Purification, Molecular Cloning, and Expression of a Novel Growth-Promoting Factor for Retinal Pigment Epithelial Cells, REF-1/TFPI-2

Yasubiko Tanaka,1 Jun Utsumi,2 Mizuo Matsui,5 Tetsuo Sudo,2 Noriko Nakamura,2 Masato Mutob,2 Akemi Kajita,2 Saburo Sone,2 Kazuteru Kigasawa,4 Masahiko Shibuya,1 Venkat N. Reddy,2 Qiang Zhang,1,6 and Takeshi Iwata1

PURPOSE. Retinal pigment epithelial (RPE) cells are known to play important roles in maintaining the homeostasis of the retina and in controlling choroidal neovascularization. The purpose of this study was to identify a factor or factors that would stimulate RPE cells to proliferate.

METHODS. To isolate such a factor, 100 L of human-fibroblast-conditioned medium underwent ion-exchange, hydrophobic, and reverse-phase chromatographies followed by sodium decyl sulfate–polyacrylamide gel electrophoresis. The growth-promoting activity of the factor was examined in a human K-1054 RPE cell line and human primary RPE cells.

RESULTS. The different chromatographic processes isolated a 31-kDa factor that had RPE cell growth-promoting properties. This factor, which we have named RPE cell factor (REF-1), promotes growth of RPE cells but not of human umbilical vein endothelial cells (HUVECs). The amino-terminal sequence and molecular cloned cDNA of REF-1 were identical with those of tissue-factor pathway inhibitor (TFPI)-2, a family of TFPIs, and placental protein (PP)-5, a serine protease inhibitor. The cDNA expression of REF-1/TFPI-2 with pcDL-psR vector in Chinese hamster ovary (CHO) cells confirmed the growth-promoting activity for RPE cells. The major component of the recombiant REF-1/TFPI-2 expressed in CHO cells had a molecular mass of 31 kDa and exerted growth-promoting activity in RPE cells but not in human endothelial cells and fibroblasts in vitro. REF-1/TFPI-2 also had protease inhibitory activity. The other family factor, TFPI-1, did not promote RPE cell growth.

CONCLUSIONS. REF-1/TFPI-2 is a novel growth-promoting factor for RPE cells but not for endothelial cells and fibroblasts. Its properties make it potentially beneficial for intracocular therapy for the repair and maintenance of RPE cells. (Invest Ophthalmol Vis Sci. 2004;45:245–252) DOI:10.1167/iovs.03-04230

Recent advances in basic and clinical research have shown that the pathogenesis of many retinal and choroidal diseases is closely related to the normal functioning of retinal pigment epithelial (RPE) cells. RPE cells play critical roles in maintaining the homeostasis of the retina and in controlling choroidal neovascularization. Because the denaturation of cellular proteins in the RPE and the loss of function of RPE cells are responsible for retinal and choroidal diseases, a factor that stimulates RPE cell growth could prove to be valuable for the treatment of RPE-related ocular diseases.

At present, various cell growth factors, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), are known to stimulate the proliferation of RPE cells. However, these factors are also known to affect the growth of vascular endothelial cells and fibroblasts, and ocular neovascularization and fibroblast proliferation can lead to serious retinal and choroidal diseases and proliferative vitreoretinopathy. The purpose of this study was to isolate and characterize a factor or factors that would promote RPE cell proliferation. We focused on the supernatant of cultured human fibroblasts as a source of the target factor, because fibroblasts function as stromal cells that are known to produce various cytokines. We have isolated a 31-kDa factor from the conditioned medium of human fibroblasts that promotes growth in RPE cells and named it RPE cell factor (REF-1). The amino terminal sequence was determined, and molecular cloning of its cDNA showed that the factor was identical with tissue-factor pathway inhibitor (TFPI)-2, a serine protease inhibitor (TFPI)-2.

