Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells

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We attempted to identify the factor that stimulated prostacyclin (PGI_2) production using conditioned medium from cultured human diploid fibroblast cells subjected to a series of purification steps using h.p.l.c. on DEAE-5PW, Heparin-5PW, Protein-Pak 300, and an insulin-like growth factor-1 ligand affinity column. The purified prostacyclin-stimulating factor (PSF) ran as a single band with a molecular mass of 31 kDa by SDS/PAGE. Analysis of the purified PSF by C4 reversed-phase h.p.l.c. showed a single

sharp peak in 31 % (v/v) acetonitrile. The material was purified 8000-fold with an overall yield of about 18 %. The purified PSF stimulated PGI_2 production by cultured bovine aortic endothelial cells at a concentration of about 10 ng/ml; maximal stimulation was achieved at a concentration of 25 ng/ml. A cDNA coding for PSF was cloned and sequenced, revealing an apparently novel protein with no obvious sequence similarity to known proteins.

INTRODUCTION

Vascular endothelial cells secrete vasoactive substances such as prostacyclin (PGI_2) [1], endothelium-derived relaxing factor(s) (EDRF) [2], and endothelin [3], which play an important role in the regulation of vascular tone. PGI_2 synthesized by the vascular wall is a potent vasodilator and an inhibitor of platelet adhesion and aggregation that contributes to the maintenance of vascular wall homeostasis. However, the detailed mechanisms of the regulation of PGI_2 production by the vascular wall have not yet been clarified. Recently, Yui et al. [4] reported that apolipoprotein A-1, a major component of high-density-lipoprotein cholesterol, was responsible for PGI_2 -stabilizing factor. MacIntyre et al. [5] demonstrated that human plasma stimulated PGI_2 production by cultured aortic endothelial cells. This stimulating activity of plasma may be crucial in the regulation of PGI_2 production by the vascular wall. Remuzzi et al. [6] also reported that this activity in plasma was reduced in patients with haemolytic-uraemic syndrome. In our previous studies, we found that the ability of plasma to stimulate PGI_2 production was reduced in patients with diabetes mellitus and reported that this activity came from relatively heat-stable, acid-labile and non-dialysable factors [7,8]. Then we found that the conditioned medium from cultured human diploid fibroblast cells also contains an activity to stimulate PGI_2 production [9]. Characterization of this activity in the conditioned medium from human diploid fibroblast cells revealed that the properties were similar to that of human plasma-derived serum, e.g. relatively heat-stable, acid-labile, trypsin-sensitive, and possessing an affinity for heparin [9]. Although many substances that stimulate PGI_2 production by vascular endothelial cells have been identified [10–16], none of these substances seems to correspond to the PGI_2 -stimulating activity in question. The present study was, therefore, undertaken to purify and clone this PGI_2 -stimulating factor (PSF) from the conditioned medium of cultured human diploid fibroblast cells.

MATERIALS AND METHODS

Assay of PSF activity

Bovine aortic endothelial cells were cultured as previously described [7,8,16]. Briefly, endothelial cells were scraped off the intimas of bovine thoracic aortas and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, U.S.A.) containing 10 % (v/v) heat-inactivated fetal-calf serum (FCS) (Gibco), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C in an atmosphere consisting of 5 % $\text{CO}_2/95$ % air. The medium was replaced every 3 days. The cultured cells showed a 'cobblestoned' appearance by phase-contrast microscopy and produced von Willebrand factor and endothelin-1 [17,18], indicating that they were vascular endothelial cells. When the cells reached confluence, they were subcultured with 0.05 % (w/v) trypsin solution. The cells were harvested from the fifth to tenth passages by trypsinization and placed in a 24-well multidish in a concentration of 1.5×10^4 cells/well in 1 ml of DMEM containing 10 % (v/v) FCS. When the endothelial cells reached confluence (1.0×10^5 cells/well), the medium was removed and test solutions were added to the wells. After 60 min of incubation, the test solution was collected and assayed for accumulated concentration of 6-keto-prostaglandin $\text{F}_{1\alpha}$ (6-keto- $\text{PGF}_{1\alpha}$) by radioimmunoassay (r.i.a.), as previously described [7,8]. The activity of PSF was determined by measuring the amount of 6-keto- $\text{PGF}_{1\alpha}$ produced in the test solution per 1×10^4 cells.

Preparation of serum-free conditioned medium from cultured human diploid fibroblast cells

Human diploid fibroblast cells (4.8×10^4 cells/ml) were cultured in 3.0 litres of DMEM containing 15 % (v/v) FCS in a roller-bottle culture flask in 5 % $\text{CO}_2/95$ % air at 37 °C for 5 days. Then the growth medium was discarded, and the cells were

Abbreviations used: PGI_2 , prostacyclin; 6-keto- $\text{PGF}_{1\alpha}$, 6-keto-prostaglandin $\text{F}_{1\alpha}$; PSF, prostacyclin-stimulating factor; IGF-1, insulin-like growth factor 1; IGFBP, insulin-like growth factor binding protein.

