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FIGURE LEGENDS

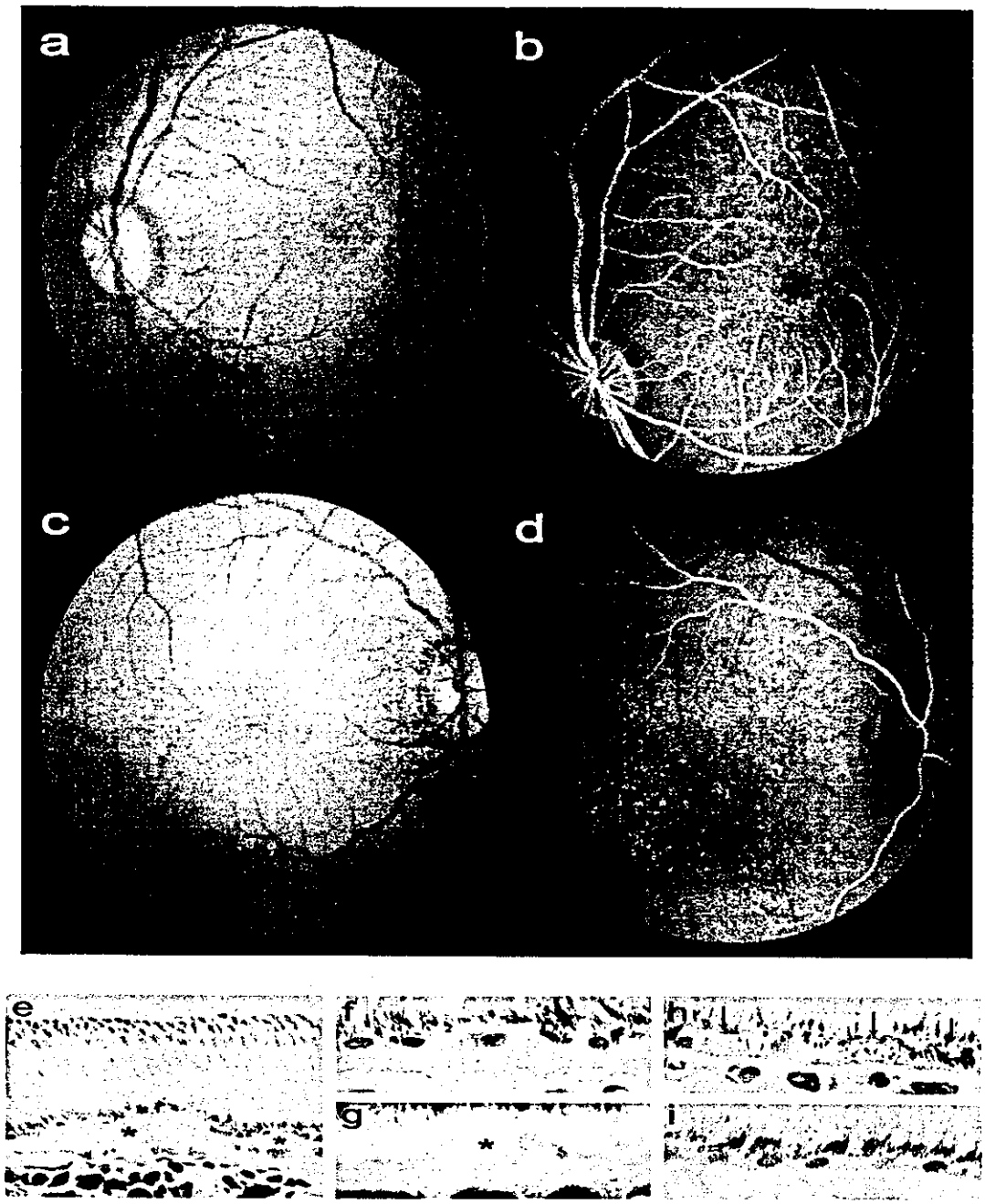

Figure 1

Drusen and degenerative changes of the RPE cells in late onset macular degeneration

monkeys.

Fundus photographs (a, c) and fluorescein angiography (FA) (b, d) of the monkeys affected with age-related form of late onset macular degeneration. Fine dots colored in yellowish-white could be observed in the maculae (a, c). Hyperfluorescein dots could be detected by FA corresponding to these spots (b, d). The fundus photograph and FA of a 17 year-old monkey that showed vacuolation and hyper- or hypopigmentation of the RPE cells (a, b). The fundus photograph and FA of another 17 year-old monkey that showed drusen (c, d). No abnormalities were found in the optic disc nor the blood vessels. (e) Various sized drusen accumulated between the RPE and choriocapillaris in the macular region (asterisks). Photoreceptor inner segments and outer segments appeared largely normal. (f) Drusen that had an eosinophilic inclusion (asterisk). (g) This spherical structure showed equivalent autofluorescence to that emitted by lipofuscin granules in the RPE (asterisk). (h) Vacuolation and hyper- or hypopigmentation of the RPE cells (arrows). (i) Intact region of the RPE in the same monkey as (h).