MATERIALS AND METHODS

Isolation of RPE Cell Growth–Promoting Factor

Human fibroblast DIP-2 cells10 were cultured for 5 days in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS) in microcarriers (Cytodex I; Amersham Biosciences, Tokyo, Japan) in 16-L glass culture vessels at 37°C. After the addition of 100 IU/mL human interferon-β (Toray Industries, Tokyo, Japan) as a priming agent and 10 μg/mL poly(I) poly(C) (Yamasa Shoyu, Choushi, Japan) as a cytokine-inducing reagent, the culture media were replaced by serum-free Eagle’s MEM and cultured at 37°C for six additional days. The cultured medium was collected and filtered to remove the cellular debris. Fractionation was started by passing 100 L of the cultured medium through an S-Sepharose column (500 mL; Amersham Biosciences), and the fraction containing growth-promoting activity (active fraction) was eluted with 200 mL of 10 mM phosphate-buffered...
concentration of 10 ng/mL (Minneapolis, MN), and bFGF (R&D Systems), was performed at a family of TFPIs, ciliary neurotrophic factor (CNTF; R&D Systems, Japan, Tokyo, Japan) or 15% FCS (Invitrogen) was used for K-1034 or human primary RPE cells, respectively. Two microliters of purified REF-1 protein was eluted by a gradient of water-acetonitrile (0% fluroacetic acid (TFA; pH 2.0). Two milliliters of the active fraction eluted from the column was concentrated to 100 µL by speed vacuum concentrator (Speed Vac Systems, Savant, NY) and applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions without 2-mercaptoethanol (2ME). Immediately after migration of the sample into the gel, the SDS-PAGE gel was cut into 1 x 2 x 4 mm slices and immersed overnight at 4°C in 0.5 mL per slice of distilled water to extract the active protein. The extracted protein was reapplied to SDS-PAGE under reducing-nonreducing conditions to examine the purity and the molecular weight of the target protein.

**Determination of Cell Growth–Promoting Activity during Purification**

Human K-1034 RPE cells or fourth-passage human primary RPE cells were used to determine RPE cell growth–promoting activity.11 K-1034 cells or human primary RPE cells were added to collagen type I coated 24-well plastic plates (Corning International, Tokyo, Japan) at a density of 1 x 10^4 cells/well. DMEM supplemented with 5% FCS (Invitrogen Japan, Tokyo, Japan) or 15% FCS (Invitrogen) was used for K-1034 or human primary RPE cells, respectively. Two microliters of purified REF-1 was added to each well and cultured at 37°C for 5 days. The number of RPE cells at each time point was determined by a cell counter (model ZM; Beckman Coulter K. K., Tokyo, Japan). The growth-promoting rate was calculated as a percentage of the control (n = 4 or 6). In the first exploratory purification, the specific concentration of REF-1 was not determined, as an REF-1 ELISA kit is not available, and REF-1 was therefore traced by the growth-promoting activity in RPE cells.

**Determination of Cell Growth–Promoting Activity Using Purified REF-1 Protein**

To examine whether purified REF-1 promotes growth in the number of vascular endothelial cells, cells were isolated from the human umbilical vein of a patient at an obstetric hospital. In accordance with the provisions of the Declaration of Helsinki, all subjects signed an informed consent after an explanation of the procedures to be used and the purpose of the studies. The human umbilical vein endothelial cells (HUVECs) were treated under conditions similar to those used for RPE cells. The expression vector RPE27-EX and the expression vector pAdDHFR (Human placenta cDNA library, CLHL1008b; BD Biosciences-Clontech Japan, Tokyo, Japan). The first PCR was performed with primers, R1 and L1, designed to flank the insert of RPE27 gene (1100-1101 bp). The second PCR was performed using 1 µL of the first PCR reaction mixture as template with three primers: 276 (5'-GATGCGACCTGGCCGACAGGCG-3') and the R2 and L2 primers. The third PCR was performed with the second PCR mixture as a template, with primer 278 (5'-CAAGAACACGAGACGG-3') and the R3 and L3 primers.

Amino Acid Analyses of REF-1/TFPI-2

REF-1 was isolated as a 31 ± 4 kDa protein on SDS-PAGE gel under nonreducing conditions. The active fraction appeared to correspond to a single band on the silver-stained gel. REF-1 was isolated from the band and subjected to amino-terminal amino acid sequence analysis (Protein sequencer model 470; Applied Biosystems Japan, Tokyo, Japan).

Amino acid composition analysis of REF-1 component was performed after hydrolysis at 110°C for 22 and 72 hours in 6 M HCl with 4% thioglycolic acid (amino acid analyzer model 835; Hitachi, Tokyo, Japan).