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washed twice with Dulbecco's PBS. The fibroblast cells were then cultured in 3.0 litres of serum-free DMEM for an additional 48 h. The serum-free culture medium was collected and sterilized with 2.5 μ m pore size filters (Millex GV, Millipore, Bedford, MA, U.S.A.). Then the medium was concentrated with an ultrafiltration membrane (molecular cut-off 10 kDa, Fuji Filter Co., Tokyo, Japan), to a final concentration of 1 mg/ml protein, and dialysed extensively against 20 mM Tris/HCl (pH 7.8) at 4 °C for 24 h.

Purification of PSF from conditioned medium of human diploid fibroblast cells

All chromatographic operations were performed using an h.p.l.c. system (Waters, Millipore, Bedford, MA, U.S.A.).

DEAE-5PW h.p.l.c.

Concentrated conditioned medium (50 ml) was pumped on to a DEAE-5PW column (25 mm $\times$ 150 mm, Toyo-Soda, Tokyo, Japan) pre-equilibrated with 20 mM Tris/HCl (pH 7.8) at a speed of 2 ml/min. The column was then washed with 500 ml of equilibration solvent and eluted at a flow rate of 6 ml/min with a linear gradient of 0–1 M NaCl in 20 mM Tris/HCl (pH 7.8). The absorbance of the column effluent was monitored at 280 nm. Fractions of 3 ml were collected and used for the PSF assay.

Heparin-5PW h.p.l.c.

The active fractions obtained from DEAE-5PW chromatography were collected and dialysed against 10 mM phosphate buffer (PB; pH 7.4). The sample was loaded on to a Heparin-5PW column (7.5 mm $\times$ 75 mm, Toyo-Soda) pre-equilibrated with 10 mM PB (pH 7.4). The column was washed with 20 ml of pre-equilibration buffer and then eluted at a flow rate of 1 ml/min with a linear gradient of 0–1 M NaCl in 10 mM PB (pH 7.4). Absorbance was monitored at 280 nm. Fractions of 1 ml were collected and used for the PSF assay.

Protein-Pak 300 gel chromatography

Active fractions obtained from Heparin-5PW chromatography were collected and concentrated to 200 μ l using a Centricon 10 microconcentrator (Amicon, Denvers, MA, U.S.A.). Aliquots were then applied to Protein-Pak 300 columns (Waters) pre-equilibrated with 10 mM PBS (pH 7.4) and eluted at a flow rate of 800 μ l/min. Fractions of 800 μ l were collected and used for the PSF assay. The column was calibrated using Blue Dextran (molecular mass, 200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa).

C4 reversed-phase h.p.l.c.

Active fractions from Protein-Pak 300 chromatography were injected on to a C4 column (Waters) equilibrated with 0.1 % (v/v) trifluoroacetic acid and 10 % acetonitrile. A linear gradient of 10–60 % acetonitrile in 0.1 % trifluoroacetic acid was applied over a period of 50 min at a flow rate of 1 ml/min. Absorbance was monitored at 280 nm in 1 ml fractions.

Insulin-like growth factor 1 (IGF-1) ligand affinity chromatography

An IGF-1 ligand affinity column was prepared according to a modification of the method of Shimokawa et al. [18]. Briefly, 2 ml

of Affi-prep 10 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was transferred to a mini-column (Bio-Rad) and washed three times with 10 ml of cold water. Then 5 ml of 0.1 M Hepes buffer (pH 7.4) containing 1 mg of recombinant IGF-1 (supplied by Fujisawa Pharmaceutical Co., Osaka, Japan) was added and the mixture was shaken for 12 h. Unreacted active sites were blocked by adding 1 ml of 1 M ethanolamine (pH 8.0) and the mixture was shaken for an additional 12 h. The reacted gel was washed sequentially with 0.1 M Hepes buffer, 10 mM PBS containing 0.5 M NaCl (pH 7.4), 0.5 M acetic acid (pH 3.0), and finally 10 mM PBS (pH 7.4). The gel was applied to a column (HR 10/10, Pharmacia P-L, Uppsala, Sweden) attached to the h.p.l.c. system, and chromatography was performed. Active fractions derived from the Protein-Pak 300 gel chromatography were applied to an IGF-1 ligand affinity column, pre-equilibrated with 10 mM PB (pH 7.4), and eluted with 0.5 M acetic acid (pH 3.0) at a flow rate of 1 ml/min. The absorbance of the column effluent was monitored at 280 nm. One millilitre flow-through fractions and eluted fractions were collected. Eluted fractions were neutralized immediately with 5 M sodium acetate to pH 6.5, and dialysed against PBS (pH 7.4). These samples were used for the PSF assay.

