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Umbilical Cord Transforming Growth Factor-β3: Isolation, Comparison with Recombinant TGF-β3 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-β1, -β2 and -β3) have overlapping yet distinct biological actions and target cell specificities, both in developing and adult tissues. The TGF-β3 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-β3 was the major TGF-β isoform in umbilical cord (230 ng/g), and was physically and biologically indistinguishable from recombinant TGF-β3 and from the tumor growth inhibitory (TGI) protein found in umbilical cord. Immunohistochemistry using antipeptide TGF-β3 specific antibody showed TGF-β3 localization in perivascular smooth muscle.

KEYWORDS: transforming growth factor beta-3, TGF-β3, 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-β1, TGF-β2 and TGF-β3 (Roberts and Sporn, 1990). TGF-βs 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 suggest the TGF-βs act on these cell types to control inflammation and epithelial cell homeostasis. In contrast, TGF-βs serve as indirect mitogens for several mesenchymal cell types, and can regulate inflammatory cell activation and chemotaxis. In addition, TGF-βs regulate the synthesis and degradation of extracellular matrix (Ignotz and Massague, 1986) and play important roles in tissue development and repair. TGF-β1, β2 and β3 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-βs 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-β3.

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TGF-β3 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-β1 and TGF-β2, pro TGF-β3 is predicted to comprise disulfide-linked monomers of 389 amino acids; a 277 amino acid prosegment and the 112 amino acid mature TGF-β3 protein (ten Dijke et al., 1988; Derynck et al., 1988). Pro TGF-β3 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-β1 or TGF-β3, 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-βs are highly conserved between species but not between individual isoforms. TGF-βs 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-βs can then be converted to a biologically active form by acid activation or by treatment with a chaotropic agent (for TGF-β3: 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-β3 from human umbilical cord, and its comparison to recombinant TGF-β3 by laser desorption mass spectrometry, protein sequencing, amino acid analysis and biological activity. TGF-β3 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-β3 was localized in umbilical cord by immunohistochemistry to perivascular smooth muscle.

MATERIALS AND METHODS

Bioassay

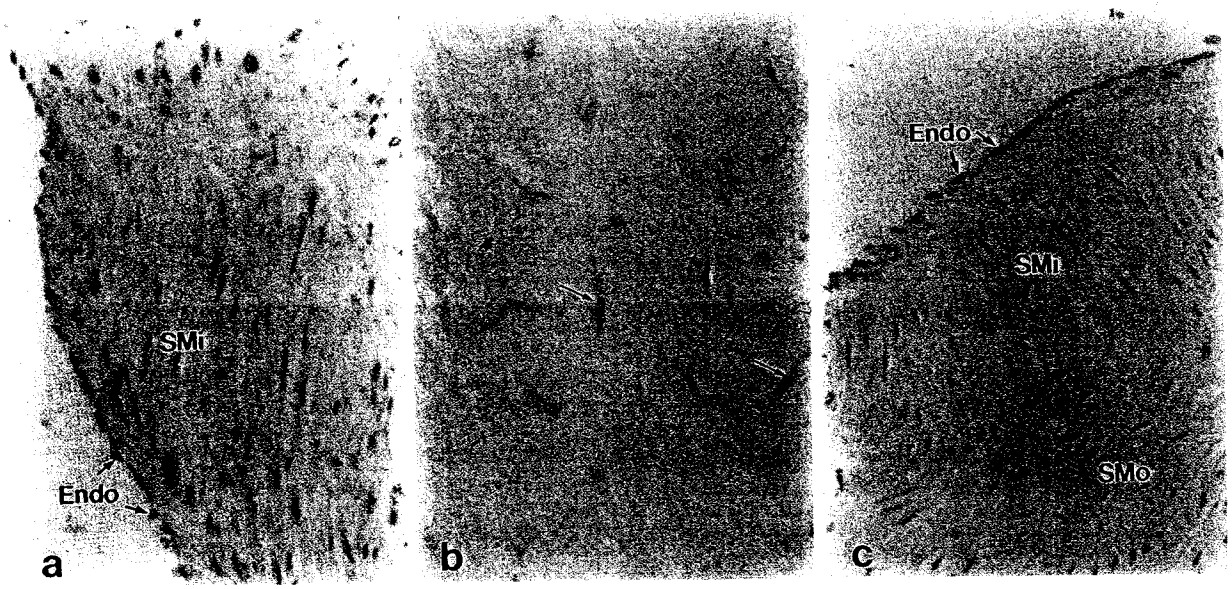
CCL64 cells, which have been shown to be highly sensitive to growth inhibition by TGF-βs (Ranchalis et al., 1987), were used for measurement of biological activity. Cells were plated on 96 well tissue culture plates (Falcon 3072) at a concentration of 3×10^3 cells per 50 µl of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Partially purified or pure TGF-β3 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 µl of MTT was added to 100 µl of CCL64 cells in culture media. Cells were incubated for 4 hours at 37°C, 5% CO_2. 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 µg/ml of the neutralizing antibody for 4 hrs at 37°C, prior to bioassay.