**Molecular Cloning of REF-1 Protein**

The primers, R1: 5'-GGAGGAGAGCACATGGG-3', R2: 5'-TATGGGGAT-3', R3: 5'-ACCTGGAGGCGGCG-3', L1: 5'-AGATATGGAAGGCGCGC-3', L2: 5'-GAGACACGAAAGAATTG-3', and L3: 5'-GGTAGCGACCGGCGC-3' were used for PCR amplification of phase insert and were designed based on the sequence of cloning vector Agt11 (Human placenta cDNA library, CLHL1008b; BD Biosciences-Clontech Japan, Tokyo, Japan). The first PCR was performed with primers, R1 and L1, designed to flank the insert of Agt11. The second PCR was performed using 1 µL of the first PCR reaction mixture as template with three primers: 276 (5'-GATGCGACCTGGCCGACAGGCG-3') and the R2 and L2 primers. The third PCR was performed with the second PCR mixture as a template, with primer 278 (5'-CAAGAACACGAGACGG-3') and the R3 and L3 primers.

Amino Acid Analyses of REF-1/TFPI-2

REF-1 was isolated as a 31 ± 4 kDa protein on SDS-PAGE gel under nonreducing conditions. The active fraction appeared to correspond to a single band on the silver-stained gel. REF-1 was isolated from the band and subjected to amino-terminal amino acid sequence analysis (Protein sequencer model 470; Applied Biosystems Japan, Tokyo, Japan).

Amino acid composition analysis of REF-1 component was performed after hydrolysis at 110°C for 22 and 72 hours in 6 M HCl with 4% thioglycolic acid (amino acid analyzer model 835; Hitachi, Tokyo, Japan).

**Construction of Expression Vector for REF-1/TFPI-2**

REF-1 cDNA was reamplified by PCR from an original Agt11 phase clone by primer set RPE27-EX1 (5'-GGAGGAGAGCACATGGG-3') and RPE27-EX2 (5'-GGAGGAGAGCACATGGG-3') to obtain the insert for the expression vector. PCR was performed for 25 cycles in a reaction mixture with 0.2 µg of Agt11 DNA, 1.6 mM dNTP, 1.0 µM of primers (RPE 27-EX1 and RPE 27-EX2), and DNA polymerase (Ex Taq; Takara, Tokyo, Japan). The PCR product was digested with EcoRI and KpnI and ligated into expression vector pcDL-SRe296 to obtain expression vector RPE27-EX (Fig. 1).12

**Recombinant REF-1/TFPI-2 by CHO Cells**

The expression vector RPE27-EX and the expression vector pADHFR containing dihydrofolate reductase (DHFR) cDNA were cotransfected.
into the DHFR gene-deficient CHO DXB11 cell strain. The surviving DHFR-positive cells were selected in αMEM without ribonucleosides and deoxyribonucleosides with 10% FCS. Highly producible cells were then selected by addition of methotrexate (MTX) to the medium. The concentration was increased stepwise from 0.0025 μM, to 0.05 μM, and finally to 1 μM, to obtain highly producible cells.12

After reaching confluence, the culture medium was replaced by serum-free αMEM and the medium was collected every 2 days, nine times. The collected medium was centrifuged at 6000 rpm at 4°C for 15 minutes, filtered, and stored at 4°C until the large-scale purification procedures.

**Preparation of Anti-REF-1/TFPI-2 Antibody and ELISA**

Peptide antibody for REF-1/TFPI-2 was generated, using peptide NH2-SSGCHRRIENRFPDE-COOH, corresponding to residues 106-120 as an antigen. Rabbit antiserum was purified on a protein A column (Prosep A; Amersham Biosciences). A sandwich ELISA system was constructed by using primary antibody (5 μg/mL) generated against whole REF-1 protein, biotinylated secondary peptide antibody (5.2 μg/mL) raised against amino acids 106 through 120, and the avidin HRP anti-rabbit antibody. During the process of REF-1 purification, protein quantification was determined by this ELISA kit with detection sensitivity of 10 ng/mL.