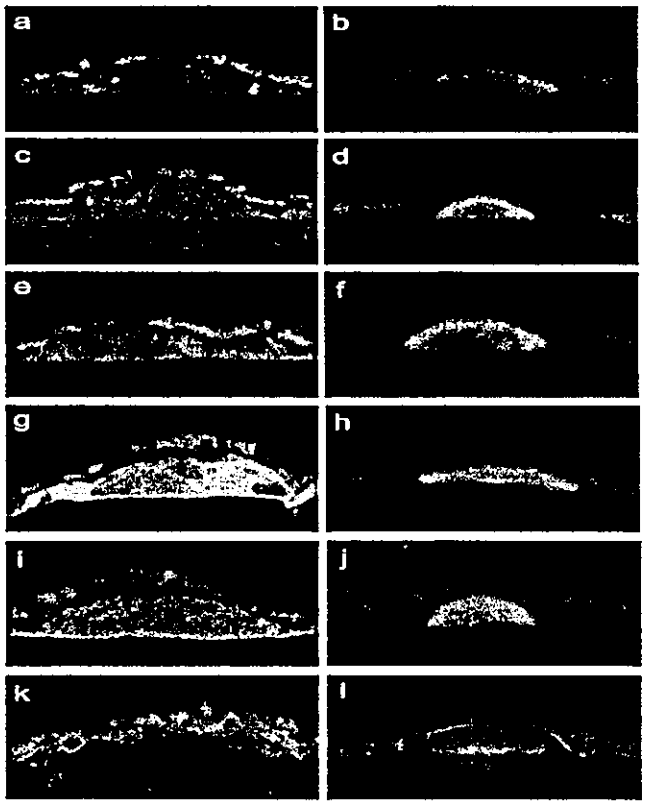

Figure 23

Drusen both in late onset and early onset macular degeneration monkeys are immuno-reactive for the protein components known in human AMD.

Drusen in late onset (a, c, e, g, i, k) and early onset (b, d, f, h, j, l) macular degeneration were heterogeneously bound by antibodies directed against apolipoprotein E (a, b), amyloid P component (c, d), complement component C5 (e, f), the terminal C5b-9 complement complex (g, h), vitronectin (i, j), and membrane cofactor protein (k, l). Double labeled images were generated by the green channel for each antigen and red channel for autofluorescence emitted by lipofuscin pigment in the RPE.

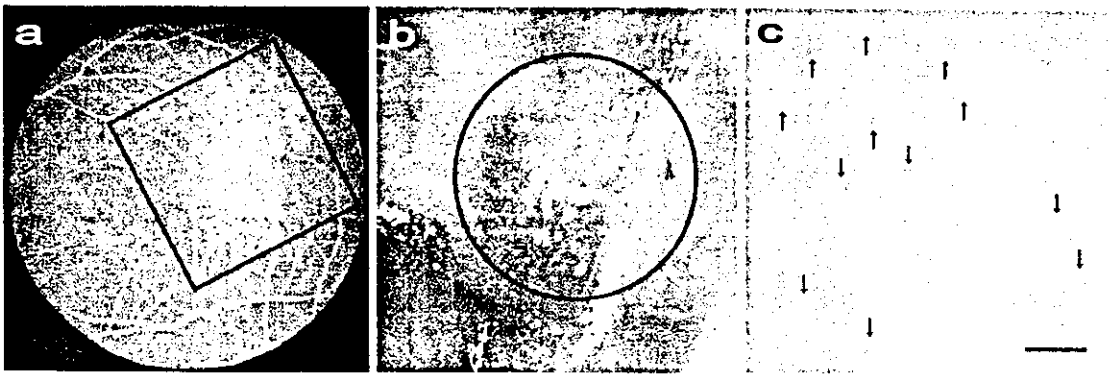

Figure 34

Isolation of drusen.

(a) The FA photograph of a monkey retina used for drusen isolation. A number of drusen that shows hyperfluorescence could be observed in the parafoveal region (indicated by a rectangle). (b) Drusen could be observed attached to the surface of Bruch's membrane at magnifications between 20 and 30 diameters under a stereoscopic microscope (white materials in a circle). (c) Isolated drusen (arrows). Diameter of a circle in (b) = 3 mm. Bar in (c) = 1mm.

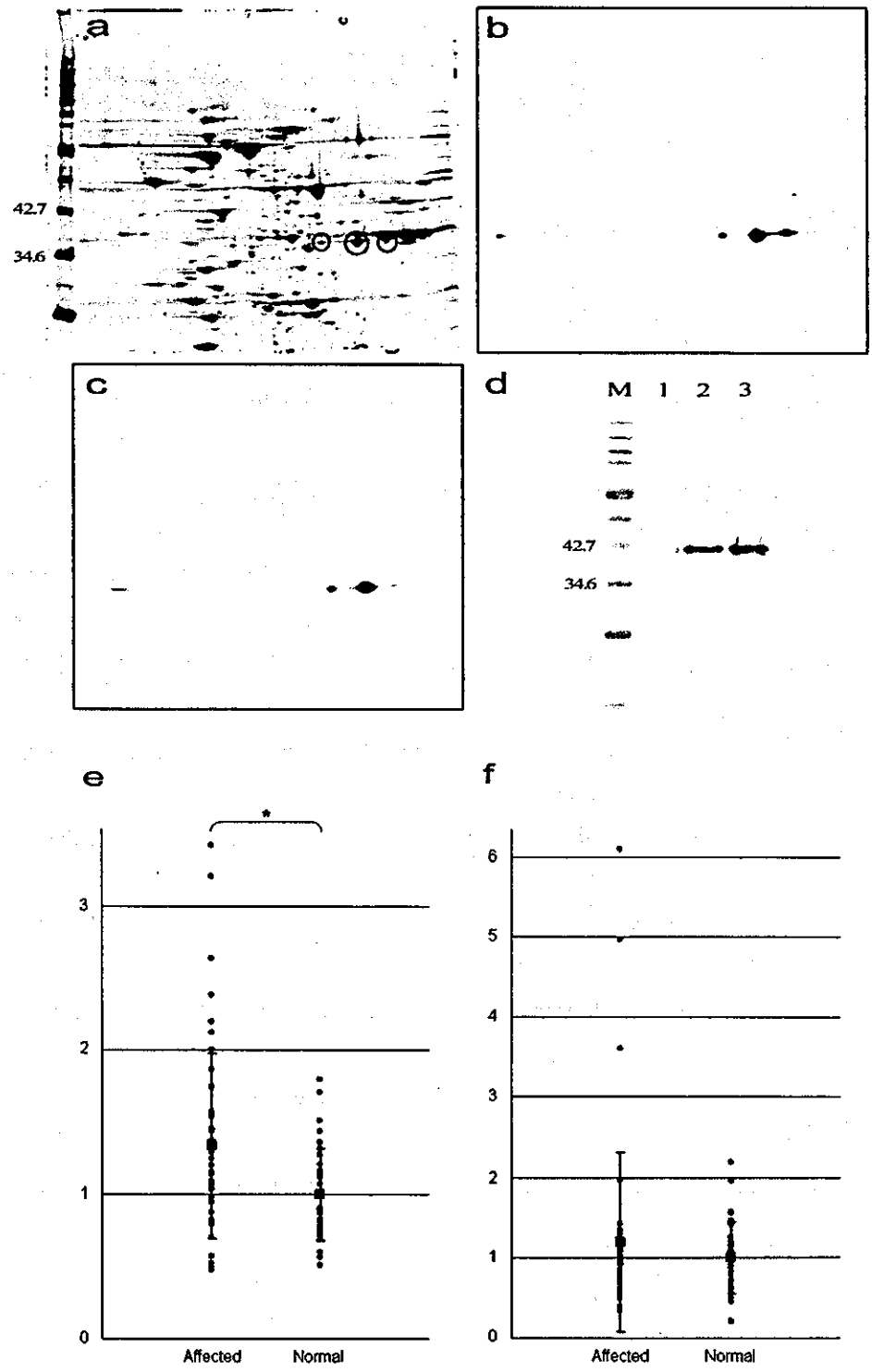
a
42.7
34.6
b
c
d
M 1 2 3
42.7
34.6
e
*
3
2
1
0
Affected
Normal
f
6
5
4
3
2
1
0
Affected
Normal

