ml. Active material was further purified by β3-V antibody affinity chromatography (80 ml resin volume in a 5 cm by 10 cm column). Approximately 30 ml of eluent was pooled, Triton X-100 was added to 1% final concentration, azide to 0.02%, EDTA to 1 mM, leupeptin

and soybean trypsin inhibitor to 10 μg/ml and debris was removed by centrifugation at 12,000 g for 30 min. The supernatant was loaded twice onto a 27 ml β3V antibody affinity column (10 mg/ml) at 4°C and washed with an excess of 0.1 M Tris-HCl pH 7.5, 10 mM EGTA, 0.1 mM PMSF, 1% Triton X-100 (Solution A at a flow rate of ~60 ml/hr), followed by solution A with 1 M NaCl, 20 mM Tris-HCl pH 7.5 and finally eluted with 50 mM glycine-HCl pH 2.3 into siliconized glass tubes. Final purification was achieved by HPLC using a Vydac C4 reverse phase column, an acetonitrile: 0.1% TFA/H₂O mobile phase and gradient elution (0–20 min: 0–34% ACN; 20–90 min.: 34–39% ACN; 90–110 min.: 39–100% ACN) monitored at 280 nm. Specific activity of TGF-β3 was determined by the CCL64 growth inhibition assay. Fractions containing mature TGF-β3 were pooled (concentration ~1 mg/ml) and dried down in siliconized glass vials under vacuum. Protein was reconstituted in 10 mM HCl, 10% ethanol or 2 mM HCl, 0.1% Tween 80.

Electrophoresis and Immunodetection

Samples were subjected to electrophoresis on 5–20% linear gradient polyacrylamide gels using the discontinuous buffer system described (Laemmli, 1970). Gels were soaked for 10 minutes in transfer buffer containing 10 mM DTT was subsequently protein was electrophoretically transferred overnight at 4°C (Towbin *et al.*, 1979). Following transfer, the membrane was washed in PBS and PBS containing 0.1% Tween 20 (PBST), incubated in blocking buffer (Tropix Inc., Bedford, MA) for 1 hr at room temperature, washed in PBST and incubated with one of the following antibodies: 1 μg/ml TGF-β1 antibody, TGF-β2 antibody, pan TGF-β antibody (R&D Sys. Inc., Minneapolis, MN) or 5 μg/ml TGF-β3 antibody (Oncogene Science Inc., Uniondale, NY) for 1 hr at room temperature. Two washes in PBST were followed by a 30 minute incubation with 200 ng/ml alkaline phosphatase conjugated goat anti-rabbit antibody (Oncogene Science Inc.) for the TGF-β2, TGF-β3 and pan TGF-β antibodies. One μg/ml alkaline phosphatase rabbit anti-chicken (Zymed Labs Inc., S. San Francisco, CA) was used for the TGF-β1 antibody. Western blots were developed using a chemiluminescent substrate AMPPD as described (Tropix Inc.) sealed in a plastic bag and exposed to X-ray film for 5–60 minutes.

Extraction of TGF- β 3 from SDS-PAGE Gels

Approximately 100 ng of TGF- β 3 was electrophoresed by non-reducing SDS-PAGE (5–20% gradient gel), stained with Coomassie, destained and washed extensively with deionized water. The sample lanes was cut into 3 mm slices, incubated in 100 ml of 50% acetonitrile, 10 mM HCl and ground using a Teflon pestle. Gel fractions were shaken overnight at 4°C, centrifuged (5', 13,000 \times g) and the supernatant analyzed for bioactivity in the CCL64 cell inhibition assay with a MTT endpoint.

N-terminal Sequence, Amino Acid and Mass Determinations

TGF- β 3 from umbilical cord and CHO cells was transferred to PVDF membrane (Matsudaira et al., 1987) visualized by Coomassie staining (1/10 normal strength), destained, washed thoroughly and the band excised. Amino acid analysis and N-terminal sequencing was performed by Edman degradation using an Applied Biosystems 470A protein sequencer with on-line PTH amino acid detection, according to the manufacturer's specifications.

The molecular mass of TGF- β 3 was measured with matrix-assisted laser desorption mass spectrometry (Hillenkamp et al., 1991) using a time-of-flight mass spectrometer as previously described (Beavis & Chait, 1989, 1990). One microliter of 7 μ M purified TGF- β 3 in 0.1% TFA/acetonitrile (2:1, v/v) was mixed with 4 μ L of 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid, 5 g/L in 0.1% TFA/acetonitrile (2:1, v/v)], and 1 μ L of this solution was placed on the mass spectrometer probe tip and dried with a stream of cold air. The probe tip was irradiated with 10 ns duration pulses of light (wavelength 355 nm) from a Nd(YAG) laser. The ions created were accelerated in an electrostatic field and their time-of-flight was measured with a LeCroy 8828D transient digitizer. To improve the statistics, 200 individual spectra were added together. The time-of-flight spectrum was converted into a mass spectrum using horse myoglobin as an internal calibrant. The sample was prepared by mixing 1 μ L of 8 μ M recombinant TGF- β 3 with 5 μ L of sinapinic acid and applying 1 μ L of the solution to the mass spectrometer probe tip.

Immunohistochemistry

Polyclonal TGF- β 3V antibody was used to localize

TGF- β 3 in human umbilical cord sections. Fresh human umbilical cord tissues were formalin-fixed paraffin-embedded or snap frozen in methyl butane. Tissue sections of 5 μ m thickness were sectioned onto Probe-on Plus slides (Fisher Scientific, Pittsburg, PA) at room temperature. Immunohistochemistry of TGF- β 3 in human umbilical cord was carried out using a capillary Microprobe System (Fisher Scientific) at room temperature. Paraffin sections were deparaffinized with Hemo-De (Fisher Scientific) for 4 min., dehydrated, and air-dried before use. Sections were preincubated with 0.5% skim milk in phosphate buffered saline (PBS), pH 7.6, and 0.5% Triton X-100 for 10 minutes and 15 to 30 μ g/ml of the TGF- β 3V primary antibody was applied for 2 hours. The primary antibody was diluted with 0.5% skim milk/PBS/0.1% Triton X-100 buffer solution. Tissue sections were drained and washed with the milk/PBS/Triton X-100 dilution buffer. Biotinylated secondary antibody (BioGenex, San Ramon, CA) was applied for 20 min., washed and followed by the incubation with avidin-phosphatase for 20 minutes. Sections were washed with the skim milk/PBS/Triton X-100 dilution buffer three times. Freshly prepared Fast red chromogen solution (Cell Analysis Systems, Inc., Elmhurst, IL) was applied as substrate to the sections for 12 minutes and the reactions were stopped by rinsing the sections in deionized water. Sections were counter-stained with hematoxylin, dehydrated, and mounted.

Frozen tissue sections were cut, air-dried and stored in desiccated boxes at -80°C. Sections were acclimated to -20°C before use. Immunostaining with frozen sections was performed similarly as described above, except that the sections were fixed in cold acetone for 20 min. and extensively washed in PBS before preincubation with the skim milk/PBS/0.5% Triton X-100. Levamisole was added to the chromogen reaction solution to inhibit endogenous alkaline phosphatase activity in the tissue sections. Negative controls for the immunostaining reactions included the use of normal non-immune rabbit serum (1:1000), no primary antibody incubation, and preabsorption of the primary antibody with the synthetic TGF- β 3 peptide.

RESULTS

Purification of TGF- β 3 from Human Umbilical Cord

TGF- β 3 (TGI) was extracted from 50 grams of