**Purification of CHO Cell–Derived Recombinant REF-1/TFPI-2**

Forty liters of culture supernatant was applied to a gel filtration column (S-Sepharose FF, 5 × 15 cm, 300 mL; Amersham Biosciences) at 2.4 L/h and the column was washed with 1.2 L of 20 mM sodium citrate buffer (pH 5.0) and 1.7 L of buffer containing 0.2 M NaCl. Protein was eluted by 20 mM sodium citrate (pH 5.0)/0.4 M NaCl. TFA was added to the eluate at a final concentration of 0.1% and further purified by reverse-phase chromatography (Resource RPC column, 0.46 × 10 cm, 3 mL; Amersham Biosciences). The elution was performed with acetonitrile gradient of 0% to 70% in 0.1% TFA (pH 2.0). REF-1 was eluted in 19 mL of 31% to 35% acetonitrile fraction. This fraction was diluted with 40 mM PBS (pH 7.2) to twofold volume and applied to a gel filtration column (SP-Sepharose FF, 1 × 1.3 cm, 1 mL; Amersham Biosciences). REF-1 was eluted with 20 mM PBS (pH 7.2) containing 0.45 M NaCl.

**Determination of Protease Inhibitor Activity**

Plasmin inhibition by REF-1 was analyzed by a method introduced previously.13 Reaction buffer (50 mM Tris-HCl [pH 7.5], 5 mM CaCl2, 0.1 M NaCl, 0.01% Tween 20) was added to 96-well plastic plates followed by the addition of 0.4 μg aprotinin (Boehringer-Yamanouchi, Tokyo, Japan) and REF-1/TFPI-2 at final concentration of 5 μg/mL. One hundred twenty-five nanograms of plasmin was added (Chromogenix, Milano, Italy) and incubated at room temperature for 30 minutes. Fifty microliters of substrate S-2251 (Val-Leu-Lys-pNA, 1 mg/mL; Chromogenix) was added and the absorbance was measured at 405 to 450 nm for 15 minutes with a microplate photometer (UV/Visible Spectrometer DU640; Beckman Coulter, Fullerton, CA) every 20 seconds. The percentage of relative activity in the inhibitor concentration was then calculated.

**Determination of RPE Cell Production of Cytokines**

The relationship between RPE cell growth and production of the growth factor bFGF, transforming growth factor (TGF)-β1, transforming growth factor (TGF)-β2, epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), and macrophage-CSF (M-CSF), and the cytokines interleukin (IL)-1α, IL-6, IL-8, tumor necrosis factor (TNF)-α by human primary RPE cells was examined. The cells were grown in DMEM with 15% FCS for 3 days and the medium then replaced by serum-free DMEM. The cytokines in the culture supernatant were determined for two additional days by ELISA kits (Amersham International, Buckinghamshire, UK; R&D Systems, Minneapolis, MN; Immuno-Biological Laboratories, Gunma, Japan).

**Western Blot Analysis of REF-1 for RPE Cell Extract**

Cellular extract was obtained from RPE cells by using M-PER (Pierce, Rockford, IL) detergent mixture. A sample amount of 7.2 μg was...
applied to each lane in 12% polyacrylamide gels. For positive control, bacteria expressing REF-1 protein was added (see Fig. 7, lane 4). After the separation, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Relliehausen, Germany), blocked for 1 hour with the blocking solution containing 10% milk diluent—blocking solution (KPL, Gaithersburg, MD) and 0.1% Tween-20 in phosphate-buffered saline (pH 7.4). The membrane was probed with a rabbit polyclonal anti-REF-1 antibody (1 μg/mL). The specific signal was detected by incubation of anti-rabbit IgG HRP secondary antibody (New England Biolabs, Beverly, MA) followed by chemiluminescence reactions with luminol reagent A and peroxide reagent B, as recommended by the manufacturer (New England Biolabs) and made visible with a chemiluminescence imager (Lumi-Imager F1; Roche Applied Science, Tokyo, Japan).

