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A NEW METHOD FOR HIGH YIELD PURIFICATION OF TYPE BETA TRANSFORMING GROWTH FACTOR FROM HUMAN PLATELETS

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SUMMARY. A new method was developed for the purification of type beta transforming growth factor from human platelets. This method is a three-step procedure including gel filtration, weak cation exchange HPLC and reverse phase HPLC. All steps are carried out at low pH using exclusively volatile acidic buffer solutions. The sterile conditions and easy removal of salt by lyophilization facilitate the quantification of the growth factor in biological assays. Based on immunological characterization the purified acid-stable, highly basic transforming growth factor beta is the β_1 form. Using the present method pure platelet TGFβ_1 is obtained in very high yield. 40 units of outdated human platelets yield 800 μg pure TGFβ_1, which is about a 10-20 fold higher yield than reported for other purification procedures.

Within the past years there has been an exponential increase in research on a family of polypeptide factors that regulate cell growth and differentiation. The prototype of this family is transforming growth factor beta (TGFβ). TGFβ is a multifunctional regulator of cellular activity because of its widespread effects on growth, differentiation, embryogenesis and tissue repair (1-5).

It is now known that genetically distinct forms of TGFβ exist with similar functional and structural features. For this reason the polypeptide originally described as TGFβ is now designated TGFβ_1 and consists of two identical disulphide-linked β_1 subunits of MW 12500. TGFβ_1 has been purified to homogeneity from several non-neoplastic tissues, transformed cells and from media conditioned by several cell lines (5). Blood platelets represent the most concentrated source of TGFβ_1. A second form, TGFβ_2, is a homodimer of subunits which share 71% amino acid homology with TGFβ_1 (6). This form has been isolated from bovine bone (7,8), from porcine platelets (9) and also from media conditioned by human glioblastoma (10) and adeno carcinoma cells (11). In addition to TGFβ_2 a rare heterodimeric form containing one TGFβ_1 and one TGFβ_2 chain has been identified in porcine platelets (9).

In most of the procedures described for the purification of TGFβ_1 and TGFβ_2, urea is used during gel filtration in order to prevent interaction of the growth factor with the column material (9,12). Furthermore, during ion exchange chromatography, gradients of non-volatile salts are often used for elution of the growth factor (13,14,22). For this reason detection of the growth factor by biological assays requires extensive dialysis of the column fractions. An additional problem is the low recovery of growth factor activity obtained by these methods.

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Recently we described the use of a weak cation exchange HPLC column (CM-2-SW) for the separation of highly basic proteins, including polypeptide growth factors (23,25). In the present report combination of this cation exchanger with gel permeation and reverse phase chromatography is presented as a new method for the purification of TGFβ_1 from human platelets. During the whole procedure the pH is kept low, the conditions are sterile and exclusively volatile solvents are used, facilitating quantification of the growth factor by biological assays. Using this purification method the yield of TGFβ_1 from human platelets is highly improved over existing procedures.

MATERIALS AND METHODS

Platelet extraction – Human platelet concentrates (40 units, 2–5 days old) were obtained from the local Red Cross Blood Bank (Amsterdam, The Netherlands). The platelets were washed with calcium- and magnesium-free phosphate-buffered saline (PBS ϕ) containing 6 mM EDTA, centrifuged (3000xg, 10 min., 4°C) to remove remaining plasma proteins and frozen at -80°C. Frozen platelets were suspended in PBS ϕ- EDTA buffer, extracted with acid/ethanol and precipitated with ether according to the method of Roberts et al. (26) as modified by Assoian et al. (12). The precipitate was suspended in 1 M acetic acid and incubated overnight at 4°C. The supernatant containing the TGFβ activity was dialyzed against 0.2 M acetic acid in Spectrapor 3 membrane tubing (molecular weight cutoff, 3500; Spectrum Medical Industries, Inc., Los Angeles, Calif.) during 48 hr at 4°C and lyophilized.

Purification of platelet-derived TGFβ

– Bio-Gel P100 chromatography. The lyophilized platelet extract from 20 units was dissolved in 7.5 ml 1 M acetic acid. A small precipitate was removed by centrifugation and the supernatant was applied to a Bio-Gel P100 column (2.5x85 cm) equilibrated in 1 M HOAc. The column was eluted at room temperature at a flow rate of 12 ml/hr: 3.5 ml fractions were collected, and aliquots of the column fractions were assayed for TGFβ and PDGF as described below. Fractions containing TGFβ were pooled and lyophilized for further purification.