Preparation of Umbilical Cord Extracts

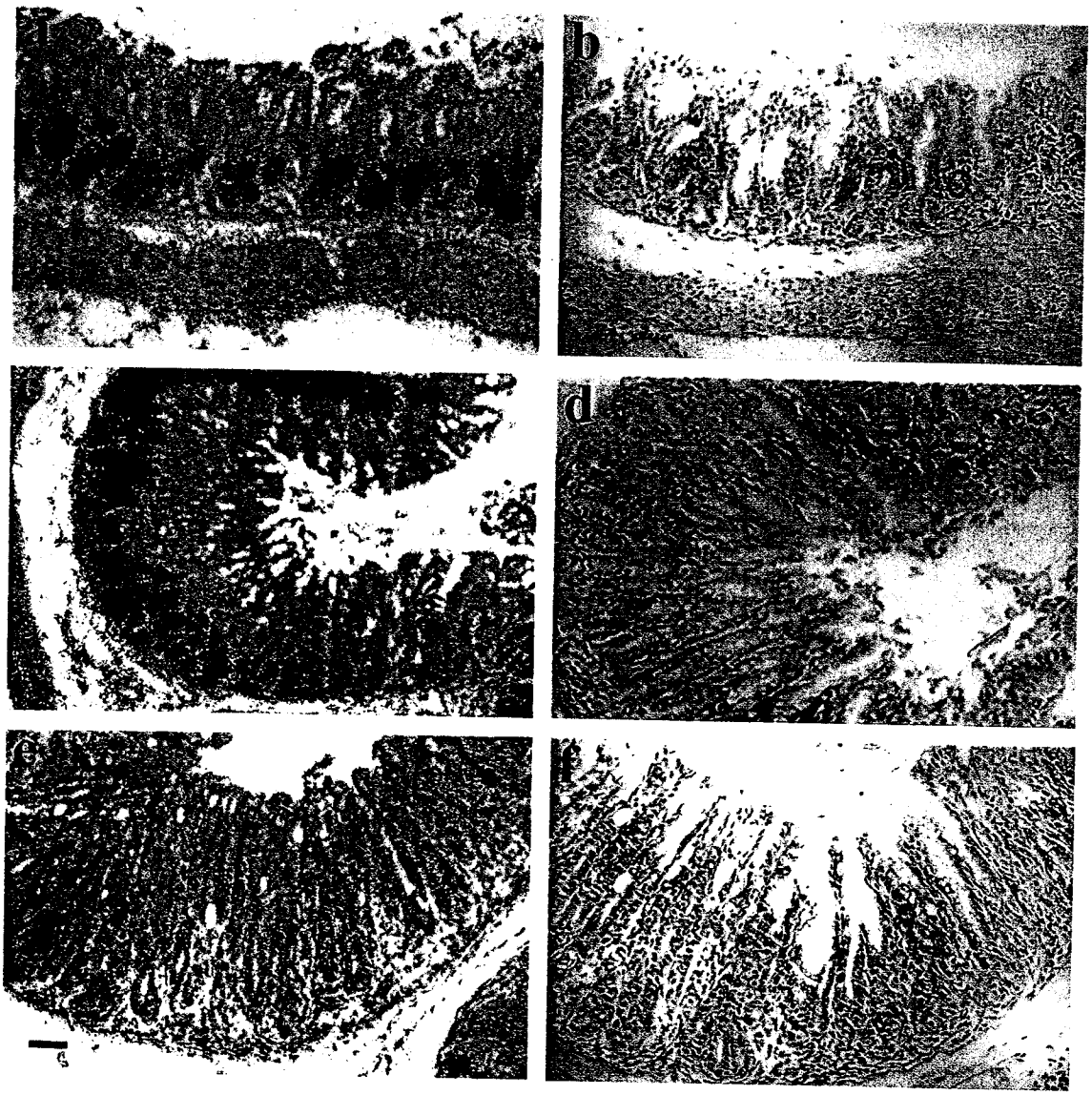
Human umbilical cord (100 g) was washed extensively to remove residual blood, extracted using a Waring Blender in acid-ethanol (375 ml of 95% (v/v) ethanol, 7.5 ml of concentrated HCl, 33 mg of phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml aprotinin, and 192 ml of water at 4°C) and stirred overnight at 4°C. The extract mixture was pelleted by centrifugation (3500 × 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 × 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 × 100 cm Biogel



Colour Plate IX
See Stewart *et al.*, Figure 7
Localization of TGF-β3 in human umbilical cord. Photomicrographs of human umbilical cord sections immunostained for TGF-β3 protein as described in the Materials and Methods section showing: (a) TGF-β3 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-β3, though some fibroblasts (arrowed) were positively stained, 640X; (c) The specificity of immunohistochemical staining was confirmed by competitive preabsorption of the primarily antibody with TGF-β3 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
TGFα mRNA and protein expression of MT-TGFα mice. *In situ* hybridization for TGFα mRNA (a,c,e) and immunohistochemistry (b,d,f) for TGFα protein were performed on sections of fundic mucosa of a 13 day old MT-TGFα 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 TGFα mRNA could be detected in normal littermate mice. Bar = 50 μm.

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; μBondapak, Waters Assoc., Waltham, MA.) and protein elution monitored at 206 nm. TGF-β3 was chromatographed using an acetonitrile (ACN): 0.05% trifluoracetic acid (TFA)/H_2O 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/H_2O (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 β3-V antibody, Protein-G Sepharose affinity matrix (as described below), with elution of TGF-β3 in 0.1% TFA.