RNA Isolation from RPE cells and RT-PCR of REF-1

Total RNA was isolated from cultured fourth-passage human primary RPE cells with a total RNA isolation kit (RNA-Bee-RNA Isolation Reagent; Tel-Test, Friendswood, TX). Total RNA samples were digested by RNase-free DNase (Roche Diagnostics Japan) to minimize the risk of genomic DNA contamination. First-strand cDNA was synthesized using random primers (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen Japan). PCR was performed using 1 μg of single-strand cDNA with 2.5U Taq DNA polymerase in a volume of 50 μL. After predenaturation at 95°C for 5 minutes, 30 cycles were performed, including denaturation at 95°C for 30 seconds, and extension at 72°C for 1 minute, followed by 1% agarose gel electrophoresis. The primers used were: 5′-ATTCTGCTGCTTTTCGTCAGC-3′ (sense primer) and 5′-CAGCTCTGCGTGTTACACTGTC-3′ (antisense primer).

RESULTS

Isolation and Identification of an RPE Cell Growth–Promoting Factor

The RPE cell growth–promoting fraction was purified from 100 L of starting material to 0.5 mL of SDS-PAGE gel extract. REF-1 was concentrated by 2 × 10^3-fold after the final step of purification. The profile of the RPE cell growth–promoting factor is shown at each step in Figure 2. The peak of RPE cell growth promotion was mainly detected in three fractions of molecular mass 46 ± 3, 31 ± 3, and 27 ± 3 kDa on the SDS-PAGE gel. The 31-kDa fraction had the highest RPE cell growth-promoting effect. This fraction showed very low growth stimulation in HUVECs for all molecular sizes detected. The 31-kDa active fraction was separated from the SDS-PAGE gel under reducing-nonreducing conditions. The 31-kDa band was made visible as a major component by silver staining (Fig. 3). There was a minor component at 61 kDa that was predicted as a dimeric form of REF-1. Amino-terminal sequence analysis was performed on the purified 31-kDa protein.

Amino-Terminal Sequence of RPE Cell Growth–Promoting Factor

Amino-terminal sequence analysis of the 31-kDa component resulted in the following sequence: NH₂-Asp-Ala-Glu-Gln-Pro-Thr-Gly-Thr-Asn-Ala-Glu-Ile-Xaa-Ala-COOH (14 amino acids).

In addition, amino-terminal sequence analysis of the 27-kDa component gave nine residues of sequences identical with the 31-kDa component. The polypeptide was named REF-1. Because the amino-terminal sequence of REF-1 was apparently identical with TFPI-28-PP5, molecular cloning of REF-1 was performed to confirm the whole sequence of the 31-kDa protein. For the 46-kDa active component isolated on SDS-PAGE gel, the amino acid sequence could not be identified because of insufficient quantity of the protein.

Molecular Cloning of REF-1

Although REF-1 was identical with TFPI-2 at the amino-terminal molecular cloning was performed to determine the complete cDNA of REF-1. One of the 16 clones isolated had an amino-terminal sequence identical with that of TFPI-2. The cloned REF-1 molecule consisted of 235 amino acids, and the theoretical molecular mass of this polypeptide was 27 kDa. The position of three tandemly arranged Kunitz-type domains and two binding sites of predicted asparagine-linked sugar chains were identical with TFPI-2. From the available evidence, we concluded that REF-1 is identical with TFPI-2. The calculated molecular mass increased by 4 to 6 kDa after possible glycosylation to molecular mass between 31 and 33 kDa.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (mL)</th>
<th>Protein Conc (μg/mL)</th>
<th>Total Protein (mg)</th>
<th>REF-1 (mg)</th>
<th>Yield (%)</th>
<th>Purity (%)</th>
<th>Purification (×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO cell CM</td>
<td>40,000.0</td>
<td>113.8</td>
<td>4552.0</td>
<td>10.8</td>
<td>100</td>
<td>0.24</td>
<td>1</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>800.0</td>
<td>82.8</td>
<td>66.2</td>
<td>8.6</td>
<td>80</td>
<td>13.00</td>
<td>54</td>
</tr>
<tr>
<td>Resource RPC</td>
<td>40.0</td>
<td>150.0</td>
<td>6.4</td>
<td>6.4</td>
<td>59</td>
<td>87.00</td>
<td>360</td>
</tr>
<tr>
<td>SP-Sepharose</td>
<td>4.2</td>
<td>915.0</td>
<td>3.8</td>
<td>3.8</td>
<td>35</td>
<td>97.00</td>
<td>400</td>
</tr>
</tbody>
</table>