Figure 45

Identification of immunogenic molecules for autoantibodies in the affected monkey sera with late onset macular degeneration.

(a) An image of 2-D electrophoresis of fractionated retinal proteins visualized by SYPRO Ruby. (b) The serum from the same monkey in Fig. 1-(c) showed three immunoreactive spots in a row at approximate size of 38 kDa. The corresponding protein spots to the chemiluminescent signals were excised (circles in a), and analyzed by LC-MS/MS. (c) Chemiluminescent signals obtained by immunoreaction with anti-annexin II monoclonal antibodies completely matched with those with the serum. (d) The recombinant annexin II purified with affinity columns on SDS-PAGE gel at approximate size of 41 kDa (lane 1). The recombinant proteins reacted with both anti-annexin IIA2 monoclonal antibodies (lane 2) and autoantibodies in the serum (lane 3). M; molecular size marker (kDa). Relative antibody valuestiters against annexin II (e) or μ-crystallin (f) in sera from affected monkeys with age-related form oflate onset macular degeneration and age-matched control animals. Relative antibody valuestiters of individual monkeys are indicated by the ratio to the mean of normal monkeys. *P value < 0.01.

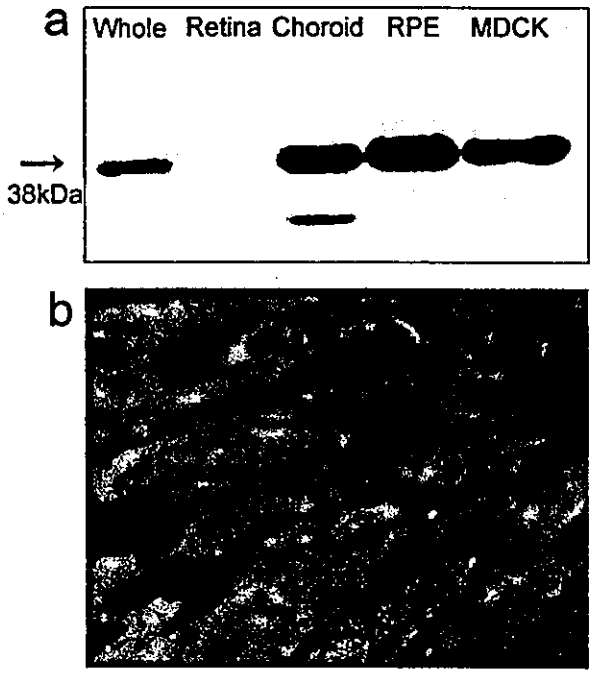

Figure 5

Expression of annexin II in the retina.

(a) The protein expression of annexin II (38kDa) was confirmed in whole retina, choroid, and most intensively in cultured human RPE cells. Protein extract from MDCK cells was used for positive control. (b) Fluorescence microscopy demonstrated that RPE cells highly expressed annexin II *in vitro*.

Table 1. Antibodies used in immunohistochemcal studies and condtions of antigen retrieval treatments.

Antigen	Retrieval treatment	Primary antibody		
		Host	Dilution	Supplier
Amyloid P Component	Pro K	Rabbit	200	Dako, Carpenteria, CA
Apolipoprotein E	-	Mouse	200	Biogenesis, Poole, UK
C5	Pro K	Rabbit	200	Dako, Carpenteria, CA
C5b-9	Pro K	Mouse	50	Dako, Carpenteria, CA
MCP	Autoclave	Rabbit	50	Santa Cruz, Santa Cruz, CA
Vitronectin	-	Mouse	100	Chemicon, Temecula, CA

Table 2. Macular status of aged monkeys.

Grade	Examined Year			Total	Percentage
	2001	2003	2004		
Normal	45	98	45	188	67.6 %
Mild	4	11	15	30	10.8 %
Moderate	5	16	10	31	11.2 %
Severe	6	17	6	29	10.4 %
Total	60	142	76	278	100.0 %

278 aged female monkeys were examined by fundus scope, and classsified into four grades. Normal-macula with no detectable pigmentary abnormalities. Mild-fewer than 5 yellowish-white spots. Moderate-5 to 20 spots. Sever-more than 20 spots.

Table 3. Protein components in monkey drusen.