– Cation-exchange HPLC. The partially purified TGFβ from the gel filtration column was dissolved in 2 ml 1 M HOAc and subjected to cation-exchange HPLC. A combination of a Bio-Sil TSK HPLC guard column (75x7.5 mm; Bio-Rad) and a Bio-Sil TSK CM-2-SW column (250x4.6 mm; Bio-Rad), equilibrated in 1 M HOAc was used. The flow rate was kept constant at 0.8 ml/min. The column was eluted with 24 ml of equilibration buffer (1 M HOAc) and subsequently with a linear one-step gradient of 0–2 M NH_4Ac in 1 M HOAc (0.01 M NH_4Ac/min.). Fractions of 1.6 ml were collected in vials containing 3.2 ml 2 M HOAc in order to keep the pH low, thereby avoiding aspecific absorption of the growth factor to the vials. Column fractions were tested for TGFβ activity and the TGFβ peak was concentrated on a Seppak C18 cartridge (Millipore-Waters) prior to reverse phase HPLC. The sample was applied to the cartridge equilibrated in 0.1% TFA and protein was eluted with 4 ml 0.1% TFA/60% acetonitrile. Acetonitrile was removed from the eluted sample by solvent evaporation using a Speed Vac concentrator.

– Reverse phase HPLC. The concentrated TGFβ sample from the Seppak C18 column was applied to a C18 Hi-Pore reverse phase HPLC column (250x4.6 mm; RP318; Bio-Rad), preceded by a micro-guard™ HPLC column (Bio-Rad). The columns were equilibrated with 0.1% TFA/18% acetonitrile (pH 2.2). Protein was eluted by applying a linear gradient of 0.1% acetonitrile per min. at a flow rate of 1 ml/min. The gradient was prepared by mixtures of 0.1% TFA (pH 2.2) and 0.1% TFA/90% acetonitrile (pH 2.2) solvents. Fractions of 2 ml were collected and screened on TGFβ activity. The final TGFβ peak was pooled and stored in small portions at -80°C.

Anti-TGFβ antibodies. –Synthetic peptides, corresponding to the amino-terminal 29 amino acids of human TGFβ_1 and TGFβ_2 were synthesized on a Sam II peptide synthesizer (Biosearch, New Brunschwig Scientific) using t-Boc protection methodology (27). The peptides were assembled on p-methylbenzhydrylamine resin, cleaved from the

solid phase resin and deprotected with a mixture of trifluoromethane sulfonic acid, thioanisole and trifluoroacetic acid. The amino acid composition was verified using the Pico-Tag amino acid analysis system (Waters). Rabbits were immunized by intramuscular injections of the uncoupled peptides biweekly for 8 weeks using 250 μg of peptide per injection. The primary immunization was in Freund's complete adjuvant and the subsequent boosts were in incomplete Freund's adjuvant. The sera were collected from blood drawn two weeks following the final boost. The specificity of the antisera was tested on immunoblots.

Electrophoresis and immunoblot assays. – SDS-polyacrylamide gel electrophoresis was carried out in 15% gels (0.75 mm) according to Laemmli (15). Proteins were stained by the silver staining technique described by Merril et al. (16). For Western blotting the proteins were transferred to nitrocellulose paper by the method of Towbin et al. (17). The nitrocellulose papers containing the protein antigens were incubated with the primary antibody (1:50 dilution) at 37°C for 1 hr. The immune complexes were visualized as described by van Zoelen et al. (18).

Bioassays. The biological activity of TGFβ was assayed by its growth inhibitory action on exponentially growing mink lung CC164 cells resulting in a reduction of [^{3}H]-thymidine incorporation (19). Furthermore, the presence of TGFβ was determined by the loss of density-dependent inhibition of growth of NRK cells. [^{3}H]-thymidine uptake was measured in the presence of EGF (5 ng/ml) and insulin (5 μg/ml) during a 2 hr time pulse between 43 and 45 hr after mitogenic stimulation of confluent serum-free cultures (20). The presence of PDGF activity in column fractions was tested by the mitogenic effect of this growth factor on quiescent 3T3 cells using a [^{3}H]-thymidine incorporation assay (21).

RESULTS

Previous studies have shown that platelets are one of the best sources for the isolation of TGFβ in sufficient amounts (5,9,12,22). For all the procedures described for the purification of TGFβ, the recovery of this growth factor is low. Furthermore, the purification methods described in the literature are complicated by the use of gradients of non-volatile salts during ion exchange chromatography (22) or by the use of urea during gel filtration, which is required for efficient removal of contaminating proteins (9,12). To avoid these problems we developed a new procedure for the purification of TGFβ from human platelets resulting in a homogeneous growth factor preparation in high yield.