SDS/PAGE

SDS/PAGE was performed according to the method described by Laemmli [19]. Samples were heated (95 °C, 3 min) and applied to a gradient gel consisting of 5–20 % polyacrylamide. After electrophoresis, the gel was fixed with 40 % (v/v) methanol/10 % (v/v) acetic acid and stained with silver [20]. Low-molecular-mass markers (Bio-Rad) consisted of phosphorylase b (97 400 Da), BSA (66 200 Da), ovalbumin (42 699 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da), and lysozyme (14 400 Da).

Protein determination

The protein concentration was estimated using a protein assay kit (Bio-Rad), with BSA (Bio-Rad) as a standard.

Amino acid analysis

Purified protein was desalted by being dialysed against distilled water and lyophilized using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.). Peptides were generated by cleavage with trypsin (Boehringer-Mannheim GmbH, Mannheim, Germany) and incubated at 37 °C for 16 h. Peptide mixtures were purified by reversed-phase h.p.l.c. on a Vydac C8 column (The Separations Group, Hesperia, CA, U.S.A.). Amino acid sequences of resulting peptides were determined by automated Edman degradation using an Applied Biosystems Model 477A protein sequencer with an on-line Model 120A analyser.

Construction of the cDNA library

Total RNA was extracted from cultured human diploid fibroblast cells by the acid guanidium thiocyanate-phenol-chloroform method [21] and poly(A)⁺ RNA was isolated using oligo(dT)-cellulose (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column. Oligo(dT)-primed and random-primed double-stranded cDNAs were prepared using cDNA Synthesis System Plus (Amersham International, Amersham, Bucks., U.K.) according to the method described by Gubler and Hoffman [22] with some modification. Then a *Bst*XI non-palindromic adapter was ligated to the oligo(dT)-primed cDNA. The cDNA fragments longer

than 500 bp were isolated by preparative agarose gel electrophoresis, and then ligated into *Bst*XI-digested pEF-BOS vector [23]. *Escherichia coli* MC1061/p3 cells were transformed with the resulting plasmids by electroporation [24], yielding approx. 6.0×10^5 independent recombinant clones. For the construction of the random-primed cDNA library, non-phosphorylated *Eco*RI/*Bam*HI/*Kpn*I/*Nco*I adapter was ligated to the cDNA and low-molecular-mass materials were removed by passage over an NIP517 column (Amersham). After phosphorylation, the cDNA were ligated into the *Eco*RI arms of λ gt10 (Amersham), followed by *in vitro* packaging (Gigapack II Packaging Extract, Stratagene, La Jolla, CA, U.S.A.). A library of approx. 6.0×10^5 independent recombinant clones was obtained.

Isolation of PSF cDNA

A partial cDNA fragment of PSF was obtained by the reverse-transcription polymerase chain reaction (RT-PCR) method using a pair of degenerate primers according to the amino acid sequence of peptide derived from the purified PSF. The two PCR primers were a sense primer consisting of a mixture of 20-mers (5'-AT^A/c/_TACXGTXGTXGA^c/_TGCX^c/_T-3') and an antisense primer consisting of a mixture of 20-mers (5'-A^A/G^c/_TTCXGCXCC^c/_TTCXCC^c/_TTT-3') where X denotes all four deoxynucleotides. Oligo(dT)-primed first-strand cDNA was synthesized by Superscript Preamplification System (Life Technologies, Gaithersburg, MD, U.S.A.) using the poly(A)⁺ RNA from the human diploid fibroblast cells, and then subjected to 30 cycles of PCR amplification with the degenerate primers. Primer-directed extension was repeatedly carried out by serial cycles of denaturation (1 min at 94 °C), annealing (2 min at 55 °C) and polymerization (3 min at 72 °C). A generated fragment was sequenced and a pair of primers for the following PCR-based screening was then designed.