Protein	Accession No.	Protein	Accession No.
Actin, alpha 2	gi|4501883|	**Hemoglobin, beta**	gi|4504349|
Albumin	gi|4502027|	*Hemoglobin, delta*	gi|70353|
Aldehyde dehydrogenase 3	gi|283971|	*Histone, H2A C*	gi|4504239|
Aldehyde dehydrogenase 5	gi|105247|	*Histone, H2A Z*	gi|4504255|
Aldolase A	gi|4557305|	*Histone, H2B F*	gi|10800140|
Alpha-1-antitrypsin	gi|1703025|	*Ig, alpha 2C*	gi|113585|
Alpha-1B-Glycoprotein	gi|46577680|	**Ig, gamma 2C**	gi|121043|
Annexin V	gi|4502107|	**Ig, lambda**	gi|87890|
Apolipoprotein E	gi|4557325|	Lactate dehydrogenase A	gi|5031857|
ATP synthase alpha chain, mitochondrial	gi|4757810|	Malate dehydrogenase 1	gi|5174539|
Calmodulin 2	gi|4502549|	Peptidylprolyl isomerase A isoform 1	gi|10863927|
Calreticulin	gi|4757900|	Phosphoglycerate kinase 1	gi|4505763|
cAMP-dependent protein kinase inhibitor, beta	gi|14210480|	Phosphoinositide 3-kinase, T96	gi|7434348|
Cell adhesion protein SQM1	gi|105595|	Plectin 1	gi|14195007|
Ceruloplasmin	gi|4557485|	Prostatic binding protein	gi|4505621|
Clusterin	gi|4502905|	Protease inhibitor 4	gi|21361302|
Collagen, alpha 1(VII)	gi|627406|	Pyruvate dehydrogenase	gi|4885543|
Complement component 5	gi|4502507|	Pyruvate kinase, M1 isozyme	gi|20178296|
Complement component 9	gi|4502511|	Ran-Binding Protein 2	gi|1709217|
Creatine kinase B	gi|125294|	Recoverin	gi|4506459
Crystallin, beta B2	gi|299263|	Retinol-binding protein 3	gi|4506453|
Crystallin, beta S	gi|345764|	Structural maintenance of chromosomes 1-like 1	gi|30581135|
Dystrobrevin, alpha isoform 8	gi|14916515|	Transferrin	gi|4557871|
Enolase 2	gi|5803011|	Triosephosphate isomerase 1	gi|4507645|
G3PDH	gi|7669492	*Tubulin, alpha 3*	gi|5174733|
Glucose phosphate isomerase	gi|18201905|	Ubiquitin and ribosomal protein L40	gi|4507761|
Glutamate-ammonia ligase	gi|2144562|	Ubiquitous mitochondrial creatine kinase	gi|10334859|
Haptoglobin	gi|4826762|	**Vimentin**	gi|14742600|
Haptoglobin-related protein	gi|123510|	**Vitronectin**	gi|72146|
Hemoglobin, alpha 2	gi|4504345|	14-3-3 protein beta/alpha	gi|4507949|

The components consistent with those of AMD drusen are shown in bold letters. The components that belong to the gene families, other members of which were known to be consituents of drusen in AMD, are shown in italic letters. National Center for Biotechnology Information database accession and version numbers are listed.

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

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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 dodecyl sulfate–polyacrylamide gel electrophoresis. The growth-promoting activity of the factor was examined in a human K-1034 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-pSRα vector in Chinese hamster ovary (CHO) cells confirmed the growth-promoting activity for RPE cells. The major component of the recombinant 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 intraocular therapy for the repair and maintenance of RPE cells. (*Invest Ophthalmol Vis Sci.* 2004;45:245–252) DOI:10.1167/iovs.03-0230

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.[1] 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.[2–4] However, these factors are also known to affect the growth of vascular endothelial cells and fibroblasts,[5–7] 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[8]-placental protein 5 (PP5).[9]

MATERIALS AND METHODS

Isolation of RPE Cell Growth–Promoting Factor

Human fibroblast DIP-2 cells[10] 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 Shouyu, Choushi, Japan) as a cytokine-inducing reagent, the culture media was 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

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saline (PBS) at pH 7.4 with 0.5 M NaCl. The eluate was added to 1 M ammonium sulfate and applied to a polypropyl A column (0.8 × 25 cm; PolyLC, Columbia, MD). The active protein was eluted by a gradient of 0 to 1 M ammonium sulfate in 10 mM PBS. Four milliliters of the active fraction from the polypropyl A column were injected into a C4 reverse-phase column (1 × 25 cm; Grace Vydac, Hesperia, CA), and the protein was eluted by a gradient of water-acetonitrile (0%–70%) including 0.1% trifluoroacetic 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, MN) 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 × 2 × 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 1×10^4 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 (n = 4 or 6). Human fibroblasts (DIP-2 cells), rabbit primary RPE cells, and human primary RPE cells were also used to characterize the growth-promoting activity (n = 6).

A comparison of the growth-promoting profile of other related factors, such as TFPI-1 (American Diagnostica, Greenwich, CT), the family of TFPIs, ciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN), and bFGF (R&D Systems), was performed at a concentration of 10 ng/mL (n = 6).

Amino Acid Analyses of REF-1/TFPI-2

REF-1 was isolated as a 31 ± 3-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).

