Purification of a growth factor related to platelet-derived growth factor and a type β transforming growth factor secreted by mouse neuroblastoma cells

A general strategy for the purification of basic polypeptide growth factors

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A general strategy was developed for the purification of basic polypeptide growth factors. This method is a combination of gel filtration, weak-cation-exchange h.p.l.c. and reverse-phase h.p.l.c., separating the proteins according to size, charge and hydrophobicity respectively. All steps are carried out at low pH with exclusively volatile acidic buffer solutions. The sterile conditions and easy removal of salt by freeze-drying facilitate the detection of the growth factors by biological assays. By using this method, homogeneous preparations of two basic growth factors were purified in high yield from mouse-neuroblastoma-Neuro-2A-cell-conditioned medium. It is shown that these purified factors are biochemically and immunologically related to platelet-derived growth factor and type β transforming growth factor from human platelets.

INTRODUCTION

Polypeptide growth factors are hormonally active polypeptides that are involved in the regulation of cell growth and differentiation (James & Bradshaw, 1984). A number of these factors that are able to stimulate or inhibit cell proliferation have been purified from various sources by a variety of methods. The best-characterized growth factors are the heat-stable and acid-stable factors, including epidermal growth factor, platelet-derived growth factor (PDGF) and related factors, insulin and insulin-like growth factors and the transforming growth factors TGF α and TGF β . No general strategy is available for the purification of these acid-stable growth factors. For example, the methods described for the purification of PDGF from human platelets and PDGF-like growth factors from conditioned media or cell lysates of transformed cells are quite different (Heldin *et al.*, 1979, 1980, 1986; Nistér *et al.*, 1984; Westermarck *et al.*, 1986; Stroobant *et al.*, 1985). Consequently the properties of these growth factors are difficult to compare, since selectivity of one or the other method for a specific form of these growth factors cannot be excluded (Heldin *et al.*, 1986). Thus for a correct identification of the nature and properties of the acid-stable growth factors produced by different cells a general purification scheme is required.

Previously we reported the chromatographic properties of a silica-based weak-cation-exchange (CM-2-SW) h.p.l.c. column at low pH (Van den Eijnden-Van Raaij *et al.*, 1987). With the use of acidic volatile solvents this column appeared to be very effective in the separation of extremely basic proteins and resulted in high protein yields. In the present paper we introduce this cation-exchange chromatography step in combination with gel permeation and reverse-phase h.p.l.c. as a general method for the purification of basic polypeptide growth

factors. In this study we apply this method for the purification of a PDGF-like growth factor and type β TGF from the conditioned medium of neuroblastoma Neuro-2A cells. During the whole procedure the pH is low, the conditions are sterile and exclusively volatile solvents are used, facilitating the detection of nanogram amounts of growth factor by biological assays. Furthermore, with the use of this purification method the yield of the acid-stable growth factors is highly improved over existing procedures.

MATERIALS AND METHODS

Collection of Neuro-2A-cell-conditioned medium

C1300 mouse neuroblastoma cells, clone Neuro 2A, were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Cells were cultured routinely in Dulbecco's modified Eagle's medium buffered with 25 mM-Hepes, pH 7.6, and supplemented with 7.5% (v/v) fetal-calf serum (GIBCO).

For the collection of conditioned medium from Neuro-2A cells the method described by Van Zoelen *et al.* (1985a) was used. In short, 10^7 cells were plated in a 640 cm² glass roller bottle in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham F 12 medium containing 15 mM-Hepes and 17.6 mM-NaHCO₃, pH 7.6 (DFBH medium), supplemented with 7.5% (v/v) calf serum [75% (v/v) newborn-calf serum/25% (v/v) fetal-calf serum; GIBCO]. After 24 h the medium was exchanged for 60 ml of serum-free DBFH medium, supplemented with 30 nM-Na₂SeO₃ and 10 μ g of human transferrin/ml. After the next 8 h the medium was discarded and the cells were then grown for 24 h in 75 ml and subsequently for 24 h in 60 ml of the latter serum-free medium. The 135 ml of Neuro-2A-cell-conditioned

Abbreviations used: PDGF, platelet-derived growth factor; TGF, transforming growth factor.

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medium collected was passed through an AP-15 pre-filter (Millipore) and freeze-dried in the presence of 1 μ g of phenylmethanesulphonyl fluoride (Sigma Chemical Col.)/ml.