In later experiments comparing biological activity of recombinant and cord TGF-β3, 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-β3 specific antibody affinity chromatography followed by reverse phase C4 HPLC (see below).

Preparation of Anti-TGF-β3 Affinity Matrix

The affinity resin was prepared as follows: β3-V antibody (ten Dijke et al., 1990) was purified by peptide affinity chromatography to the cognate peptide [NH_2-YLRSADTTHSTVLGLYNTLNPEASASY-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-β3

The TGF-β3 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 dipepetide within the signal peptide to an Arg-Ala dipeptide. Briefly, the TGF-β3 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′-CCTTTGCAAGTGCATGGTGGTGTGTGAGGACCCTCCG-3′]. The signal peptide mutation (Leu-Ser to Arg-Ala) was directed with the oligonucleotide [5′-GGTGCAAGTGGACAGGGCGCGGCTGACCGTGGCAA-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-β3, 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-β3 expression plasmids were transfected into the COS-1 cell line (SV40 transformed African Green Monkey fibroblast line) by DEAE-dextran transfection. Briefly, 2×10^6 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 μl of diluted DNA was added to 80 μl 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 μg/ml

chloroquine diphosphate (100 mg/ml H_2O; Sigma Chemical Company, St. Louis, MO) and 1X Nutridoma HU serum supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 3 hr at 37°C, 5% CO_2. 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. 1×10^6 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% CO_2. 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/H_2O 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-β3 from SDS-PAGE Gels

Approximately 100 ng of TGF-β3 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 × 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-β3 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-β3 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 μM purified TGF-β3 in 0.1% TFA/acetonitrile (2:1, v/v) was mixed with 4 μL of 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid, 5 g/L in 0.1% TFA/acetonitrile (2:1, v/v], and 1 μL 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-light 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 μL of 8 μM recombinant TGF-β3 with 5 μL of sinapinic acid and applying 1 μL of the solution to the mass spectrometer probe tip.

Immunohistochemistry

Polyclonal TGF-β3V antibody was used to localize TGF-β3 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 μm thickness were sectioned onto Probe-on Plus slides (Fisher Scientific, Pittsburg, PA) at room temperature. Immunohistochemistry of TGF-β3 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% Trition X-100 for 10 minutes and 15 to 30 μg/ml of the TGF-β3V 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-β3 peptide.

RESULTS

Purification of TGF-β3 from Human Umbilical Cord

TGF-β3 (TGI) was extracted from 50 grams of

human umbilical cord as described in Materials and Methods. Immunoblot analysis of the crude extract indicated that TGF-β3 was the major isoform of TGF-β present (results not shown). Based on bioassay and immunoblot measurements, the TGF-β3 concentration in the ether/ethanol precipitate was estimated to be 230 ng/g of starting cord material. The ether/ethanol precipitate was dissolved and chromatographed by P10 gel filtration in 1 M acetic acid. Under these conditions the considerable purification achieved is due both to the molecular sieving effect and a hydrophobic interaction of TGF-β3 with column resin, resulting in elution of TGF-β3 at a smaller apparent molecular weight (~14–15kD). Bioactivity was measured by growth inhibition of the mink lung epithelial cell line CCL64. Bioactive fractions were pooled and coincided with a single peak of TGF-β protein as detected by pan-TGF-β or TGF-β3 antibody immunoblots. The P10 TGF-β3 pool was further purified by C18 reverse-phase chromatography using an acetonitrile gradient. A single peak of activity was eluted at approximately 34% acetonitrile, which coincided with the peak of TGF-β3 immunoreactivity, as detected by TGF-β3 and pan TGF-β antibodies. No peak was detected at 31% acetonitrile, where TGF-β1 would be expected to elute (Stam et al., 1995). Bioactivity also co-eluted with the TGF-β protein peak after a second C18 chromatography using a 2-propanol gradient. SDS-PAGE analysis of the fractions reveals a silver-staining band at the same molecular weight (~25 kDa) and fraction numbers as the immunoblot bands. This material was characterized by non-reducing SDS-PAGE, stained with Coomassie Blue, and protein extracted from the sliced gel as described in Materials and Methods. Activity co-eluted with a protein with an apparent molecular weight of 25 kDa (not shown). Approximately 25% of the bioactivity applied was recoverable from this band and no activity was detectable elsewhere in the gel, under conditions where a 4000 fold dilution of the extracted band was required for bioassay linearity. It is important to note that the TGF-β present in the assay (estimated by immunoblot or silver-stained SDS-PAGE analysis using TGF-β standards) is sufficient to account for all of the observed bioactivity, with an IC_{50} of ~0.05 ng/ml. The yields of activity from the P10 and C18 chromatography steps are also in excess of 90%, suggesting that the major transforming growth factor activity of the extract is being analyzed.