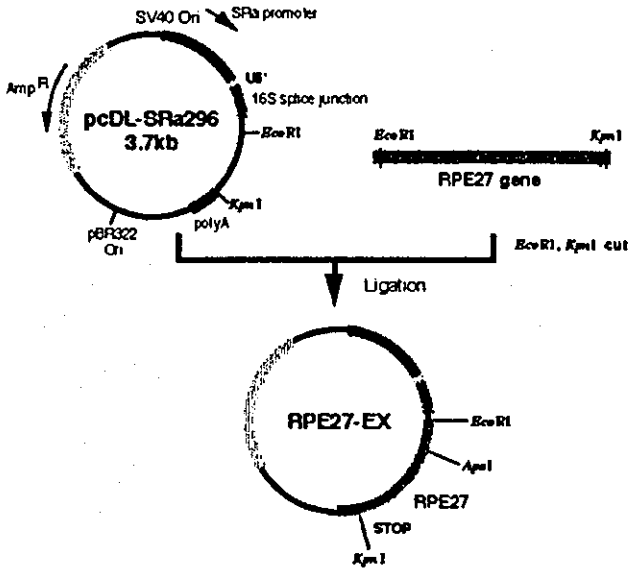

FIGURE 1. The construction of the REF-1 expression vector. The RPE27-EX1 expression vector was obtained by ligation with pcDL-SRα296 vector (3.7 kb) and RPE27 gene (750 bp).

Molecular Cloning of REF-1 Protein

The primers, R1: 5′-GGAAGAAGGCACATGGC-3′, R2: 5′-TATGGGGATTGGTGGCG-3′, R3: 5′-ACTCCTGGAGCCCGTC-3′, L1: 5′-AGACATGGCCTGCCCG-3′, L2: 5′-GACACCAGACCAACTGG-3′, and L3: 5′-GGTAGCGACCGGCGC-3′ were used for PCR amplification of phage insert and were designed based on the sequence of cloning vector λgt11 (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 λgt11. The second PCR was performed using 1 μL of the first PCR reaction mixture as template with three primers: 2751 (5′-GATGCIGAACAAGAACCIACIG-3′) and the R2 and L2 primers. The third PCR was performed with the second PCR mixture as a template, with primer 2752 (5′-CAAGAACCIACIGGIACIAATGC-3′) and the R3 and L3 primers.

The PCR conditions were initiated at 94°C for 5 minutes, then 25 cycles at 94°C for 30 seconds, 56°C for 2 minutes, and 72°C for 8 minutes, followed by 1 cycle at 72°C for 7 minutes. The amplified DNA fragment from the third PCR was separated by 1% agarose gel electrophoresis and the DNA fragment was purified from gel by the electroelution method. Purified DNA fragment was cloned using a kit (Sure Clone; Amersham Bioscience). The nucleotide sequence was determined for 16 clones containing full-length cDNA on a DNA sequencer (model 373A; Applied Biosystems).

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

REF-1 cDNA was reamplified by PCR from an original λgt11 phage clone by primer set RPE27-EX1 (5′-GGGGAATTCCTTTCTCGGACGGCTTGC-3′) and RPE27-EX2 (5′-GGGGGTACCTAAAAATTGCTTCTTCCG-3′) to obtain the insert for the expression vector. PCR was performed for 25 cycles in a reaction mixture with 0.2 μg of λgt11 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 *Eco*RI and *Kpn*I and ligated into expression vector pcDL-SRα296 to obtain expression vector RPE27-EX (Fig. 1).[12]

Expression of Recombinant REF-1/TFPI-2 by CHO Cells

The expression vector RPE27-EX and the expression vector pAdDHFR 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 NH_2-SGGCHRNRIENRFPDE-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 $CaCl_2$, 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

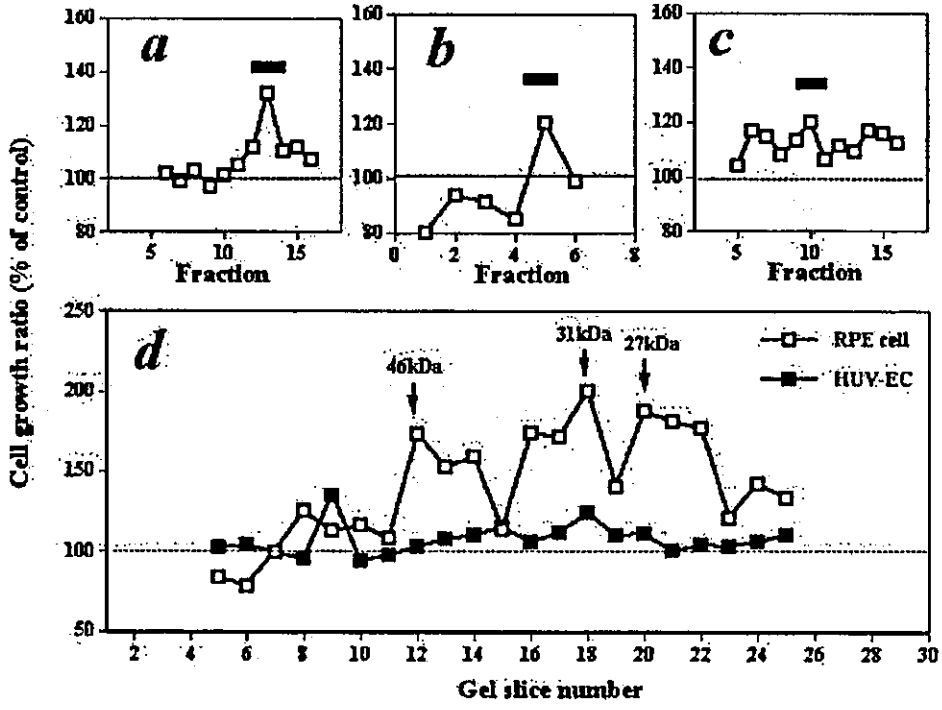

FIGURE 2. Chromatography was used to determine RPE cell growth-promoting activity. Profiles of the activity on S-Sepharose (a), polypropyl-A (b), and Vydac-C4 (c) columns and on a nonreducing SDS-PAGE gel (d). Data denote active fractions collected. In the SDS-PAGE fraction, the growth-promoting activity was examined in RPE cells and HUVECs.