Purification of the PDGF-like growth factor and a type β TGF from Neuro-2A-cell-conditioned medium

Conditioned medium from Neuro-2A cells was freeze-dried and subsequently extracted with 1 M-acetic acid. The extract was dialysed against 0.2 M-acetic acid in Spectrapor 3 membrane tubing (molecular-mass cut-off 3500 Da; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) at 4 °C and freeze-dried again. The residue was subjected to a number of chromatography steps that are based on the following principles for protein separation: molecular mass (Bio-Gel P-100), charge (Bio-Sil TSK CM-2-SW h.p.l.c.) and hydrophobicity (reverse-phase h.p.l.c.) of proteins.

Bio-Gel P-100 chromatography. Freeze-dried extract from 4 litres of Neuro-2A-cell-conditioned medium was dissolved in 7 ml of 1 M-acetic acid. A small precipitate was removed by centrifugation and the supernatant was applied to a Bio-Gel P-100 (100–200 mesh; Bio-Rad Laboratories) column (850 mm $\times$ 25 mm) equilibrated in 1 M-acetic acid (Van Zoelen *et al.*, 1984). The column was eluted at room temperature at a flow rate of 12 ml/h: 3.5 ml fractions were collected. Samples of the even-numbered fractions were assayed for mitogenic activity on Swiss 3T3 fibroblasts as a bio-assay for PDGF-like growth factors (Van Zoelen *et al.*, 1984) and for growth-inhibitory activity on mink lung CC164 cells as a bio-assay for TGF β (Van Zoelen *et al.*, 1986a). The mitogenically active and growth-inhibiting fractions were separately pooled and freeze-dried for further purification.

Weak-cation-exchange h.p.l.c. Cation-exchange h.p.l.c. was carried out on equipment from Millipore-Waters comprising a model 680 automated gradient-controller supplied with two 510 solvent pumps and a 481 variable-wavelength detector linked to a BD 41 chart recorder from Kipp. A combination of a Bio-Sil TSK h.p.l.c. guard column (75 mm $\times$ 7.5 mm; Bio-Rad Laboratories) and a Bio-Sil TSK CM-2-SW column (250 mm $\times$ 4.6 mm; Bio-Rad Laboratories), equilibrated in 1 M-acetic acid, was used. The freeze-dried pool of mitogenically active (or growth-inhibiting) fractions from the Bio-Gel column was dissolved in 1 ml of 1 M-acetic acid and applied to the column with the use of a loop injector (U6K; Millipore-Waters). The flow rate was kept constant at 0.8 ml/min. The column was eluted by 24 ml of equilibration buffer (1 M-acetic acid) and subsequently with a linear one-step gradient (for the growth-inhibitory fractions; 0.01 M-ammonium acetate/min) or three-step gradient (for the mitogenically active fractions) of 0–2 M-ammonium acetate in 1 M-acetic acid. For the three-step gradient, the initial steep gradient of 0–0.36 M-ammonium acetate (0.01 M-ammonium acetate/min) was followed by a slow gradient of 0.36–1 M-ammonium acetate (0.002 M-ammonium acetate/min) and a steep gradient of 1–2 M-ammonium acetate (0.01 M-ammonium acetate/min), respectively. Fractions (1.6 ml) were collected in vials containing 3.2 ml of 2 M-acetic acid in order to keep the pH low, thereby avoiding aspecific adsorption of growth-factor activity on the walls of the

vials. The absorbance was measured at 280 nm. Mitogenic activity or growth inhibition was determined in the even-numbered fractions and the active peaks were concentrated on a Sep-Pak C₁₈ cartridge (Millipore-Waters).

The Sep-Pak cartridge was activated with 5 ml of acetonitrile (Rathburn Chemicals) and then washed successively with 5 ml of each of the following wash fluids: distilled water, 1 M-acetic acid, 1 M-acetic acid/1 M-ammonium acetate, 1 M-acetic acid, 0.1 % (v/v) trifluoroacetic acid (Merck), 0.1 % (v/v) trifluoroacetic acid/50 % (v/v) acetonitrile, 0.1 % (v/v) trifluoroacetic acid and 1 M-acetic acid. Active fractions from the Bio-Sil TSK CM-2-SW column were applied to the cartridge at a flow rate of 20 ml/h. The cartridge was washed with 5 ml of 0.1 % trifluoroacetic acid, and protein was eluted with 4 ml of 0.1 % trifluoroacetic acid/50 % acetonitrile. Acetonitrile was removed from the eluted sample by solvent evaporation in a Speed Vac concentrator (Savant).