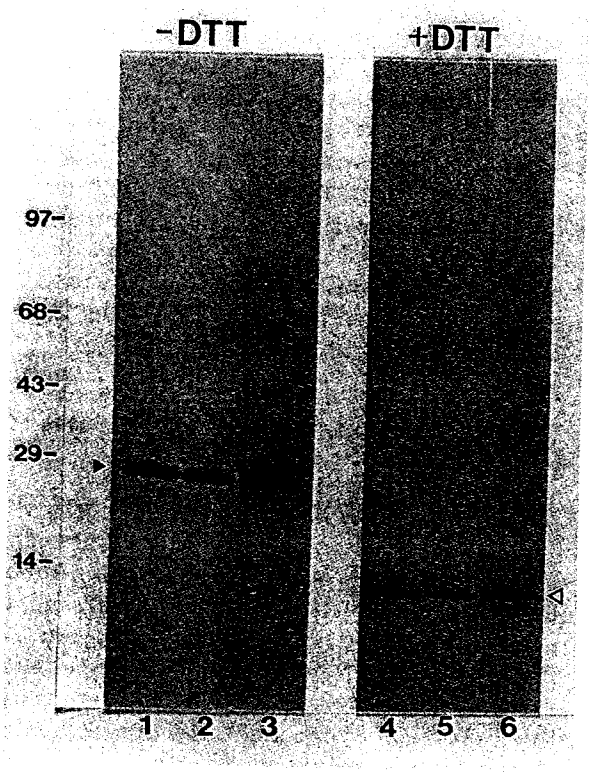


FIGURE 1. Electrophoretic mobility of cord TGF-β3 and recombinant TGF-β3 under reducing and non-reducing conditions. Recombinant TGF-β3 (lanes 1 and 4), bioactive gel filtration fraction (lanes 2 and 5) or bioactive acetonitrile C18 HPLC fraction (lanes 3 and 6) were electrophoresed under non-reducing or reducing (10 mM DTT) conditions.

Immunoblot and Antibody Neutralization of Cord TGF-β3

Bioactive cord samples were subjected to SDS-PAGE under reducing and non-reducing conditions and immunoblotted. Under reducing conditions, the TGF-β immunoreactive band changed in size from ~25 kDa to ~12.5 kDa (Fig. 1), as expected for the reduction of TGF-β dimer to monomer.

To identify the specific isoform(s) of TGF-β responsible for CCL64 growth inhibitory activity derived from umbilical cord, antibodies selectively recognizing the TGF-β1, β2 and β3 isoforms were used to (1) immunoblot bioactive Biogel P-10 and C18 HPLC purification fractions and to (2) neutralize growth inhibitory activity (Fig. 2). Aliquots of the bioactive pools from the P10 gel filtration and C18 reverse phase chromatography steps were subjected to SDS-PAGE and immunoblotted as described in Materials and Methods. Four antibody preparations

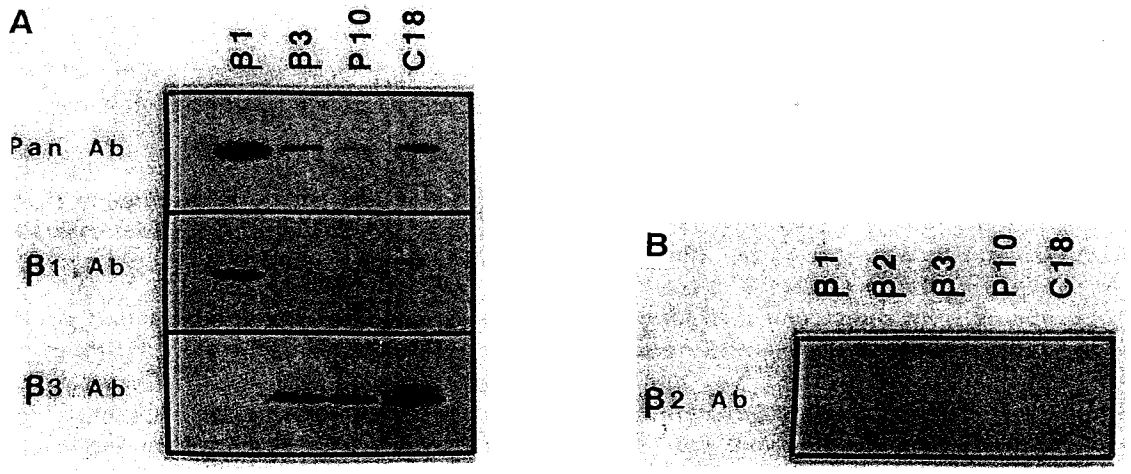


FIGURE 2. Recognition of tumor growth inhibitor TGI factor by a TGF-β3 specific monoclonal antibody. Samples from the TGF-β bioactive pools from the Biogel P-10 (~8.5 ng TGF-β3) and uBondapak C18 (~15 ng TGF-β3), TGF-β1 standard (10 ng), TGF-β2 standard (10 ng) and TGF-β3 standard (10 ng) were subjected to SDS gel electrophoresis under non-reducing conditions, transferred to nitrocellulose and incubated with A. pan TGF-β, TGF-β1 specific, TGF-β3 specific antibodies or B. TGF-β2 specific antibody. The TGF-β3 levels in the chromatography fractions were estimated by quantitative immunoblotting using TGF-β3 specific antibody and recombinant TGF-β3 standard.

with different specificities (anti-panTGF-β, anti-TGF-β1, anti-TGF-β2 and anti-TGF-β3) were used on immunoblots (Stam et al., 1995; Haley, unpublished). Antibody specificities were confirmed by immunodetection of 10 ng of purified TGF-β1, -β2 or -β3 (Fig. 2). Both P10 gel filtration and C18 reverse phase bioactive cord fractions exhibited strong immunoreactivity with TGF-β3 antibody, weak reactivity with pan-TGF-β antibody and no reactivity with TGF-β1 or TGF-β2 antibodies (Fig. 2), consistent with the majority of the TGF-β being the TGF-β3 isoform.