To obtain full-length PSF cDNA, two kinds of libraries, the oligo(dT)-primed cDNA library in pEF-BOS and the random-primed cDNA library in λ gt10, were screened. Approx. 6.0×10^5 recombinant clones from the pEF-BOS cDNA library in *E. coli* MC1061/P3 were plated (1.4×10^4 clones each). The plasmid DNAs were prepared, digested with *Xba*I and subjected to 30 cycles of PCR amplification with the PCR-based screening primers. Primer-directed extension was repeatedly carried out by serial cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and polymerization (1 min at 72 °C). The λ gt10 cDNA library was screened using the radiolabelled *Pvu*II/*Sma*I fragment of the cDNA obtained from a positive clone in the pEF-BOS cDNA library. Plaque hybridization was performed overnight on nylon filters at 42 °C in a hybridization solution containing 6 × SSPE (SSPE: 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA, pH 7.4), 1 × Denhardt's, 50 % formamide, 10 % (w/v) dextran sulphate, 100 µg/ml denatured salmon sperm DNA, 0.1 % SDS and 1×10^6 c.p.m./ml of the labelled probe. The filters were washed with 0.1 × SSC (SSC: 0.15 M NaCl/0.015 M sodium citrate) containing 0.1 % SDS at 44 °C for 1 h. The cDNA inserts were isolated from the phage purified from positive plaques, and subcloned into pUC vector for nucleotide sequencing.

Viability of cultured bovine aortic endothelial cells

Cultured bovine aortic endothelial cells were stimulated by PSF partially purified by a DEAE-5PW anion-exchange column for 60 min. Lactic dehydrogenase activity in the medium was meas-

ured by the method of Wroblewski and LaDue [25] with some modification and a number of the cells that excluded Trypan Blue solution was counted by light microscopy.

[³H]Thymidine incorporation into cultured bovine aortic endothelial cells

After stimulation with partially purified PSF, the medium was removed and the cells were further incubated with DMEM containing methyl-[³H]thymidine (0.25 µCi/ml, specific radioactivity 20 Ci/mmol) for 4 h at 37 °C. The cells were washed with PB (pH 7.4), lysed by 1 M NaOH and the radioactivity incorporated into the cells was measured by a liquid-scintillation counter [26].

Western-blot analysis of PSF in human serum and conditioned medium from human diploid fibroblast cells

Polyclonal antibodies against PSF were raised in rabbits by multiple intracutaneous injections of authentic 12 amino acids corresponding to the C-terminal of PSF in Freund's adjuvant. Two weeks after the last immunization blood was collected and anti-PSF antibody was prepared. For Western-blot analysis, samples of conditioned medium from human diploid fibroblast cells and human serum (20 µg of protein/lane) were placed in a loading buffer (963 mM Tris/HCl, pH 6.8, 2 % SDS, 10 % glycerol, 0.05 % Bromophenol Blue) and boiled for 3 min. The samples were loaded on 5–20 % SDS/PAGE in a running buffer (25 mM Tris, 192 mM glycine, and 0.1 % SDS). Molecular-mass markers (Bio-Rad) were run on each gel. The proteins were then transferred electrophoretically to nitrocellulose membrane at 1 mA/cm² of paper for 45 min using 20 % (v/v) methanol/25 mM Tris/192 mM glycine/0.05 % SDS. For blocking, the membrane was first incubated with 100 mM PB (pH 7.5), containing 100 mM NaCl, 0.1 % Tween 20 and 5 % (w/v) non-fat milk powder for 1 h at room temperature. Then anti-PSF antibody was added for 1 h at room temperature. The membrane was washed three times with washing buffer (100 mM PB, 100 mM NaCl, 0.1 % Tween 20, pH 7.5) and then incubated with horseradish peroxidase-labelled antibody against rabbit IgG (Amersham enhanced chemiluminescence system) for 45 min. The membrane was washed five times with the same buffer, and the specific band corresponding to PSF was visualized by enhanced chemiluminescence (Amersham ECL system, Amersham).

RESULTS

Purification of PSF

The purification of PSF was performed using the concentrated serum-free conditioned medium of human diploid fibroblast cells. We found that the PSF in this conditioned medium resides in an acid-labile, trypsin-sensitive, anionic, and heparin-binding factor with an apparent molecular mass of 30000 Da by gel-filtration chromatography [9]. Using these characteristics of PSF, a purification scheme was designed. The concentrated medium was first subjected to anion-exchange h.p.l.c. on a DEAE-5PW column. The bulk of the PSF activity bound to this column and a single PSF activity peak was eluted between 50 and 150 mM NaCl (Figure 1a), resulting in an 8-fold increase in specific activity, and a calculated recovery of 49 % (Table 1). No PGI₂-stimulating activity was detected in the fractions above 150 mM NaCl. In this purification step, we examined the kinetics and dose dependency of PGI₂ production. The PSF partially purified by the DEAE-5PW column significantly stimulated 6-

Figure 1 Chromatography on (a) a DEAE-5PW column and (b) a Heparin-5PW column

(a) Concentrated conditioned medium was applied to a DEAE-5PW column and eluted with a linear gradient of 0–1.0 M NaCl (—). Absorbance was measured at 280 nm (—). PSF activity (●) was determined. Fractions under the horizontal bar were collected. (b) Active fractions from the DEAE-5PW column were applied to a Heparin-5PW column and eluted with a linear gradient of 0–1.0 M NaCl (—) as described. Absorbance at 280 nm (—) and PSF activities (●) were measured. Fractions under the horizontal bar were collected.