Reverse-phase h.p.l.c. For reverse-phase h.p.l.c. the same equipment was used as described for cation-exchange h.p.l.c. After concentration on a Sep-Pak C₁₈ column the partially purified growth-factor activity was applied to a C₁₈ Hi-Pore reverse-phase h.p.l.c. column (250 mm $\times$ 4.6 mm; RP 318; Bio-Rad Laboratories) preceded by a Micro-Guard h.p.l.c. column (Bio-Rad Laboratories). The columns were equilibrated with 0.1 % trifluoroacetic acid/18 % acetonitrile, pH 2.2. Protein was eluted by applying a linear gradient of 0.1 % acetonitrile/min at a flow rate of 1 ml/min. The gradient was prepared by mixtures of 0.1 % trifluoroacetic acid, pH 2.2, and 0.1 % trifluoroacetic acid/90 % acetonitrile, pH 2.2, solvents. Fractions (2 ml) were collected and screened for mitogenic or growth-inhibitory activity. The absorbance was monitored at 229 nm.

Metabolic labelling of neuroblastoma cells

Confluent cultures of Neuro-2A cells were incubated with [³⁵S]cysteine (0.1 mCi/ml) for 22 h in cysteine-free RPMI-1640 medium (Flow Laboratories). This medium was collected and immunoprecipitated according to the method of Betsholtz *et al.* (1983). First, medium was preincubated with normal rabbit serum for 20 h at 4 °C, followed by an incubation with Protein A-Sepharose beads for 2 h at 4 °C. The supernatant was then incubated with PDGF antiserum for 20 h at 4 °C, and the immune complexes adsorbed on Protein A-Sepharose were analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions. Autoradiographs were exposed to Kodak X-Omat AR films at –70 °C.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out in 15 % gels (0.75 mm thick) according to the procedure of Laemmli (1970). The stacking gel contained 5 % polyacrylamide. Samples for SDS/polyacrylamide-gel electrophoresis were solubilized in 5 % SDS in the absence or in the presence of reducing agents by heating at 100 °C for 5 min. Proteins were fixed in a solution of 50 % (v/v) methanol/12 % (v/v) acetic acid overnight and stained by using the silver staining technique described by Merrill *et al.* (1980).

Isolation of mRNA and Northern blotting

RNA was extracted from confluent N_2A cultures grown on 150 cm² tissue-culture flasks. After aspiration of the medium, the cells were lysed directly on the plates with 2.5 ml of 4 M-guanidine thiocyanate. RNA was isolated by the procedure of Chirgwin *et al.* (1977). Polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography and quantified by measuring the absorbance at 260 nm. Samples (7.5 μ g) were denatured in 50 % (v/v) formamide in 2.2 M-formaldehyde in MEN buffer (20 mM-Mops / 1.0 mM-EDTA / 5.0 mM-sodium acetate buffer, pH 7.0) for 15 min at 65 °C and electrophoresed in 0.8 % agarose gels containing 2.2 M-formaldehyde. After electrophoresis, RNA was stained with ethidium bromide and transferred to nitrocellulose filters in 20 $\times$ SSC (1 $\times$ SSC is 0.15 M-NaCl / 15 mM-sodium citrate buffer, pH 7.0). After transfer, the filters were rinsed in 2 $\times$ SSC and baked at 80 °C for 2 h under vacuum.

Filters were pre-hybridized for 4 h in a buffer composed of 5 $\times$ SSC, 2 $\times$ Denhardt's solution, 50 mM-sodium phosphate buffer, pH 6.8, 10 mM-EDTA, 0.1 % SDS and 0.1 mg of sheared salmon sperm DNA/ml containing 50 % (v/v) formamide and hybridized in the same buffer with 1 $\times$ 10⁶–2 $\times$ 10⁶ c.p.m. of ³²P-labelled probe/ml for 16–24 h. Probes were obtained by nick translation of DNA fragments with [α -³²P]dCTP (> 3000 mmol) and DNA polymerase I to a specific radioactivity of 0.5 $\times$ 10⁹–1 $\times$ 10⁹ c.p.m./ μ g. After hybridization, the filters were washed twice in 2 $\times$ SSC and once in 0.1 $\times$ SSC for 30 min at room temperature followed by a single wash in 0.1 $\times$ SSC at 55 °C. Filters were autoradiographed at –70 °C with Kodak X-Omat AR film and intensifying screens.

The following plasmids were used to isolate DNA fragments for nick translation: PDGF-A, a 1.3 kb *Eco*R1 fragment from the A-chain clone D1 (Betsholtz *et al.*, 1986); v-sis, a 1.0 kb *Sst*I–*Xba*I fragment from pC60 (Germann *et al.*, 1981); TGF β , a 1.0 kb *Eco*R1 fragment from pbC1 (Derynck *et al.*, 1985).