The identify of the TGF-β isoform(s) in the umbilical cord C18 fraction was also assessed by TGF-β isoform selective antibody neutralization of the growth inhibitory activity, under conditions previously described (Stam et al., 1995). The TGF-β3 antibody effectively neutralized C18 fraction bioactivity (Fig. 3), but had no effect on the bioactivity of purified TGF-β1 or TGF-β2 (not shown). In contrast, the TGF-β1 antibody had no effect on cord bioactivity under conditions which completely neutralize TGF-β1. The TGF-β2 specific antibody was shown to completely neutralize TGF-β2 bioactivity under the conditions used. However, the TGF-β2 antibody only weakly inhibited both the umbilical cord C18 fraction and purified recombinant TGF-β3 bioactivities to a similar extent, again suggesting that the majority of the bioactivity present in umbilical cord was derived from TGF-β3.

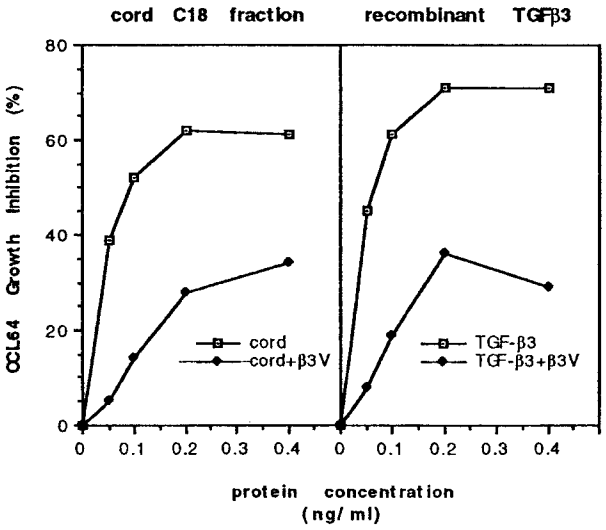


FIGURE 3. Specific neutralization of the umbilical cord bioactive C18 fraction by the TGF-β3 selective antibody, β3-V. Inhibition of CCL64 cell proliferation as a function of TGF-β concentration in the presence or absence of TGF-β3 selective neutralizing antibody (25 μg/ml). In control experiments (not shown) β3-V antibody failed to blot equivalent amounts of TGF-β1 or TGF-β2. Anti-TGF-β1 antibody (which effectively neutralized TGF-β1) failed to neutralize the bioactive umbilical cord C18 fraction. Neutralizing anti-TGF-β2 antibody weakly cross-reacted with recombinant TGF-β3, and weakly inhibited umbilical cord bioactivity.

High Level Expression of Recombinant TGF-β3

Prepro TGF-β3 contains an N-terminal sequence including N-met-lys-met-his. Site directed mutagenesis was used to remove the first methionine in the TGF-β3 expression plasmids. Expression levels with translation initiating from either the first or second methionine residue were measured following transient transfection into COS–1 fibroblasts. Consistent with previous studies (Graycar et al., 1990), expression from the second methionine resulted in a 2-fold higher level of expression and was used for all expression studies.

TGF-β3 contains a 23 amino acid signal peptide sequence which serves to translocate pro TGF-β3 across the endoplasmic reticulum. TGF-β3 and TGF-β2 signal peptides are most likely removed by cleavage of a Leu-Ser bond, which differs considerably from a consensus secretion cleavage signal (Walter and Blobel, 1982). Mutation of this cleavage sequence to the consensus cleavage sequence Arg-Ala had no measurable effect on efficiency of TGF-β3 expression (not shown).

Chinese hamster ovary cells were engineered to express TGF-β3 under the control of CMV promoter and copy number of the integrated expression plasmid was increased by co-transfection with dihydrofolate reductase (dhfr) and stepwise amplification with methotrexate. The resultant cell line CHO-M6 was adapted to suspension culture and stably secreted TGF-β3 (1.0–1.5 mg/l) over a period of several months in the absence of methotrexate. Both mature TGF-β3 homodimer with an apparent molecular weight of 25 kDa and uncleaved dimeric precursor were found in the culture supernatant. TGF-β3 was purified by a rapid three-step purification using cation exchange, antibody affinity and reverse phase (C4) HPLC chromatography steps. Overall yield was 61% with an 1867-fold purification.

Comparison of native and recombinant TGF-b3 by N-terminal sequence, amino acid composition, mass spectrometry and bioactivity

Bioactive material (TGI) was further purified by reverse phase chromatography (C18) using a 2-propanol, 0.1% TFA gradient. The peak fractions were pooled and proteins resolved on a SDS-PAGE gel under non-reducing conditions, electrophoretically transferred to a PVDF membrane and lightly stained with Coomassie Blue. The TGF-β band at 25 kDa was clearly resolved from other proteins. This material was excised and subjected to Edman degradation. As a positive control, recombinant TGF-β3 (~1 μg) was similarly electrophoresed, electroblotted, excised and sequenced.