Table 1 Purification of PSF

Results are means of four different experiments. Maximal stimulation was determined from the peak of the dose–response curves (as in Figure 7). Protein concentration was estimated by protein assay kit (Bio-Rad) up to the Heparin-5PW h.p.i.c. step and by relative intensities of bands in silver-stained SDS gels in subsequent steps.

Purification step	Protein (μg)	Maximal stimulation (ng/ml)	Purification (fold)	Recovery (%)
Conditioned medium	52350	200000	1	100
DEAE-5PW	3234	25000	8	49
Heparin-5PW	650	10000	20	25
Protein-Pak 300	5.5	100	2000	21
IGF-1 affinity	1.2	25	8000	18

keto-PGF_{1α} production by cultured bovine aortic endothelial cells in a time- and dose-dependent manner (Figure 2). The maximal accumulated 6-keto-PGF_{1α} production was induced by

Figure 2 Time-course and dose–response curve of 6-keto-PGF_{1α} production

(a) Time course of 6-keto-PGF_{1α} production by cultured bovine aortic endothelial cells stimulated with the PSF purified by DEAE-5PW anion-exchange column from the conditioned medium of human diploid fibroblast cells (25 μg/ml). (b) Dose–response curve of 6-keto-PGF_{1α} production. Cultured bovine endothelial cells were incubated with the PSF purified by DEAE-5PW anion-exchange column for 60 min. Values are expressed as means ± S.E.M. of four different experiments.

a concentration of 25 μg/ml of partially purified PSF at 60 min. The production had ceased by 60 min. In further studies, incubation was therefore carried out for 60 min and PGI₂-stimulating activity was determined by using cultured bovine aortic endothelial cells as indicator cells.

The viability of the cells was confirmed by the measurement of lactate dehydrogenase activity in the medium, Trypan Blue exclusion test, and [³H]thymidine incorporation into the cells after stimulation by the partially purified PSF. As shown in Table 2, lactate dehydrogenase was not detected in either control or PSF-stimulated cells. The percentage of cells which excluded Trypan Blue and the extent of [³H]thymidine incorporation were not different between control and PSF-stimulated cells. Indomethacin completely inhibited the 6-keto-PGF_{1α} production stimulated with the partially purified PSF.

Table 2 Viability of cultured bovine aortic endothelial cells after stimulation by the partially purified PSF and effect of indomethacin on 6-keto-PGF_{1α} production stimulated with the partially purified PSF

Cultured bovine aortic cells were stimulated with the partially purified PSF or DMEM for 60 min. Measurement of lactate dehydrogenase (LDH) activity, Trypan Blue exclusion test and [³H]Thymidine incorporation were done as described in the Materials and methods section. Detectable limit of LDH was 12 i.u./l. To examine the effect of indomethacin on 6-keto-PGF_{1α} production, the cells were preincubated with indomethacin (15 μM) or DMEM alone for 30 min before the addition of the stimulants. Values are mean ± S.E.M. of three different experiments. n.d., not detected.

Assay	DMEM	DMEM + PSF
Lactate dehydrogenase activity (i.u./l)	n.d.	n.d.
Cells that excluded Trypan Blue (%)	96.2 ± 2.6	95.8 ± 3.3
[³ H]Thymidine incorporated into the cells (d.p.m./1 × 10 ⁵ cells)	130115 ± 5860	145726 ± 4823
6-Keto-PGF _{1α} production (pg/1 × 10 ⁴ cells per h)		
Preincubation with DMEM alone	4.3 ± 0.2	409.3 ± 73.8
Preincubation with DMEM plus indomethacin	1.8 ± 0.1	6.25 ± 1.0

Figure 3 Gel chromatography on Protein-Pak 300 columns

(a) Active fractions from the Heparin-5PW column were concentrated and applied to a Protein-Pak 300 column. Molecular-mass markers are indicated by the arrows. Absorbance at 280 nm (—) and PSF activities (●) were determined. (b) Analysis of SDS/PAGE and silver staining of the fractions from the Protein-Pak 300 gel chromatography under non-reduced conditions.