Other procedures

Protein determinations were carried out by the method of Sandwick & Schray (1985) with ovalbumin as a standard. The protein samples were freeze-dried and then dissolved in 0.1 % (v/v) trifluoroacetic acid/50 % (v/v) acetonitrile. After removal of the acetonitrile the residue was assayed for protein content by measurement of the alkaline phosphatase activity in a competitive binding assay utilizing the non-specific adsorption of proteins on polystyrene latex tubes.

Mitogenic activity of column fractions was tested by [³H]thymidine incorporation in quiescent 3T3 cells as described by Van Zoelen *et al.* (1984). Human platelet PDGF was used as a standard for quantification of the mitogenic activity. Growth-inhibitory activity was quantified by the decrease in [³H]thymidine incorporation in exponentially growing mink lung CC164 cells, with human platelet TGF β as a standard (Van Zoelen *et al.*, 1986a). For testing the mitogenic or growth-inhibitory activity, fractions from the Bio-Sil TSK CM-2-SW h.p.l.c. and reverse-phase h.p.l.c. columns were freeze-dried in the presence of 0.01 % bovine serum albumin, thereby avoiding irreversible adsorption of growth-factor activity on the tubes during freeze-drying.

RESULTS

Purification of the Neuro-2A-cell-derived PDGF-like and TGF β -like growth factors

Previous studies have shown that mouse neuroblastoma Neuro-2A cells secrete both a PDGF-related growth factor and a type β TGF (Van Zoelen *et al.*, 1984, 1985a). In order to characterize these growth factors and compare their properties with those of PDGF and TGF β from human platelets, attempts were made to purify these factors from serum-free medium, conditioned by the Neuro-2A cells. By use of conventional gel-permeation chromatography followed by an additional reverse-phase h.p.l.c. step a partially purified preparation of the PDGF-like growth factor was obtained, as described by Van Zoelen *et al.* (1985a). We now introduce a weak cation-exchange h.p.l.c. chromatography as an additional step in the purification procedure for the Neuro-2A-cell-derived PDGF-like growth factor. The new procedure also appeared to be applicable for the purification of a type β TGF from the Neuro-2A-cell-conditioned medium and appeared to be a general method for the purification of basic polypeptide growth factors, resulting in homogeneous growth-factor preparations in good yield.

Bio-Gel P-100 chromatography. Fig. 1 represents the first chromatography step (Bio-Gel P-100) of Neuro-2A-cell-conditioned medium, extracted with 1 M-acetic acid. In Fig. 1(a) the absorbance at 280 nm and the stimulation of DNA synthesis in quiescent 3T3 cells ([³H]thymidine incorporation, in d.p.m./well) are shown. Transferrin and other proteins with molecular masses above 29 kDa are separated from the mitogenic activity. Most of the activity is present in fractions 40–55. Van Zoelen *et al.* (1985a) have shown by immunoblotting experiments with anti-PDGF antibodies that the mitogenic peak contained a PDGF-like growth factor with a molecular mass of 32 kDa, which is slightly higher than that of PDGF from human platelets. Charge interactions of the highly basic PDGF and PDGF-like growth factors with the column material resulted in retardation of the growth factor on the column and elution at an aberrant molecular size of 15–20 kDa (Westermarck *et al.*, 1983; Van Zoelen *et al.*, 1985a). The nature of the small mitogenic peak (fractions 34–40) is still unclear. The fractions indicated in Fig. 1(a) were pooled, freeze-dried, dissolved in 1 M-acetic acid and used for further purification.

Fig. 1(b) represents the amount of ng equivalents of TGF β of the Bio-Gel P-100 column fractions (same as in Fig. 1a) quantified by means of the CC164-cell growth-inhibition assay (Van Zoelen *et al.*, 1986a). We refer to this peak as the growth-inhibitory activity or inhibition peak. TGF β is known to be eluted as a broad peak, probably because of the strong interaction of this growth factor with the column material (Assoian *et al.*, 1983). The apparent inhomogeneity and complexity of the TGF β peak in this step and the following purification steps is due to the high sensitivity of the CC164-cell growth-inhibition assay for TGF β (60–70 % inhibition at 0.5 ng of TGF β /ml) rather than microheterogeneity of the TGF β preparation. Small variations in the size of the test samples might result in fluctuation of the inhibition values. There is slight overlap between the inhibition peak and the mitogenic activity. However, during the

Fig. 1. Bio-Gel P-100 chromatography in 1 M-acetic acid of the crude acetic acid extract of Neuro-2A-cell-conditioned medium (4 litres)

(a) ●—●, A_{280} ; ○---○, [^3H]thymidine incorporation into quiescent Swiss 3T3 fibroblasts (400 μl portions tested in triplicate). (b) Elution profile of TGF β . The amount of TGF β -like growth factor is expressed in ng equivalents of TGF β determined from growth-inhibitory activity towards CC164 cells (400 μl portions in triplicate) as described by Van Zoelen *et al.* (1986a).

further purification procedure these activities are effectively separated from each other.