2ME

TABLE 1. Purification of CHO-Cell-Derived Recombinant REF-1

Downloaded from iovs.arvojournals.org on 10/06/2019
We developed a large-scale purification procedure for CHO cell-derived recombinant REF-1. From 40 L of conditioned medium of recombinant REF-1-CHO cells, recombinant REF-1 was purified by the combination of cation exchange chromatography and reverse-phase high-performance liquid chromatography (HPLC) as shown in Table 1. The purity of the final recombinant REF-1 was more than 97% on SDS-PAGE gel and was free of pyrogen. The reverse-phase HPLC profile and SDS-PAGE pattern of purified CHO cell-derived recombinant REF-1 are shown in Figure 4.

**Molecular Heterogeneity of REF-1**

A molecular heterogeneity of the CHO-cell-derived recombinant REF-1 was observed. Three forms of REF-1 at molecular masses of 31 ± 1, 27 ± 1, and 17 ± 1 kDa were found. The ratios for each size were approximately 40% for 31 kDa, 50% for 27 kDa, and 10% for 17 kDa. The 31- and 27-kDa components were major and appeared to be different because of attached sugar chains. The 17-kDa component was smaller than the theoretical molecular mass by approximately 10 kDa. This form was possibly produced by extracellular protease digestion after the secretion of the mature form based on the amino acid composition analysis. The molecular mass of 10 kDa was calculated to match the 28-kDa component lacking the C-terminal portion.

Currently, data are not available for the differences in biological effects of the different molecular forms. TFPI-2 also demonstrated molecular heterogeneity of 31 and 27 kDa, and it has been suggested that this may be due to different glycosylated forms.14

**Cell Growth–Promoting Activity of Recombinant REF-1**

The growth-promoting activity of REF-1 in K-1034 cells was dose dependent, with a bell-shaped curve (Fig. 5a), perhaps because of the downregulation of receptor at a higher REF-1 concentration.

The growth-promoting activities of other relevant cytokines, TFPI-1, CNTF, and bFGF on RPE cells were compared at a 10-ng/mL concentration. TFPI-1 is a member of the TFPI family with 35% amino acid sequence homology with TFPI-2; however, RPE cells did not respond to TFPI-1. CNTF, a human ciliary nerve nutritional factor, also did not stimulate RPE cell proliferation. However, the growth stimulation of bFGF was stronger than that of REF-1 (Fig. 5b).

Growth stimulation of HUVECs, human fibroblasts, rabbit primary RPE cells, and fourth-passage human primary RPE cells was also examined (Fig. 5c). A 12% and 25% increase after
stimulation by REF-1 was observed in rabbit primary RPE cells and human primary RPE cells, respectively. Significant proliferation was observed in human primary RPE cells cultured in medium with 15% FCS.

Proteinase Inhibitory Activity
REF-1 inhibited plasmin (Fig. 6), and it was confirmed that it inhibited serine protease.

Determination of REF-1 in Human Primary RPE Cells
The existence of REF-1 was determined in human primary RPE cells by Western blot analysis and RT-PCR (Fig. 7). REF-1 was not detected in RPE cells by Western blot under the conditions we used; however, REF-1 mRNA was detected in total RNA extracted from human primary RPE cells by 30 cycles of PCR.

Effect of REF-1 Treatment on Cytokine Production of RPE Cells
Eleven cytokines and growth factors were measured in serum-free culture medium of fourth-passage human primary RPE cells treated with 10 ng/mL of REF-1 for 2 days. TGF-β1 and GM-CSF were significantly induced by 4.7- and 2.4-fold, respectively. bFGF, IL-6, IL-8, and M-CSF showed no or only a moderate increase with REF-1 treatment. TGF-β2, IL-1β, G-CSF, TNF-α, and EGF were undetectable (Table 2).