Figure 4 (a) Reversed-phase h.p.l.c. on a C4 column and (b) chromatography on IGF-1 ligand affinity column

(a) Active fractions from the Protein-Pak 300 gel chromatography were injected into a C4 column and eluted with a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid. (—) Absorbance at 280 nm; (---) acetonitrile gradient. (b) Active fractions from the Protein-Pak 300 gel chromatography were applied to an IGF-1 ligand affinity column and eluted with 0.5 M acetic acid. For the preparation of the IGF-1 ligand affinity column and experimental details, see the Materials and methods section. Absorbance at 280 nm (—) and PSF activity (●) were measured.

As the next step, the pooled active fractions obtained from the DEAE-5PW column were injected into a Heparin-5PW column. Much protein with undetectable PSF activity was passed through the column, and a single peak of PSF activity was eluted between 450 and 500 mM NaCl (Figure 1b). The biologically specific activity of PSF increased by a further 2.5-fold, with a recovery of 25% (Table 1). Then h.p.l.c. was performed on a Protein-Pak 300 column for further purification, and a single peak of PSF activity was detected at a position corresponding to a molecular mass of 29 kDa (Figure 3a), leading to a further 100-fold purification with 21% recovery (Table 1). Analysis of the protein composition of biologically active fractions of PSF from the Protein-Pak 300 gel chromatography by SDS/PAGE and silver staining showed that PSF migrated as a single band in a position corresponding to a molecular mass of 31 kDa under non-reduced conditions (Figure 3b).

In order to determine the purity of PSF, the active fractions (retention times 22 and 23 min) from the Protein-Pak 300 gel chromatography were subjected to C4 reversed-phase h.p.l.c. Two major peaks of protein were eluted; one at 28% concentration of acetonitrile (fraction I) and the other at 31% acetonitrile (fraction II) (Figure 4a). No PSF activity was recovered from these fractions because acetonitrile or low pH

Figure 5 Reversed-phase h.p.l.c. on a C4 column

Flow-through fractions (a), and eluted fractions (b) from IGF-1 ligand affinity chromatography were injected into a C4 column and eluted with a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid.

Figure 6 Analysis of SDS/PAGE and silver staining of the purified PSF

inactivated the biological activity of the PSF. However, the N-terminal ends of the proteins isolated from the C4 column could be analysed. Fraction I yielded a single N-terminal amino acid sequence, RAPGPGQGVQAGAPG, which is nearly identical with the N-terminal sequence of the insulin-like growth factor-binding proteins (IGFBPs) purified from human cerebrospinal fluid [27], human transformed fibroblast cells [28] and human serum [29].

Final purification was therefore achieved by an IGF-1 ligand affinity column. PSF activity was detected in flow-through fractions of the IGF-1 ligand affinity chromatography, but not in the fractions eluted with 0.5 M acetic acid (Figure 4b). Flow-

Figure 7 Dose-dependent effects of PSF at each purification step on 6-keto-PGF_{1α} production by cultured bovine aortic endothelial cells

Key to symbols: ◆, conditioned medium; ◇, DEAE-5PW; □, Heparin-5PW; ●, Protein-Pak 300; ○, IGF-1 affinity. Values are expressed as means of two different experiments.

through fractions applied to a C4 column showed a single protein peak at 31% acetonitrile (Figure 5a), and eluted fractions showed a peak at 28% acetonitrile (Figure 5b). In this step, the biological activity of PSF increased 4-fold, with a recovery of 18%. SDS/PAGE showed that PSF migrated with apparent molecular mass of 31 kDa under non-reduced conditions (Figure 6). Dose-dependent effects of PSF at each purification step on 6-keto-PGF_{1α} production by cultured bovine aortic endothelial cells are shown in Figure 7. Table 1 summarizes the results of the purification steps performed with 52.35 mg of protein of serum-free conditioned medium from human diploid fibroblast cells. The overall increase in specific activity of PSF was about 8000-fold, with a recovery of 18%.

The purified PSF was digested with trypsin and the resulting polypeptides were separated by reversed-phase h.p.l.c. on a Vydac C8 column. Major peaks on the C8 column were subjected to amino-acid-sequence analysis, revealing amino acid sequences of ITVVDALHEIPVKKGEGAEI (peptide 1), GHYGVQR (peptide 2), and TELLPGDRDNLAIQTR (peptide 3). The information on the sequence of peptide 1 was used in following experiments to clone the PSF cDNA.

cDNA cloning

To isolate cDNA encoding PSF, we first performed RT-PCR with a pair of degenerate primers as described in the Materials and methods section, generating a 59-bp fragment (5'-ATTACGGTGGTTGATGCGTTACATGAAATACCAAGTG-AAAAAAGGCGAAGGCGCCGAATT-3'). The amino acid sequence deduced from the nucleotide sequence of this fragment agreed absolutely with that of peptide 1, one of the peptide fragments derived from purified PSF. We subsequently synthesized a pair of PCR primers (5'-TTGATGCGTTACATGAAATA-3', 5'-CCTTCGCCTTTTTCACCTGG-3'), both of which are parts of the 59-bp fragment, to screen the human diploid fibroblast-derived oligo(dT)-primed cDNA library in pEF-BOS. We examined 46 pools, each of which contained approx. 1.4×10^4 clones, and detected the expected PCR product in 27 pools. Two of them were subpooled and then screened again with the PCR, yielding two positive clones named pM950 and pM951 which have 557-bp and 802-bp cDNA inserts