Weak-cation-exchange h.p.l.c. The next fractionation step, using a weak-cation-exchange h.p.l.c. column (Bio-Sil TSK CM-2-SW) at low pH, is an additional one to the existing purification procedure of the PDGF-like growth factor. Separation of proteins on this column is based on charge properties of the proteins (Van den Eijnden-Van Raaij *et al.*, 1987). Extremely basic proteins differing only slightly in pI value are base-line-separated under acidic conditions. Application of the crude highly basic PDGF-like growth-factor preparation from the Bio-Gel P-100 column and subsequent elution with a three-step linear gradient of ammonium acetate in 1 M-acetic acid resulted in a mitogenic peak being eluted at an ammonium acetate concentration of 0.64–0.68 M (Fig. 2a). The mitogenic activity is well separated from the protein peak. The recovery of the applied activity is 70% and the growth-factor preparation is purified about 70-fold (Table 1). Fractions containing the mitogenic activity were pooled as indicated in Fig. 2(a) and concentrated on

Fig. 2. Cation-exchange h.p.l.c. chromatography (Bio-Sil TSK CM-2-SW column) of (a) the freeze-dried mitogenic peak from the gel-filtration column (Fig. 1a, horizontal bar) and (b) the freeze-dried growth-inhibitory activity from the Bio-Gel P-100 column (Fig. 1b, horizontal bar)

The column was equilibrated in 1 M-acetic acid, and mitogenic or inhibitory activity was eluted by applying a linear gradient of 0–2 M-ammonium acetate in 1 M-acetic acid at a flow rate of 0.8 ml/min. —, A_{280} ; ○---○, [^3H]thymidine incorporation into quiescent Swiss 3T3 fibroblasts (400 μl portions in triplicate) (a); ●—●, ng equivalents of TGF β (400 μl portions in triplicate) (b). Fractions indicated by the horizontal bars were concentrated on top of a Sep-Pak C_{18} cartridge and eluted with 0.1% (v/v) trifluoroacetic acid/50% (v/v) acetonitrile, pH 2.2.

a Sep-Pak C_{18} cartridge (see the Materials and methods section). The recovery of activity was about 80% in this concentration step.

For further purification of the growth-inhibitory activity secreted by Neuro-2A cells, fractions 55–80 from the Bio-Gel P-100 column (Fig. 1b) were pooled, freeze-dried and subjected to the cation-exchange h.p.l.c. column. As shown in Fig. 2(b) the TGF β -like growth factor is well separated from the bulk protein during a linear gradient of ammonium acetate in 1 M-acetic acid. The growth-inhibitory activity was eluted as a broad peak at 0.8–1.6 M-ammonium acetate, indicating a strong interaction of this growth factor with the cation-exchange column material. Fractions containing the growth-inhibitory activity were pooled as indicated in Fig. 2(b) and concentrated on a Sep-Pak C_{18} cartridge before reverse-phase h.p.l.c.

Table 1. Purification of PDGF-like growth factor from Neuro-2A-cell-conditioned medium

For experimental details see the Materials and methods section. Protein determination was carried out by the method of Sandwich & Schray (1985).

	Protein recovered ($\mu\text{g/l}$ of conditioned medium)	Activity recovered [ng (%)]	Specific activity (ng/ μg)	Degree of purification (fold)
Bio-Gel P-100	1.15×10^3	750*(100)	0.65	1
CM-2-SW h.p.l.c.		525 (70)		
Sep-Pak C_{18} concentration	8.9	420 (80)	47	73
C_{18} Hi-Pore h.p.l.c.		252 (60)		1540†

* A similar amount of mitogenic activity was found in the acetic acid extract (Van Zoelen *et al.*, 1984).
† Based on an assumed 100 % purity of the factor.

Fig. 3. Reverse-phase h.p.l.c. purification of the mitogenic activity (a; from Fig. 2a) and the growth-inhibitory activity (b; from Fig. 2b) after Bio-Sil TSK CM-2-SW h.p.l.c. (Fig. 2) and concentration on a Sep-Pak C_{18} column

After removal of the acetonitrile the Sep-Pak material was injected on to a C_{18} Hi-Pore reverse-phase h.p.l.c. column, equilibrated in 0.1 % (v/v) trifluoroacetic acid/18 % (v/v) acetonitrile. Mitogenic activity (a) and growth-inhibitory activity (b) were eluted by a linear gradient of 0.1 % (v/v) acetonitrile/min at a flow rate of 1 ml/min. —, A_{229} ; ○—○, [^3H]thymidine incorporation in quiescent Swiss 3T3 fibroblasts (200 μl portions tested in triplicate) (a); ●—●, ng equivalents of TGF β (200 μl portions tested in triplicate) (b).