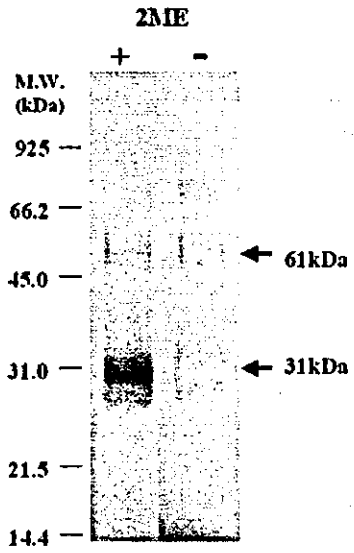

FIGURE 3. SDS-PAGE pattern of the RPE cell growth-promoting factor REF-1 isolated from the conditioned medium of human fibroblasts. Approximately 1 μg/lane of the purified protein was loaded and made visible with silver staining after electrophoresis under reducing (+2ME) and nonreducing (−2ME) conditions. A major band at 31 kDa and a minor band at 61 kDa correspond to the monomeric and dimeric forms of REF-1, respectively.

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, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute, followed by 1% agarose gel electrophoresis. The primers used were: 5′-ATTCTGCTGCTTTTC-CTGAC-3′ (sense primer) and 5′-CAGCTCTGCGTGTACCTGTC-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 2×10^5-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: NH_2-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-2[8]-PP5,[9] 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 1. Purification of CHO-Cell-Derived Recombinant REF-1

Purification Step	Volume (mL)	Protein Conc (μg/mL)	Total Protein (mg)	REF-1 (mg)	Yield (%)	Purity (%)	Purification (×)
CHO cell CM	40,000.0	113.8	4552.0	10.8	100	0.24	1
S-Sepharose	800.0	82.8	66.2	8.6	80	13.00	54
Resouce RPC	40.0	159.0	6.4	6.4	59	87.00	360
SP-Sepharose	4.2	913.0	3.8	3.8	35	97.00	400

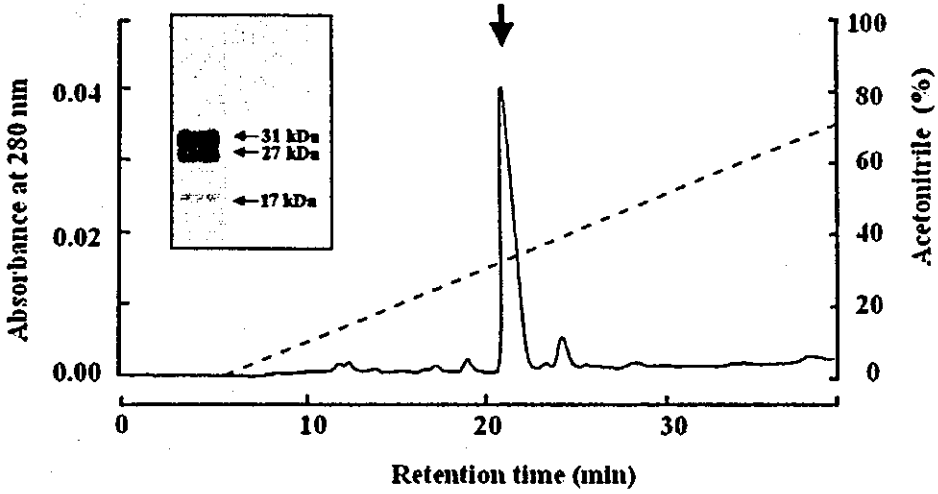

FIGURE 4. Reverse-phase chromatographic profile of CHO-cell-derived recombinant REF-1 in a final purification step. SDS-PAGE was used to amplify recombinant REF-1. Approximately 5 μg of the purified protein was loaded and made visible with Coomassie blue R-250 staining after electrophoresis under reducing (+2ME) conditions. Major components at 31 and 27 kDa and a minor component at 17 kDa were observed.

Purification of CHO Cell–Derived Recombinant REF-1

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

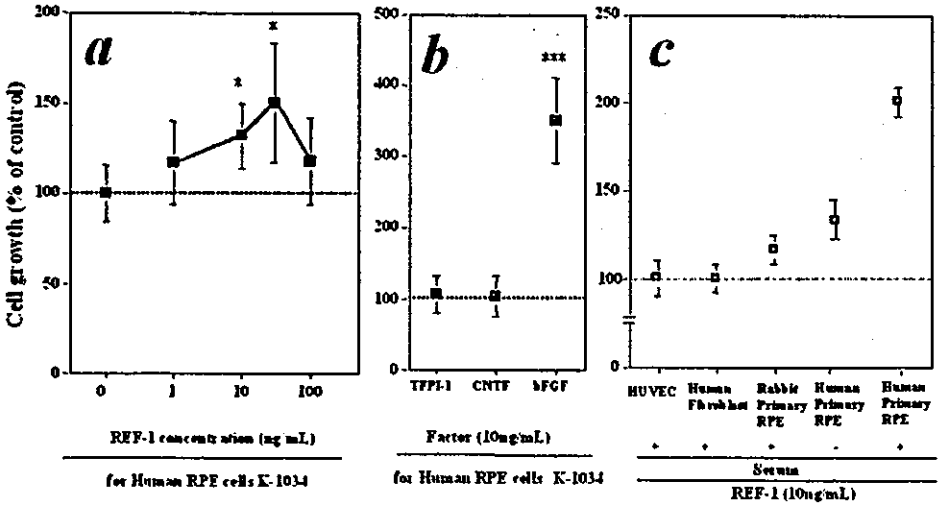

FIGURE 5. Cell proliferation activity of recombinant REF-1. Dose-dependent REF-1 activity in human K-1034 RPE cells (a), growth-promoting activities of TFPI-1, CNTF, and bFGF in human K-1034 RPE cells (b), and proliferative response of HUVECs, human fibroblast, rabbit primary RPE cells, and human primary RPE cells to 10 ng/mL REF-1 (c).