With a *PvuII/Smal* fragment of pM951 cDNA insert as a probe, we then screened a random-primed cDNA library in λ gt10 and isolated eight positive cDNA clones. One clone, named pM952, has a 1124-bp cDNA insert containing an open reading frame which codes 282 amino acids from the putative first methionine with a potential N-glycosylation site at position 171 (Figure 8). Analysis of the hydrophobicity of the deduced amino acid sequence predicts that a hydrophobic stretch of the N-terminal region is a signal peptide.

cultured non-transformed fibroblast cells also contained IGFBP-4. The above-mentioned N-terminal amino acid sequence of the fraction (I) reveals a high degree of sequence similarity with that of IGFBP-4, apart from two residues in positions 1 and 13. The success of the purification of PSF was thus due to the use of an IGF-I ligand affinity column.

Many substances, including arachidonic acid, ionophore A23187, phorbol ester [10], platelet-derived growth factor [11], interleukin 1 [12], tumour necrosis factor [13], epidermal growth factor [14] and transforming growth factor [15], have been found to stimulate PGI_2 production by cultured vascular endothelial cells. However, the chemical properties of PSF purified from serum-free conditioned medium of human diploid fibroblast cells differ from the properties of these substances. Arachidonic acid, A23187, and phorbol ester cause rapid stimulation of PGI_2 production; the remaining substances cause a delayed stimulation of PGI_2 production. PSF also caused rapid stimulation of PGI_2 production. The stimulation of PGI_2 production by PSF was completely inhibited by pre-incubation of endothelial cells with indomethacin. These results suggest that the PGI_2 stimulation by PSF is caused by the activation of enzymes in the arachidonic acid cascades. The kinetics of PGI_2 production stimulated by the partially purified PSF was somewhat different from that by human serum. 6-Keto- $\text{PGF}_{1\alpha}$ production stimulated by human serum reached a maximum extent faster (10 min) than that stimulated by partially purified PSF (60 min). Furthermore, a maximum extent of 6-keto- $\text{PGF}_{1\alpha}$ production by partially purified PSF was greater than that by human serum [8]. These differences may be explained by the existence of many substances that affect PGI_2 production in serum. A computer search of the May 1993 edition of the GenBank database for sequences show no similarity between the PSF and any other known proteins, suggesting that PSF is a novel factor.

We previously detected the presence of PGI_2 -stimulating activity in human plasma-derived serum and found that this activity was reduced in patients with non-insulin-dependent diabetes mellitus and in streptozotocin-induced diabetic rats [7,8]. This circulating activity for PGI_2 production has also been found to be reduced in thromboembolic disorders, such as the haemolytic-uraemic syndrome [6], thrombotic thrombocytopenic purpura [37], sickle cell anaemia [38,39] and acute myocardial infarction [40]. Our results indicate that the PSF purified from human diploid fibroblast cells is also present in human serum. However, it remains to be verified as to how much PSF in plasma contributes to the PGI_2 production by vascular endothelial cells *in vivo* because many factors in plasma have been reported already to stimulate PGI_2 production as mentioned above. Expression of PSF in appropriate cell systems should allow isolation of sufficient amounts of pure material to perform further studies on the actions of PSF *in vitro* and *in vivo*.

In conclusion, we have confirmed the presence of the PGI_2 -stimulating activity in the conditioned medium of cultured human diploid fibroblast cells, and succeeded in the complete purification and molecular cloning of the novel factor corresponding to this activity.