Reverse-phase h.p.l.c. For the final purification of the PDGF-like growth factor from Neuro-2A cells the sample from the Sep-Pak C_{18} column was chromatographed on a Hi-Pore $\mu\text{Bondapak } C_{18}$ column with an acetonitrile/0.1 % (v/v) trifluoroacetic acid gradient. The elution profile is shown in Fig. 3(a). The single mitogenic peak observed after the cation-exchange h.p.l.c. step (Fig. 2a) was split into two distinct mitogenic activities by the reverse-phase h.p.l.c. step. The first, more hydrophilic, peak was eluted between 27 and 29 % acetonitrile. The second, mitogenically less active, peak was eluted as a broad band between 32 and 37 % acetonitrile. As shown by Van Zoelen *et al.* (1985a), the position of the first mitogenic peak corresponds to the PDGF-like growth factor. The strong absorbance peaks at 229 nm migrating close to this mitogenic activity, as observed by the latter authors, are completely absent from the elution pattern of Fig. 3(a). This result indicates the effectiveness of the Bio-Sil TSK CM-2-SW cation-exchange column in removing contaminating proteins from the PDGF-like growth factor. The recovery of mitogenic activity in the first peak is 60 % (Table 1). In agreement with the results obtained by Van Zoelen *et al.* (1985a, 1986a) the second, more hydrophobic, mitogenic peak most probably represents TGF β , which has poor mitogenic activity on Swiss 3T3 cells. Probably this peak results from co-migration of a small fraction of TGF β with the PDGF-like growth factor on the gel-filtration and cation-exchange columns.

The reverse-phase C_{18} h.p.l.c. column was also used as the final step for the purification of the TGF β -like growth factor. As shown in Fig. 3(b), additional protein impurities and traces of PDGF-like growth-factor activity were finally removed from this growth factor on the reverse-phase column. The inhibitory activity was eluted between 32 and 37 % acetonitrile, which is very similar to the elution position of TGF β_1 from human platelets (Van den Eijnden-van Raaij *et al.*, 1988). Comparison of the fractions containing this inhibitory activity with the TGF β -containing fractions observed in Fig. 3(a) reveals slight differences in elution position of the growth factor, but it is not yet known whether this is the result of different forms of TGF β . The overall recovery of inhibitory activity is 12 % relative to the Bio-Gel P-100 column.

Biochemical characterization of the PDGF-like and TGF β -like growth factors

The purity of the final PDGF-like growth-factor preparation was shown by SDS/polyacrylamide-gel electrophoresis (Fig. 4a, lane 2). In the absence of a reducing agent a single polypeptide band with an apparent molecular mass of 32 kDa was observed. This is in complete agreement with the results obtained after immunoprecipitation by anti-PDGF serum of cysteine-free medium conditioned by Neuro-2A cells in the presence of [^{35}S]cysteine. As shown in Fig. 4(b) (lane 1) under non-reducing conditions a protein with a molecular mass of 32 kDa was precipitated by anti-PDGF serum but not by control serum. Upon reduction this protein could be split into two closely migrating bands of 16.5 kDa and 17 kDa (Fig. 4b, lane 2). The presence of other proteins than the PDGF-like growth factor in the immunoprecipitate is a general phenomenon, which was observed for many cell types (Betsholtz *et al.*, 1986). As these proteins are recognized by both normal rabbit serum and anti-PDGF

Fig. 4. Biochemical characterization of the PDGF-like and TGF β -like growth factors purified from Neuro-2A-cell-conditioned medium