The sequence obtained unequivocally identified the umbilical cord TGI 25 kDa protein as TGF-β3 (Figure 4). Upon reduction and alkylation with 4-vinyl pyridine, the repetitive yield comparing Leu_2 with Leu_{11} was 94%, with an absolute yield of 62% (5.0 pmol applied with 3.1 pmol recovered [Leu_2]). In the absence of reductive alkylation, the absolute yield was 69% (5.0 pmol applied with 3.4 pmol recovered). Recombinant TGF-β3 was sequenced in parallel. Upon reduction and alkylation the repetitive yield of Leu_2 and Leu_{11} was 97.2%, with an absolute yield (Leu) of 83.3% (31.7 pmol applied with 22.5 pmol recovered). In the absence of reductive alkylation the repetitive yield (Leu_2, Leu_{11}) was

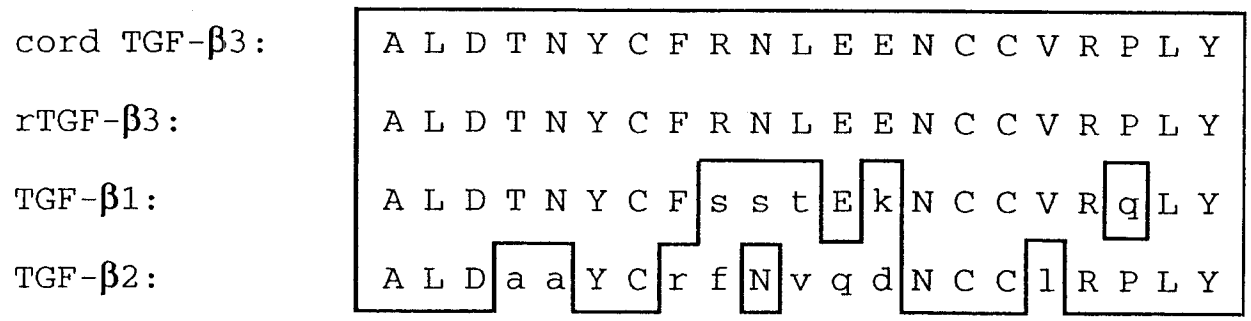


FIGURE 4. N-terminal sequence comparison of umbilical cord TGF-β3 (TGI), recombinant TGF-β3, TGF-β1 and TGF-β2. Lower case letters indicate amino acid substitutions.

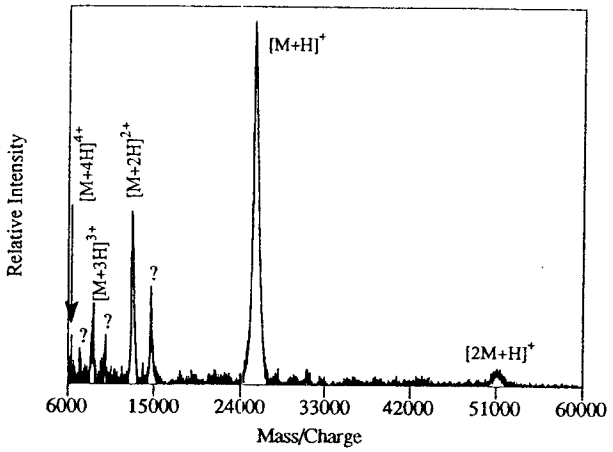


FIGURE 5. Molecular mass determination of cord TGF-β3 by matrix-assisted laser desorption mass spectrometry. $[M+nH]^{n+}$ designates the intact molecule with n protons attached to it. The peak labeled $[2M+H]^+$ corresponds to the singly protonated dimer of TGF-β3.

92.7%, with an absolute yield of 83.3% (15.9 pmol applied with 13.2 pmol recovered). The first 21 residues of the bioactive TGI fraction were identical with the N-terminal sequence of TGF-β3, but distinct from that of TGF-β1 and TGF-β2 (Fig. 4). Similarly, amino acid composition data was consistent with the predicted composition of TGF-β3 (not shown) and indicated that TGF-β3 was the major protein in the sequenced fraction.

The molecular weight of cord TGF-β3(TGI) was determined by mass spectrometry (Fig. 5). The peaks correspond to singly, doubly, triply, and quadruply protonated cord TGF-β3. The measured molecular mass of cord TGF-β was 25,426±7 Daltons (average of the values obtained from the peaks corresponding to the singly and doubly protonated molecule), which is in agreement (within the experimental error of the determination) with the measured and calculated molecular masses of recombinant TGF-β3, respectively 25,426±3 Daltons and 25,428 Daltons.

Both purified native and recombinant TGF-β3 showed similar mobility by SDS-PAGE under reducing conditions (Figure 6A). Bioactivity of purified native and recombinant TGF-β3 were compared by serial dilution and measurement of CCL64 cell growth inhibition (Figure 6B). Both protein preparations exhibited half maximal growth inhibition at ~35 pg/ml.

Immunolocalization of TGF-β3 in Umbilical Cord

The vascular smooth muscle cells of the human umbilical cord were the major cell type immunostaining for TGF-β3 (Fig. 7A). Both the inner longitudinal and outer circular muscle layers surrounding the two umbilical arteries and the vein showed reactivity with the TGF-β3V antibody. Similar patterns of immunostaining were observed in the formalin-fixed, paraffin-embedded as well as in the frozen umbilical cord sections. Slight immunostaining was detectable in some endothelial cells in frozen sections but not in paraffin-embedded ones.