TGFβ has been purified from the platelet extract in a three-step procedure using filtration, weak cation-exchange HPLC and reverse phase HPLC. Fig. 1 represents the elution profile of the acid-ethanol platelet extract after Bio-Gel P100 gel chromatography in 1 M acetic acid. In first instance the position of TGFβ was determined by its growth inhibitory activity on ML-CC164 cells. However, at this stage of purification the ML-CC164 assay is unsatisfactory because of its high sensitivity for TGFβ, resulting in very broad peaks (not shown). For a sharper cutoff of the TGFβ peak the position of this growth factor was determined by SDS polyacrylamide gel electrophoresis as also described by Assoian et al. (12). Analysis of the TGFβ peak by SDS-PAGE was possible by the retarded elution of TGFβ on the gel filtration column, which was due to aberrant interaction of this growth factor with the column material (12). As shown in Fig. 1 (inset) TGFβ was separated from most of the contaminating bulk proteins. In addition, TGFβ was partially separated from platelet-derived growth factor (PDGF), which is strongly mitogenic for Swiss 3T3 fibroblasts. Fractions 74-100 of crude TGFβ were pooled, lyophilized and subjected to further purification.

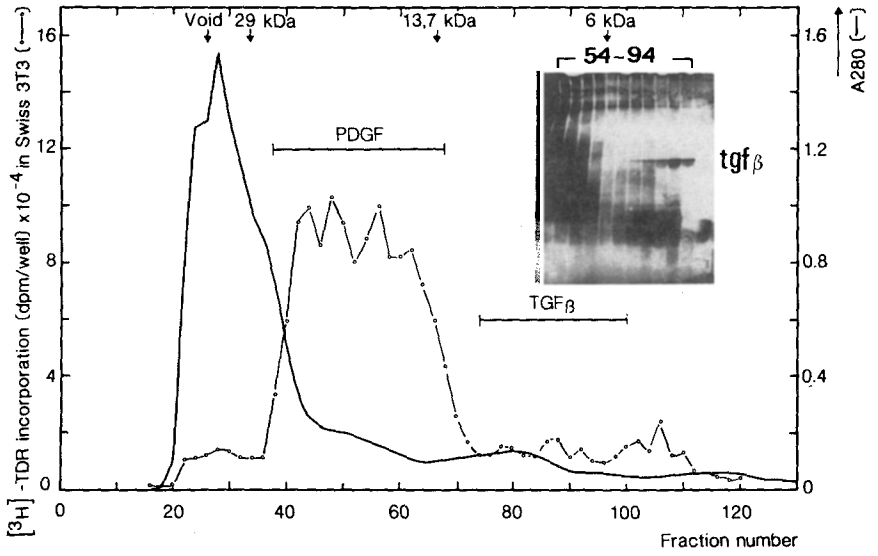

Figure 1. Bio-Gel P100 chromatography of the crude platelet extract (20 units). Absorbance at 280 nm (–) and [^{3}H]-thymidine incorporation into quiescent Swiss 3T3 fibroblasts (o-o). Inset: Silver-stained SDS-gel under non-reducing conditions of 100 μl aliquots from selected column fractions.

The crude TGFβ from the gel filtration step was applied to a cation-exchange column and eluted with a linear gradient of ammoniumacetate in 1 M acetic acid (Fig. 2). The position of TGFβ was determined by its capacity to induce loss of density-dependent inhibition of growth of NRK cells in the presence of EGF and