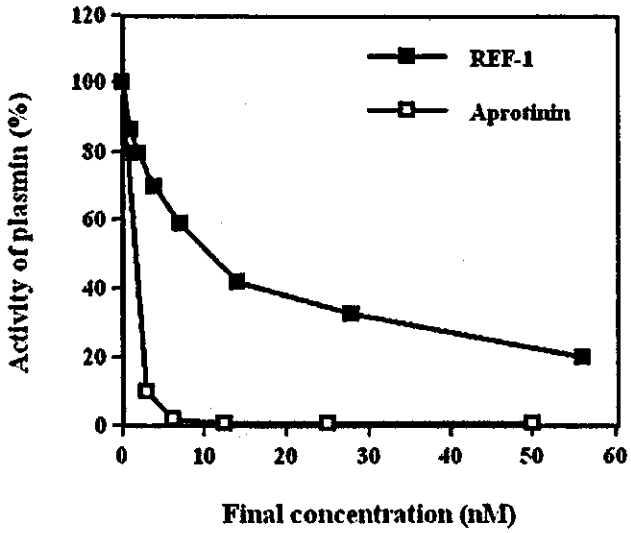

FIGURE 6. Protease inhibitory activity of REF-1. The residual activities of plasmin with aprotinin (positive control, 4 μg/mL) or REF-1/TFPI-2 (5 μg/mL) were determined, in 96-well plastic plates, with S-2251 (Val-Leu-Lys-pNA, 1 mg/mL) used as a substrate. The percentage of relative activity in the inhibitor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

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

Determination of REF-1 in RPE Cells by Western Blotting Analysis and RT-PCR

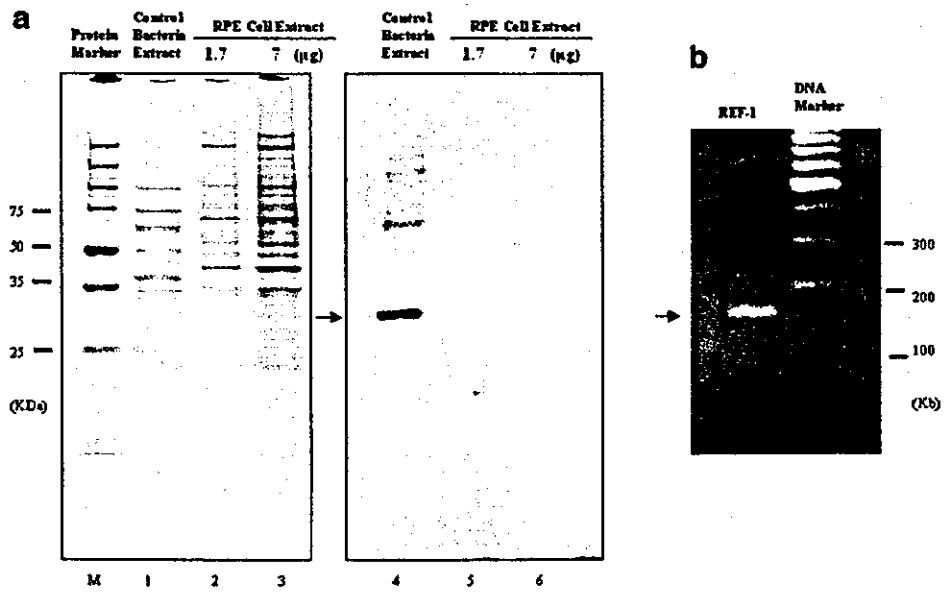

FIGURE 7. Determination of REF-1 in human primary RPE cells. REF-1 in human primary RPE cells was determined by Western blot analysis or RT-PCR. (a) Western blot of REF-1 for human primary RPE cell extract. *Lane 1*: Coomassie staining of bacteria extract expressing recombinant REF-1 (34 kDa); *lane 2*: Coomassie staining of human RPE cell extract (1.7 μg); *lane 3*: Coomassie staining of RPE cell extract (7 μg); *lane 4*: Western blot of bacteria extract expressing recombinant REF-1 (34 kDa); *lane 5*: Western blot of RPE cell extract (1.7 μg); *lane 6*: Western blot of RPE cell extract (7 μg). (b) RT-PCR of REF-1 transcript in total RNA extracted from human primary RPE cells. A single band was observed after 30 cycles of amplification.

TABLE 2. Production of Cytokines by Human Primary RPE Cells Treated with REF-1 (10 ng/mL)

Cytokine	REF-1 (pg/10^5 cells)		
	−	+	+/−
bFGF	508	546	1.0
TGF-β1	673	3163	4.7
TGF-β2	Nondetectable	Nondetectable	—
IL-1β	Nondetectable	Nondetectable	—
IL-6	1770	1922	1.1
IL-8	556	837	1.5
G-CSF	Nondetectable	Nondetectable	—
GM-CSF	817	1959	2.4
M-CSF	420	509	1.2
TNF-α	Nondetectable	Nondetectable	—
EGF	Nondetectable	Nondetectable	—

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-α,[24] IL-1α,[25] or IL-1β[26] and downregulated by IFN-γ.[26] 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[8] and serine protease inhibitor.[9] 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 epithelium[20] 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.

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Analysis of Porcine Optineurin and Myocilin Expression in Trabecular Meshwork Cells and Astrocytes from Optic Nerve Head

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PURPOSE. To determine the cDNA sequences and analyze the expression of porcine optineurin and myocilin in trabecular meshwork cells (TMCs) and astrocytes from the optic nerve head under normal and experimental conditions.

METHODS. Both porcine optineurin and myocilin were cloned to determine the cDNA sequences. Porcine TMCs and astrocytes were isolated and treated with dexamethasone (500 nM) for 2 weeks, incubated under hypoxic conditions (7% O_2) for 72 hours, or exposed to 33 mm Hg hydrostatic pressure for 72 hours. A 10% mechanical stretch for 24 hours was also performed on TMCs. The expression level of the optineurin and myocilin transcripts was analyzed by real-time quantitative PCR.