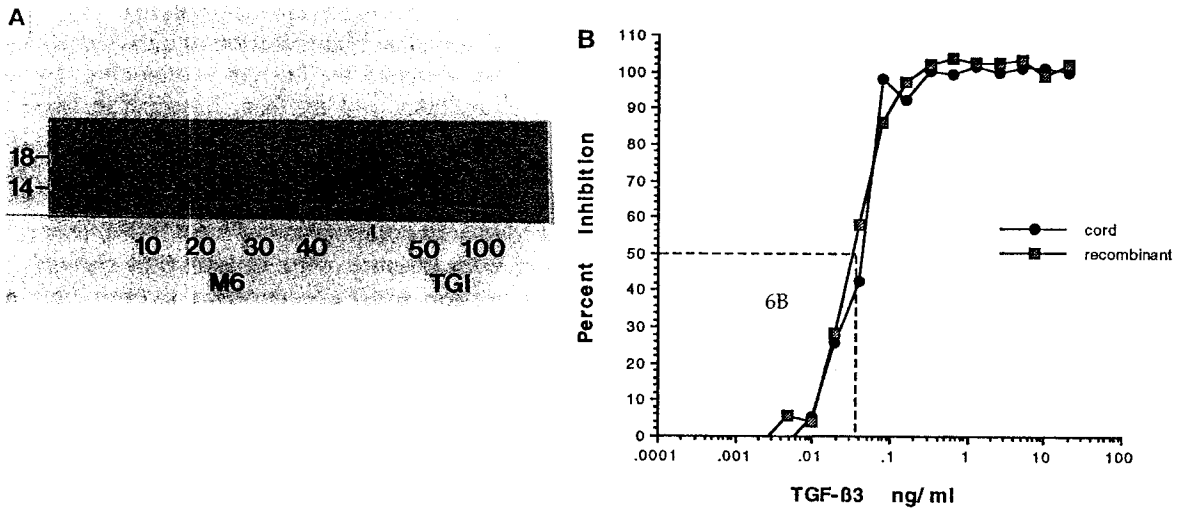


FIGURE 6. Comparison of umbilical cord and recombinant TGF-β3. A. Silver stained SDS electrophoresis gel of serially diluted recombinant TGF-β3 (M6) and cord TGF-β3 (TGI), under reducing conditions. B. TGF-β3 CCL64 growth inhibitory activity of umbilical cord and reference standard recombinant TGF-β3 (CIBA-Geigy). Half maximal growth inhibitory activity of both protein preparations was ~35 pg/ml.

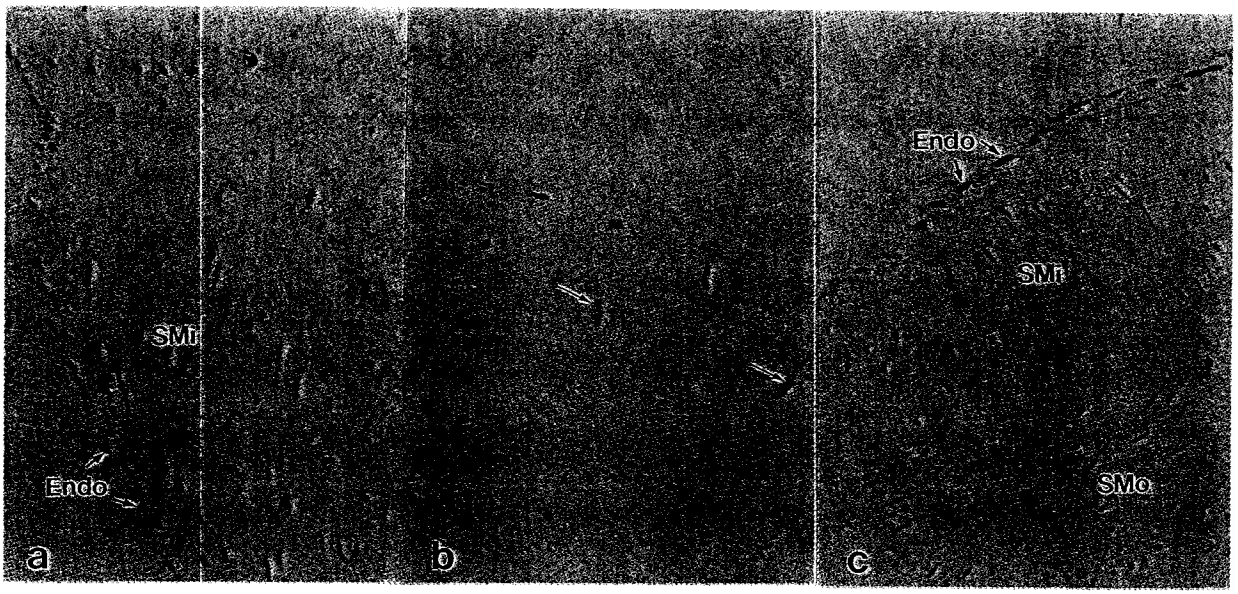


FIGURE 7. (See Colour Plate at back of issue.) Localization of TGF-β3 in human umbilical cord. Photomicrographs of human umbilical cord sections immunostained for TGF-β3 protein as described in the Materials and Methods section showing: (a) TGF-β3 positive vascular smooth muscle layers (SMi) surrounding 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-β3, though some fibroblasts (arrowed) were positively stained, 640X; (c) The specificity of immunohistochemical staining was confirmed by competitive preabsorption of the primarily antibody with TGF-β3 cognate synthetic peptide for both the inner longitudinal (SMi) and outer circular (SMo) layers of vascular smooth muscle around the umbilical vessel, 400X and the fibroblasts in the stromal layer (not shown).