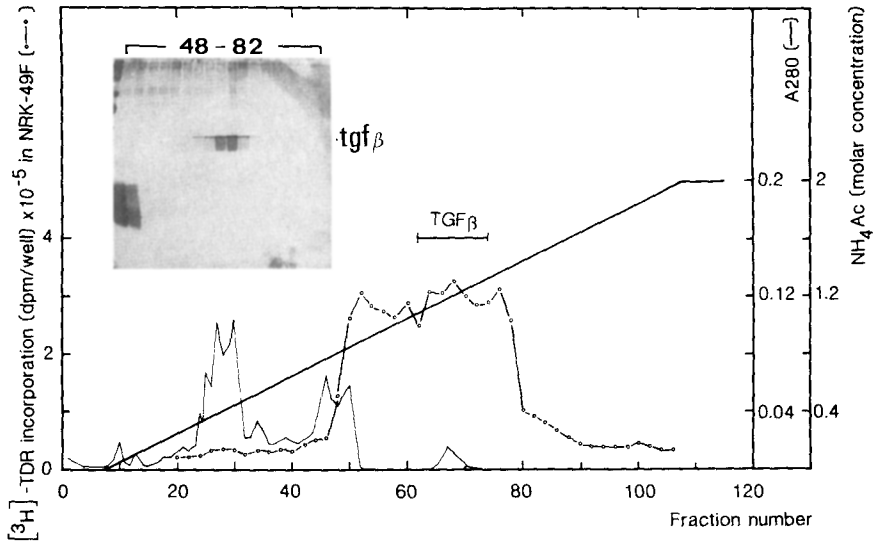

Figure 2. Cation-exchange HPLC chromatography (Bio-Sil TSK CM-2-SW column) of the lyophilized TGFβ pools from two runs on the P100 column (40 units). The column was equilibrated in 1 M HOAc and TGFβ was eluted by applying a linear gradient of 0-2 M NH_4Ac in 1 M HOAc. Absorbance at 280 nm (–) and loss of density-dependent inhibition of growth of NRK cells determined in the presence of EGF (5 ng/ml) and insulin (5 μg/ml) by [^{3}H]-thymidine uptake during a 2 h time pulse between 43 and 45 h after mitogenic stimulation (20). Inset: Silver-stained SDS-gel (non-reducing) of 125 μl aliquots from selected column fractions.

insulin as determined by [^{3}H]-thymidine uptake (Fig. 2; 20). However, as NRK-transforming activity has also been observed for PDGF in this assay the position of TGFβ was further confirmed by SDS polyacrylamide gel electrophoresis. As shown in Fig. 2 (inset) the silver-stained gel of the fractions with NRK-transforming activity revealed that TGFβ eluted at 1.1-1.3 M ammoniumacetate indicating a strong interaction of this growth factor with the column material. Furthermore, this column appeared to be very effective in separating TGFβ from the contaminating low molecular weight smear. The presence of NRK-transforming activity in fractions 45-60, lacking significant amounts of TGFβ on SDS-gel, might be explained by the occurrence of reminiscent PDGF activity in these fractions (20).

Final purification of TGFβ from human platelets was achieved by reverse phase HPLC chromatography. The pooled fractions of TGFβ activity from the cation-exchange column were concentrated on a Seppak column (see Materials and Methods) and applied to a reverse phase C18 HPLC column. TGFβ eluted between 32 and 34% acetonitrile as judged by SDS-gel electrophoresis (Fig. 3a, inset), loss of density-dependent growth of NRK cells (Fig. 3a) and growth inhibitory activity towards ML-CC164 cells (Fig. 3b). The difference in TGFβ peak width observed with the latter two bioassays can be explained by the higher sensitivity of the ML-CC164 assay than the NRK assay towards TGFβ. The peak fractions of pure TGFβ were pooled and the TGFβ activity was quantitated in the ML-CC164 growth inhibition assay by comparison to a dose response curve of human platelet TGFβ_1 obtained from Dr. M.B. Sporn (Fig. 3b, inset; 19). On this basis the yield of TGFβ by the present purification procedure is 800 μg of TGFβ from 40 units of washed outdated platelets. This is a 10-20 fold higher yield than reported for other procedures. The TGFβ preparation was stored in small portions in the HPLC solvent at -80°C at a concentration of 40 μg/ml.

The purity of the final TGFβ solution was shown by SDS polyacrylamide gel electrophoresis (Fig. 4). Under non-reducing conditions a single protein band with a molecular weight of 25000 dalton was observed (lane 1). Reduction of TGFβ prior to electrophoresis resulted in a 13000 dalton polypeptide band (lane 2). Even when loading microgram amounts of TGFβ only one single TGFβ band was detected thus indicating the absence of contaminating proteins in the TGFβ preparation.

In order to test the nature of TGFβ purified from human platelets by the present method, electroblots of SDS-gels containing pure TGFβ were incubated with antibodies raised against different forms of TGFβ. Polyclonal antibodies raised against the N-terminal amino acid residues 1-29 of TGFβ_1, that specifically recognize TGFβ_1 but do not recognize TGFβ_2 on immunoblots (Fig. 5, lane 1 and 3), form complexes with human platelet TGFβ on blots (Fig. 5, lane 5). The human platelet TGFβ is not recognized by polyclonal antibodies raised against the N-terminal residues 1-29 of TGFβ_2 (Fig. 5, lane 6) whereas TGFβ_2 purified from porcine platelets is recognized well (Fig. 5, lane 4). As these antibodies appeared to be highly specific for TGFβ_2 (Fig. 5, lane 2 and 4; van den Eijnden-van Raaij et al., manuscript in preparation) the platelet TGFβ purified by the new procedure is most likely the TGFβ_1 form.