DISCUSSION
We have isolated and identified a biologically active protein that stimulated RPE cell to proliferate and consider it to be a potential therapeutic agent. This factor has growth-promoting properties that it exerts on RPE cells and was identified as REF-1 protein. Molecular cloning showed that this factor was homologous to the TFPI-2/PP5 protein. The RPE cell growth-promoting effect of REF-1/TFPI-2 was found to be more specific to RPE cells than to fibroblasts and HUVECs. Currently, there are no reports of factors that specifically stimulate the growth of RPE cells, although several growth factors such as bFGF, EGF, PDGF, and VEGF are growth promoters. These factors also have other properties, such as angiogenesis and potential stimulation of endothelial cell growth and can cause proliferative vitreoretinopathy by fibroblast proliferation. These undesirable properties do not allow them to be used for the treatment of retinal diseases. Although REF-1/TFPI-2 has a relatively weaker growth-promoting action than bFGF in vitro, it did not stimulate endothelial cell growth or fibroblast proliferation. Thus, the specificity of REF-1/TFPI-2 to RPE cells is greater than that of other growth factors (Fig. 5).

We determined the growth-promoting activity of REF-1/TFPI-2 using 10th- to 20th-passage human K-1034 RPE cells, primary HUVECs, primary rabbit RPE cells, and 4th-passage primary human RPE cells. Early-passage RPE cells responded

![Figure 6. Protease inhibitory activity of REF-1. The residual activities of plasmin with aprotinin (positive control, 4 μg/mL) or REF-1/TFPI-2 (5 μg/mL) were determined, in 96-well plastic plates, with S-2251 (Val-Leu-Lys-pNA, 1 mg/mL) used as a substrate. The percentage of relative activity in the inhibitor concentration was calculated from absorbance at 405 to 450 nm.](image)

![Figure 7. Determination of REF-1 in human primary RPE cells by Western blotting analysis and RT-PCR.](image)
satisfactorily to REF-1; however, aged K-1034 RPE cells did not (data not shown), whereas primary rabbit RPE cells and primary HUVECs responded poorly to REF-1. Aged K-1034 RPE cells retained their response to basic FGF as well as early-passaged cells. These observations indicate that the growth-promoting effect of RPE-1 may be age-related and that it probably stimulates growth by a pathway different from that used by other growth factors such as bFGF. Although, growth stimulation was observed for human primary RPE cells in both serum-free and serum-added medium, REF-1 favored the latter condition, resulting in fourfold proliferation. Exogenous factor(s) may be involved in this effect.

Our experiments showed that at least 2 of 11 cytokines were stimulated by REF-1 treatment. To our surprise, TGF-β1 production was significantly induced (4.7-fold) in REF-1-treated compared with nontreated cells. A possible explanation for this phenomenon is that TGF-β1 production is stimulated to suppress and balance the rapid growth rate of RPE cells. This suggestion may be supported by the inhibitory effect of TGF-β1 on RPE cell proliferation.21

Another cytokine, increased by 2.4-fold, was colony-stimulating factor GM-CSF. GM-CSF is known to be an important regulator of macrophage, granulocyte, dendritic cell, and eosinophil behavior.22,23 RPE cells have properties similar to macrophages—that is, to phagocytose and generate different cytokines, including GM-CSF.24 In RPE cells, GM-CSF has been reported to be upregulated in response to TNF-α25, IL-1α,26 or IL-1β26 and downregulated by IFN-γ.25 The signal transduction mechanism for upregulation of GM-CSF by REF-1 is currently under investigation.

REF-1 was detected by RT-PCR in human primary RPE cells after 30 cycles of PCR; however, Western blot analysis failed to detect REF-1 in the experimental conditions we used. REF-1 mRNA may require specific stimulation to produce protein in RPE cells.

TFPI-2 has been shown to act as an anticoagulant and serine protease inhibitor. It is unclear whether these activities are correlated with growth promotion. Recent studies on TFPI-2 have shown that it has novel biological effects, such as inhibition of matrix metalloproteinase (MMP).15,16 Promotion of smooth muscle growth,17 and modulation of melanoma and glioma invasion.18,19 The relationship between these activities and promotion of RPE cell proliferation is still unknown. TFPI-2/REF-1 has been found in human ciliary epithelium20 and may play an important role in the normal RPE environment. It also has potential for therapeutic use for ocular tissue damage. To confirm these possibilities further pharmacological evaluations in vivo are needed, using suitable animal models and effective drug delivery methods to the damaged sites.

## Novel Growth-Promoting Factor for RPE Cells

### References