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REFERENCES

- Moncada, S., Gryglewski, R. J., Bunting, S. and Vane, J. R. (1976) *Nature* (London) **263**, 663–665.
- Furchgott, R. F. and Zawadzki, J. V. (1980) *Nature* (London) **288**, 373–376.
- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) *Nature* (London) **323**, 411–415.
- Yui, Y., Aoyama, T., Morishita, H., Takahashi, M., Takatsu, Y. and Kawai, C. (1988) *J. Clin. Invest.* **82**, 803–807.
- MacIntyre, D. E., Pearson J. D. and Gordon, J. L. (1978) *Nature* (London) **271**, 549–551.
- Remuzzi, G., Misioni, R., Marchesi, D., Livio, M., Mecca, G., Gaetano, G. D. and Donati, M. B. (1978) *Lancet* **ii**, 871–872.
- Inoguchi, T., Umeda, F., Watanabe, J. and Ibayashi, H. (1986) *Haemostasis* **16**, 447–452.
- Inoguchi, T., Umeda, F., Ono, H., Kunisaki, M., Watanabe, J. and Nawata, H. (1989) *Metabolism* **38**, 837–842.
- Yamauchi, T., Umeda, F., Masakado, M., Ono, Y. and Nawata, H. (1993) *Biochem. Mol. Biol. Int.* **1**, 65–71.
- Weksler, B. B., Ley, C. W. and Jaffe, E. A. (1978) *J. Clin. Invest.* **62**, 923–930.
- Coughlin, S. R., Moskowitz, M. A., Zetter, B. R., Harry, N. A. and Levine, L. (1980) *Nature* (London) **288**, 600–602.
- Rossi, V., Breviario, F., Ghezzi, P., Dejana, E. and Mantovani, A. (1985) *Science* **229**, 174–176.
- Kawakami, M., Ishibashi, S., Ogawa, H., Murase, T., Takaku, F. and Shibata, S. (1986) *Biochem. Biophys. Res. Commun.* **141**, 482–487.
- Ristimäki, A., Ylikorkala, O., Perheentupa, J. and Viinikka, L. (1988) *Thromb. Haemostasis* **59**, 248–250.
- Ristimäki, A. (1989) *Biochem. Biophys. Res. Commun.* **160**, 1100–1105.
- Ohnaka, K., Takayanagi, R., Yamauchi, T., Okazaki, H., Ohashi, M., Umeda, F. and Nawata, H. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1128–1136.
- Yamauchi, T., Ohnaka, K., Takayanagi, R., Umeda, F. and Nawata, H. (1990) *FEBS Lett.* **267**, 16–18.
- Shimokawa, M., Schroeder, R., Shimasaki, S. and Ling, N. (1989) *Biochem. Biophys. Res. Commun.* **165**, 189–195.
- Laemmli, U. K. (1970) *Nature* (London) **227**, 189–198.
- Oakley, B. R., Kirsch, D. R. and Morris, N. R. C. (1980) *Anal. Biochem.* **105**, 361–363.
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
- Gubler, U. and Hoffman, B. J. (1983) *Gene* **25**, 263–269.
- Mizushima, S. and Nagata, S. (1990) *Nucleic Acids Res.* **18**, 5322.
- Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988) *Nucleic Acids Res.* **16**, 6127–6145.
- Wroblewski, F. and LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90**, 210–213.
- Hadrava, V., Tremblay, J. and Hamet, P. (1989) *Hypertension* **13**, 589–597.
- Roghani, M., Hossenlopp, P., Lepage, P., Bolland, A. and Binoux, M. (1989) *FEBS Lett.* **255**, 253–258.
- Martin, J. L., Willets, K. E. and Baxter, R. C. (1990) *J. Biol. Chem.* **265**, 4124–4130.
- Kiefer, M. C., Masair, F. R., Bauer, D. M. and Zapf, J. (1991) *J. Biol. Chem.* **266**, 9043–9049.
- Shing, Y., Folkman, J., Sullivan, R., Butterfield, R., Murray, J. and Klagsbrun, M. (1984) *Science* **223**, 1296–1299.
- Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A. and Bohlent, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6963–6967.
- Maciag, T., Mehlman, T., Triesel, R. and Schreiber, A. (1984) *Science* **225**, 932–935.
- Sullivan, R. and Klagsbrun, M. (1985) *J. Biol. Chem.* **260**, 2399–2404.
- Nakamura, T., Nawa, K., Ichihara, A., Kaise, N. and Nishio, T. (1987) *FEBS Lett.* **224**, 311–316.
- Seid, J. M., Jones, P. B. B. and Russell, R. G. G. (1983) *Clin. Sci.* **64**, 387–394.
- Umeda, F., Yamauchi, T., Inoguchi, T. and Nawata, H. (1992) *Diabetes Res. Clin. Pract.* **16**, 109–115.
- Hensby, C. N. and Lewis, P. J. (1979) *Lancet* **ii**, 748.
- Fitzgerald, G. A., Maas, R. L., Stein, R., Oates, J. A. and Roberts, L. J. (1981) *Ann. Intern. Med.* **95**, 319–322.
- Stuart, M. J. and Sills, R. H. (1981) *Br. J. Haematol.* **48**, 545–550.
- Yui, Y., Takatsu, Y., Hattori, R., Sakaguchi, K., Susawa, T., Ikeda, N. and Kawai, C. (1984) *Prostaglandins Leukotrienes Med.* **16**, 389–394.