Positive immunostaining was also found in the epithelial cells surrounding the umbilical cord. Some specific immunoreactivity could be detected within the stromal fibroblasts of the umbilical cord (Fig. 7B). Incubation with non-immune rabbit serum consistently yielded low-level, non-specific immunostaining. No background immunostaining was observed in the absence of the primary antibody. The preincubation of the TGF-β3V antibody with cognate TGF-β3 peptide eliminated immunoreactivity (Fig. 7C).

DISCUSSION

We have demonstrated by several criteria that TGF-β3 is the major isoform of TGF-β in umbilical cord. Under reducing and non-reducing conditions cord TGF-β migrated on SDS-PAGE with identical mobility as TGF-β3 (Fig. 1). Cord TGF-β was selectively recognized by the TGF-β3 specific antibody β3-V, but not by TGF-β1 or TGF-β2 specific antibodies (Fig. 2). Western blot analysis indicated that the cord bioactivity co-eluted with TGF-β3 immunoreactivity in both gel filtration and reverse phase HPLC chromatography steps and that no other TGF-β isoforms were present in significant amounts (data not shown). Cord bioactivity was abolished by preincubation with the TGF-β3 specific neutralizing antibody (Fig. 3), but not with TGF-β1 or TGF-β2 neutralizing antibodies (not shown).

The physical identification of native cord TGF-β3 was established by Edman sequencing, by amino acid analysis and by laser desorption mass spectrometry. Purified umbilical cord TGF-β (TGI) had protein sequence identify with TGF-β3 over the N-terminal 21 amino acids (Fig. 4). The measured amino acid composition of cord TGF-β was consistent with identity with TGF-β3 (not shown). Laser desorption mass spectroscopy experiments established the mass of cord TGF-β to be 25,426±7 Daltons (Fig. 5) and the mass of recombinant TGF-β3 to be 25,426±3 Daltons, both in good agreement with the predicted molecular mass of TGF-β3 (25,428 Daltons). Both cord TGF-β3 and recombinant TGF-β3 exhibited similar specific activity in the CCL64 growth inhibition assay (Fig. 6B). Finally, the correspondence between the molecular masses and bioactivity of cord TGF-β3 and recombinant TGF-β3 is in accordance with the hypothesis that the two

species are identical and that mature, native TGF-β3 is not post translationally modified.

Despite the fact that before extraction, the umbilical cord was washed to remove blood, one might have expected that a proportion of the 230 ng of TGF-β extracted per gram of cord would have been TGF-β1, since platelets are such a rich source of the latter (Frolik et al., 1983). TGF-β1 has been observed in human serum (84.1 ng/ml ±32.4), while TGF-β2 was generally undetectable (Wakefield et al., 1995). We readily detected TGF-β1 (10 ng) by using TGF-β1 specific antibodies in our immunoblot assays (Fig. 2) and bioactivity neutralization assays (Stam et al., 1995), but no TGF-β1 was detected in bioactive umbilical cord purification fractions by ether immunoblot or neutralization assays. If the cord tissue examined contained 100% blood by volume, then ~37% of the cord TGF-β which we analyzed in our immunoblot experiments should have been TGF-β1, an amount which we would have readily detected. Therefore, with a detection limit of ~1 ng TGF-β1 by immunoblot analysis, we estimate that the volume of blood within the cord sample must have been less than 18%. Similar conclusions have been reached by other investigators when extracting TGF-β from human placenta (Assoian et al., 1983).

The individual TGF-β isoforms can be differentially expressed in a tissue specific manner during development and in the adult. For example, in situ hybridization studies showed messenger RNA encoding TGF-β3 to be expressed in murine lung, heart, brain, liver, perichondrium, bone, olfactory lobe and testis during fetal development. TGF-β3 mRNA localizes strongly to the mesenchymal tissue adjacent to organs and to the spinal cord suggesting a role in mesenchymal condensation (Lehnert and Akhurst, 1988; Wilcox and Derynck, 1988).

The cellular localization of TGF-β3 in umbilical cord was examined by immunohistochemistry, in order to gain insight into its potential role in fully formed umbilical cord. Expression of TGF-β3 was confined mainly to the vascular smooth muscle cells. The expression of TGF-β3 in vascular smooth muscle in the umbilical cord suggests several possible roles TGF-β3 may play a role in the maintenance of the cord vasculature, or, alternatively, if the umbilical cord production of TGF-β3 also extends into the adjoining fetal placenta, TGF-β3 may serve an immunosuppressive function to prevent fetal rejection. In this regard, the immunosuppressive properties of the TGF-β family have been well characterized (Roberts and Sporn, 1990; Schull et al., 1992) and the TGF-β3 gene has been shown to be regulated by estrogens (Yang et al., 1993, 1994).

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