DISCUSSION

In this paper we described a highly improved method for the purification of TGFβ from human platelets. Using a three-step sequence of chromatographic columns pure TGFβ was obtained from the crude acid-ethanol platelet extract in a yield of 20 μg of TGFβ per unit of platelets. This is a 10-20 fold increase over previous purifi-

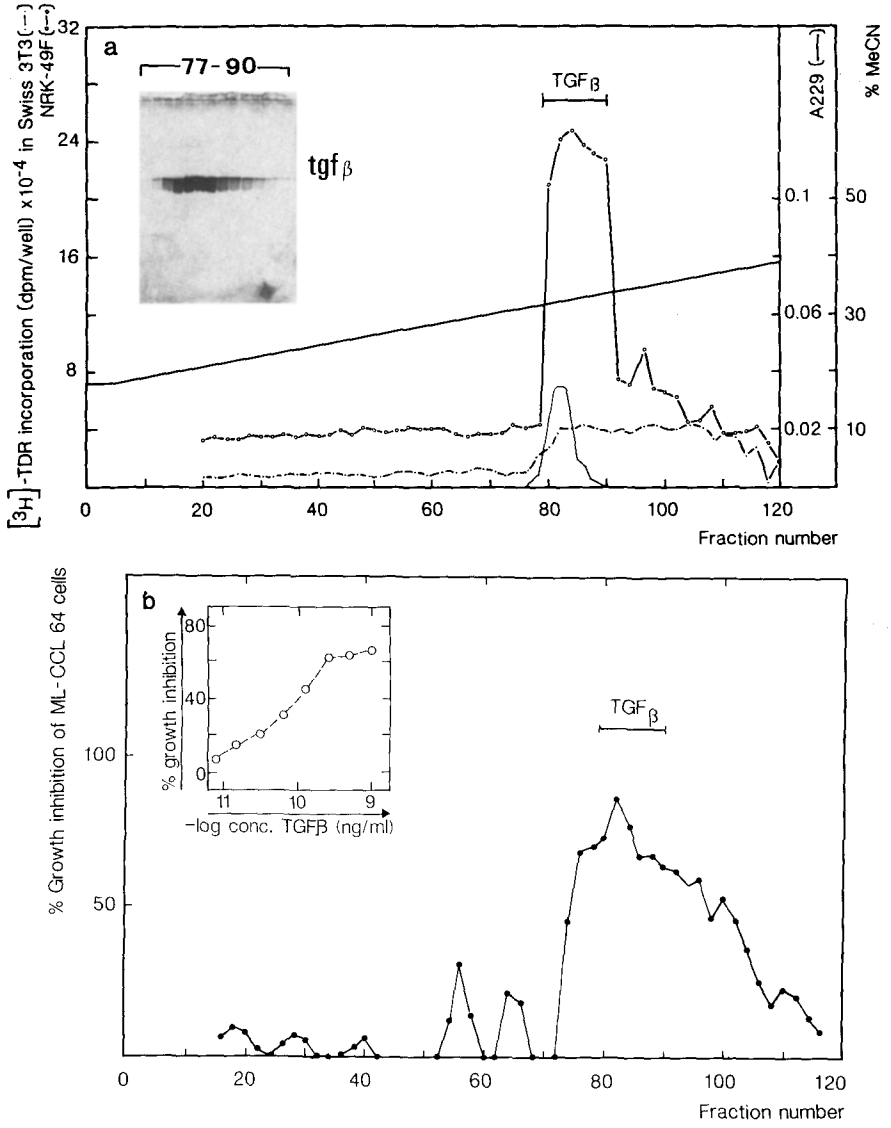

Figure 3. Reverse phase HPLC purification (C18 Hi-Pore, RP318, Bio-Rad) of TGFβ after cation-exchange HPLC and concentration on a Seppak C18 column. The column was equilibrated in 0.1% TFA-18% acetonitrile (pH 2.2) and TGFβ was eluted by a linear gradient of 0.1% acetonitrile/min. (a) Absorbance at 229 nm (—), [^{3}H]-thymidine incorporation in quiescent Swiss 3T3 fibroblasts (•—•) and loss of density-dependent inhibition of growth of NRK cells in the presence of EGF and insulin (0-0). Inset: Silver-stained SDS-gel (non-reducing) of 100 μl aliquots from selected column fractions. (b) Percentage of growth inhibition of exponentially growing mink lung ML-CC164 epithelial cells determined from [^{3}H]-thymidine incorporation by addition of 100 μl aliquots of the column fractions. Inset: Dose response curve for induction of growth inhibition of ML-CC164 cells by human platelet TGFβ$_1$ (o o) (Dr. M.B. Sporn).