(a) Silver-stained SDS/polyacrylamide gel (0.75 mm 15% polyacrylamide gel) of (lane 1) calibration proteins, (lane 2) 70 ng of purified PDGF-like growth factor from Neuro-2A-cell-conditioned medium (peak of Fig. 3a) and (lane 3) 60 ng of purified Neuro-2A-cell-derived TGF β -like growth factor under non-reducing conditions. The band at 68 kDa was present as an artificial band throughout the whole gel. (b) Immunoprecipitation of the Neuro-2A-cell-derived PDGF-like growth factor. Medium conditioned by Neuro-2A cells in the presence of [35 S]cysteine was preincubated with normal rabbit serum and Protein A-Sepharose, and the supernatant was immunoprecipitated by anti-PDGF serum. The immunoprecipitate was analysed by electrophoresis on an SDS/15% polyacrylamide gel under non-reducing (lane 1) or reducing (lane 2) conditions. Exposure was for 24 h at -70°C . (c) Immunoblot of the gel shown in lane 3 of panel (a) after incubation with anti-TGF β serum. The immune complexes were detected with horseradish-peroxidase-labelled goat anti-(rabbit IgG) antibody as described by Van Zoelen *et al.* (1985a). Incubation with normal rabbit serum was negative (results not shown).

serum, it is very unlikely that they represent PDGF-like growth factors.

The final TGF β -like growth-factor preparation appeared to be homogeneous on a silver-stained SDS/polyacrylamide gel (Fig. 4a, lane 3) and has a molecular mass of 24 kDa under non-reducing conditions, which is very similar to that of TGF β obtained from human blood platelets. Reduction of the type β TGF from Neuro-2A cells resulted in the appearance of a single 12 kDa band (results not shown). The 68 kDa band is an artifact, as it is present throughout the whole gel. As shown in Fig. 4(c), the TGF β -like growth-factor preparation is recognized by antibodies raised to a synthetic oligopeptide identical with the *N*-terminal residues 1–29 of TGF β from human platelets (A. J. M. Van den Eijnden-Van Raaij, I. Koornneef, R. P. de Groot & E. J. J. Van Zoelen, unpublished work). The specificity of this antiserum for the β_1 form of human platelet TGF β on Western blots indicates the presence of TGF β_1 chains in the Neuro-2A-cell-derived type β TGF preparation.

Because of the difficulties in accurately measuring the protein concentration of the PDGF-like growth factor and TGF β -like growth factor eluted from the reverse-phase C_{18} column, calculation of an exact specific-activity value is not possible. Owing to the low absolute amounts of the PDGF-like and TGF β -like growth factors the criteria of purity of these factors are only based on the

silver-stained SDS/polyacrylamide gel, taking into account that these factors are poorly stained by silver. For this reason the presence of poorly staining impurities in the growth-factor preparations could not be excluded. A purity of 100% would indicate that the overall purification of the PDGF-like growth factor from Neuro-2A-cell-conditioned medium by the use of Bio-Gel P-100 gel filtration, Bio-Sil TSK CM-2-SW h.p.l.c. and reverse-phase h.p.l.c. is about 1500-fold with a total recovery of 30% of the mitogenic activity relative to the Bio-Gel P-100 material (Table 1). Thus an amount of about 200 ng of the PDGF-like growth factor can be obtained from 1 litre of Neuro-2A-cell-conditioned medium. The yield of TGF β -like growth factor is 250 ng per litre of conditioned medium.

Expression of the genes encoding TGF β and the A and B chains of PDGF in Neuro-2A cells

Expression of the genes encoding PDGF-A, PDGF-B and TGF β was investigated by Northern blotting on polyadenylated RNA. As shown in Fig. 5, Neuro-2A cells express the gene encoding PDGF A, predominantly as a 1.9 kb transcript (lane 1; Betsholtz *et al.*, 1986), whereas the gene encoding PDGF B was not found to be expressed under these conditions (lane 2). These results are in agreement with the observation that the Neuro-2A-cell-derived PDGF-like growth factor migrates as a discrete 32 kDa band on SDS/polyacrylamide-gel electrophoresis

Fig. 5. Northern blot of Neuro-2A-cell polyadenylated RNA hybridized with probes for PDGF-A (lane 1), PDGF-B (lane 2) and TGF β (lane 3)

The positions of 28 S rRNA and 18 S rRNA are indicated.

(Fig. 4a), which probably is a homodimer of two PDGF A chains. This is in line with the nature of the PDGF-like secretion product of other tumour-cell lines (Nistér *et al.*, 1984; Heldin *et al.*, 1986; Westermarck *et al.*, 1986).

As shown in Fig. 5 (lane 3), Neuro-2A cells express a TGF β_1 -specific transcript of 2.4 kbp, which is identical in size with the 2.4 kbp transcript present in a number of normal and transformed cells (Derynck *et al.*, 1985, 1987). This is in close agreement with the production of a TGF β_1 -like growth factor at the protein level by Neuro-2A cells. In conclusion, the data both from the Northern blots and from the biochemical and immunological studies revealed the production and secretion of a PDGF A-A and a TGF β_1 -like growth factor by a neuroblastoma cell line. The relevance of these factors for the growth of the producer cell itself needs further investigation.

DISCUSSION

In the present paper we have described a general strategy for the purification of highly basic acid-stable polypeptide growth factors. This procedure was used for the preparation of highly pure PDGF-like growth factor and TGF β -like growth factor in high yield from medium conditioned by exponentially growing neuroblastoma Neuro-2A cells. The present method has many advantages over the existing procedures for the purification of these basic growth factors. During the whole procedure the pH is kept at a low value (pH < 4), thereby minimizing non-specific interactions of growth factors with the column material. This is in contrast with other methods that omit acidification of the conditioned medium and use several column-chromatography steps at neutral pH before switching to acidic conditions (Heldin *et al.*, 1979, 1980, 1986; Stroobant *et al.*, 1985). A general problem of these methods is the low recovery of growth-factor activity after most of the purification

steps. For this reason large amounts of starting material should be used in order to obtain sufficient amounts of the desired growth factor. As shown in the present paper the loss of growth-factor activity during purification under permanent acidic conditions is very limited. Recoveries of 60–80 % are obtained after each step, resulting in a high overall recovery of the growth factors. This high yield of growth-factor activity per step argues against selective purification of a specific form of the growth factor being purified. High concentrations of urea and other non-volatile salts, which are usually applied for the separation of growth factors from impurities (Frolik *et al.*, 1983; Assoian *et al.*, 1983; Nistér *et al.*, 1984; Heldin *et al.*, 1986), are not required. Exclusively volatile solvents and sterile conditions are used in each of the purification steps, facilitating the detection of growth factors in the column fractions (Van den Eijnden-Van Raaij *et al.*, 1987).

Protein separation via the present method is based on a combination of molecular mass, charge and hydrophobicity of the proteins. The use of the Bio-Sil TSK CM-2-SW weak-cation-exchange h.p.l.c. column appeared to be the most effective step in separating the Neuro-2A-cell-derived PDGF-like and TGF β -like growth factors from most of the contaminating proteins present. Recently it has been shown that other basic polypeptide growth factors also, including the basic form of fibroblast growth factor and a PC13-derived heparin-binding growth factor, can be separated well by this column procedure (Van Veggel *et al.*, 1987). This result illustrates the general usefulness of the cation-exchanger in separating basic polypeptide growth factors. In contrast with the data published for highly basic globular proteins (Van den Eijnden-van Raaij *et al.*, 1987), the basic polypeptide growth factors are not eluted according to their pI values. All of the basic growth factors tested on the cation-exchanger, e.g. PDGF, TGF β and fibroblast growth factor, are eluted later from the column than the highly basic globular protein lysozyme (pI 11.0) in spite of their lower pI values. The individual growth factors can be separated in order of basicity in this system. Probably the chromatographic behaviour of the non-globular basic growth factors on the cation-exchanger is dependent on both surface charge density and protein conformation. However, whatever the reason is for the retardation of the basic growth factors on the Bio-Sil TSK CM-2-SW column, it is the basis for the good separation of these factors from contaminating proteins in the acidic system. Preliminary experiments have shown that, in addition to basic growth factors, also acidic acid-stable growth factors, such as insulin-like growth factor II and TGF α , can be separated on the cation-exchange column by a modification of the present gradient (E. J. J. Van Zoelen, unpublished work). These results indicate that there is a strong tendency that acid-stable growth factors can be purified by a general strategy, implicating purification by Bio-Gel P-100, gel filtration, Bio-Sil TSK CM-2-SW h.p.l.c. and reverse-phase h.p.l.c. under acidic conditions.

The present study provides the first example of purification of a PDGF-like growth factor from a mouse cell source. The recovery of PDGF-like growth factor obtained by the present method is approx. 10-fold higher than by previously described methods (Heldin *et al.*, 1980; Stroobant *et al.*, 1985). Earlier investigations have shown that this neuroblastoma-derived PDGF-like

growth factor can induce phenotypic transformation of normal rat kidney cells (Van Zoelen *et al.*, 1984, 1985*a,b*, 1986*a,b*, 1988). Sufficient amounts of pure growth factor are now available to compare this property with PDGF from human platelets or other PDGF-like growth factors.

Further, we have shown that PDGF and TGF β can be purified from human platelets basically according to a similar scheme to that described in the present paper (A. J. M. Van den Eijnden-Van Raaij *et al.*, 1988). Because of the high recovery obtained by the present method, this will allow analysis of all possible forms of PDGF and PDGF-like growth factors present in human platelets. In addition, this method enables direct comparison of the structural characteristics of PDGF and TGF β from normal cells with their related counterparts produced by transformed cells.

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