cation procedures. Another advantage of the present method is the use of exclusively volatile solvents in each of the purification steps, facilitating the quantification of TGFβ by biological assays. Furthermore, the pH is constantly kept at a

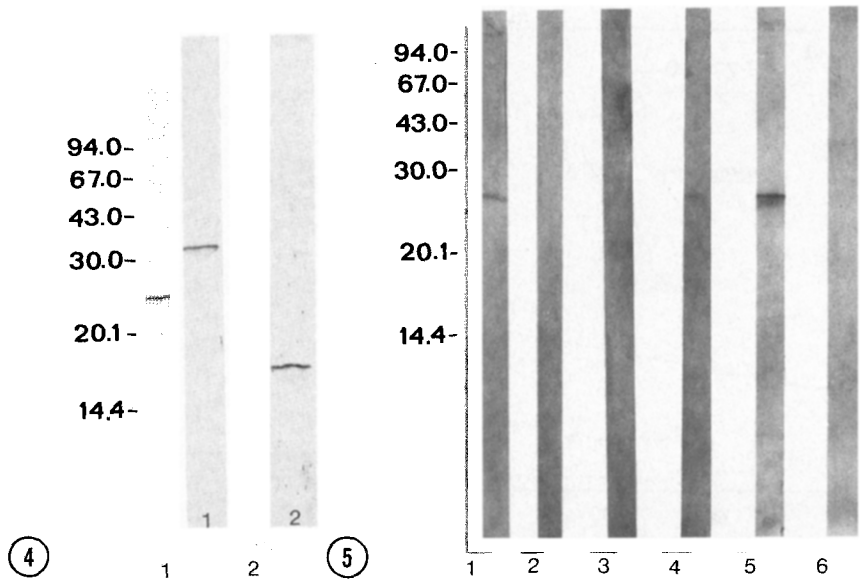

Figure 4. Silver-stained SDS polyacrylamide gel (0.75 mm, 15% gel) of 250 ng purified TGFβ from human platelets under non-reducing (1) and reducing conditions (2). Numbers denote molecular weight of calibration proteins in kilodalton.

Figure 5. Immunoblot of SDS-polyacrylamide gel (non-reducing conditions) after incubation with anti-TGFβ_1 (1-29) peptide antiserum (odd-numbered lanes) and anti-TGFβ_2 (1-29) peptide antiserum (even-numbered lanes). The immune complexes were detected with horse-radish peroxidase-labeled goat anti-rabbit antibody. Under reducing conditions the same specificity of the antibodies was observed. (1) and (2) 150 ng TGFβ_1 from human platelets (R and D Systems, Inc. Minneapolis); (3) and (4) 150 ng TGFβ_2 from porcine platelets (R and D Systems); (5) and (6) 150 ng TGFβ from human platelets (van den Eijnden-van Raaij et al., this paper).

low value (pH<4), thereby minimizing loss of growth factor activity by strong aspecific interactions of the growth factor with the column material.

The CM-2-SW weak cation-exchange HPLC column appeared to be the most effective step in separating TGFβ from the low molecular weight contaminating proteins present. Recently it was shown that in this system highly basic globular proteins could be separated according to their isoelectric point (23). Compared to the highly basic protein lysozyme (pI 11.0), TGFβ and also other basic polypeptide growth factors, including PDGF and FGF, elute later from the column, despite their lower pI values (24,25). Thus the retardation of TGFβ on the cation exchanger is the basis for the good separation of this growth factor from other impurities.

In work to be published elsewhere we will show that PDGF can be purified from human platelets in addition to TGFβ, according to a similar scheme as described in this paper (van Zoelen et al., unpublished results). Furthermore, the purification procedure may be used for the isolation of TGFβ- and PDGF-like growth factors from cell-conditioned media (25) and from other tissues containing these factors. In view of these results the present method, which is based on the separation of proteins according to size, charge and hydrophobicity at low pH, might be applied as a general method for the simultaneous purification of acid-stable basic polypeptide growth factors in high yield.

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