RESULTS. The sequences of porcine optineurin and myocilin cDNA were determined, and the expression of both genes was confirmed in both TMCs and astrocytes. Amino acid sequences of porcine optineurin and myocilin were homologous to those of humans by 84% and 82%, respectively, and shared protein motifs and modification sites. The expression of myocilin mRNA by TMCs and astrocytes was increased by 8.0- and 5.5-fold, respectively, after exposure to dexamethasone. In contrast, the expression of optineurin was suppressed to 68% in TMCs and 48% in astrocytes after exposure to dexamethasone. A significant reduction of myocilin expression was observed after 72 hours of incubation under hypoxic conditions in both types of cells, whereas optineurin was not affected. Hydrostatic pressure for 72 hours and mechanical stretching for 24 hours had minimal affects on gene expression of both optineurin and myocilin.

CONCLUSIONS. The high homology of porcine optineurin and myocilin to the comparable human genes indicates that pigs can be used to study changes in gene expression in hypertensive eyes. The alterations in expression of myocilin but not of optineurin under stress suggest that different mechanisms in the phenotype of glaucoma associated with the two genes are involved in development of glaucoma. (*Invest Ophthalmol Vis Sci.* 2004;45:2652-2659) DOI:10.1167/iovs.03-0572

Characteristic degeneration and excavation of the optic nerve head are found in glaucomatous eyes. These changes are considered to be due to ocular hypertension with the intraocular pressure (IOP) continuously more than 21 mm Hg. In contrast, there are patients with normal ocular tension who show glaucomatous changes in the optic nerve head. These patients, in whom there is no evidence of an elevation of IOP at any time, are said to have normal-tension glaucoma (NTG).

Currently three genes—myocilin (*MYOC*),[1,2] cytochrome P4501B1 (*CYP1B1*),[3,4] and optineurin (*OPTN*)[5]—are associated with glaucoma. Optineurin is the most recent gene to be identified and is responsible for 16.7% of families with hereditary NTG.[5] It has been identified and studied by different groups under various names: NRP, NF-κB essential modulator (NEMO)-related protein[6]; FIP-2, adenovirus E3-14-kDa interacting protein 2[7]; Huntingtin interacting protein L (HYPL)[8]; and transcription factor IIIA interacting protein (TFIIIA-INTP).[9] Optineurin is homologous to NEMO, a structural and regulatory subunit of the high molecular weight kinase complex (IKK) that is responsible for the phosphorylation of NF-κB inhibitors.[6]

Some of the functions of optineurin are known. They include inhibition of the tumor necrosis factor (TNF)-α pathway,[7] interaction with transcription factor IIIA,[9] and mediation of the interaction of Huntingtin and Rab8 for regulation of membrane trafficking and cellular morphogenesis.[8] Optineurin is induced by TNF-α and binds to an inhibitor of TNF-α and the E3-14.7-kDa protein.[7]

The optineurin protein contains two leucine zippers (LZs); an N-terminal LZ responsible for the association with Rab8, and a C-terminal LZ required for Huntingtin. The gene is mapped to 10p14 and contains 16 exons encoding a 66-kDa protein. It contains two putative bZIP transcription factor motifs, a C2H2 type zinc finger, and two LZ domains.

Recently, Vittitow and Borrás[10] reported that elevated IOP, and exposure to TNF-α and dexamethasone (DEX) led to an upregulation of optineurin expression in an organ culture system. However, it is still unclear how mutations of the optineurin gene lead to glaucoma.

Another gene associated with glaucoma is myocilin, which is found in 36% of juvenile-onset POAG and 4% of adult-onset POAG.[11-15] Myocilin is a 57-kDa protein that contains motifs homologous to the olfactomedin domain where nearly all mutations in patients with POAG have been identified.[1,11-15]

Pigs and miniature pigs are readily available and have been used for a wide variety of medical studies, including tissue transplantation.[16,17] Because their eyes are similar in size and

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anatomy to human eyes,[18] pigs have often been used to study the aqueous outflow system and the regulation of IOP.

The purpose of this study was to clone both the porcine optineurin and myocilin genes to determine their cDNA sequences, and then to use the sequences to determine the transcriptional response of isolated porcine trabecular meshwork cells (TMCs) and astrocytes from the optic nerve head after exposure to dexamethasone (DEX), increased hydrostatic pressure, hypoxia, and mechanical stretching.

Materials and Methods

Cell Cultures

Pig eyes were obtained within 3 hours of death from a local abattoir. The eyes were disinfected in 0.2% povidone iodine for 10 minutes followed by soaking in 70% alcohol for 30 seconds. The eyes were washed several times in phosphate-buffered saline (PBS) and cut into halves along the equator.

After the lens and iris were removed from the anterior half, the trabecular tissue was trimmed from the cornea at the Schwalbe's line and then from the sclera, as described.[19,20] The optic nerve head was separated from the sclera and surrounding tissues. The prelaminar region was dissected from the optic nerve head and cut into three or four pieces.[21,22] The trabecular and prelaminar tissues were placed separately in 35-mm plastic Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (Invitrogen-Gibco).

The tissues were incubated for 1 to 2 weeks at 37°C in humidified 5% CO_2 and 95% air until cells migrated from the tissue onto the surface of the culture dish. Cells were isolated, and fourth-passage cells were obtained for experimental use. The cells that migrated from the optic nerve head were confirmed to be astrocytes by immunostaining with anti-glial fibrillary acidic protein (GFAP), a protein marker for astrocytes (Sigma-Aldrich).

Cloning of Porcine Optineurin cDNA

mRNA was isolated from cultured TMCs using mRNA isolation kits (MicroPoly(A)Pure; Ambion, St. Austin, TX). Primers (sense primer, 5′-ATGTCCCATCAACCTCTGAGCT-3′, antisense primer 5′-TGTCCTCGGCTCGTCTTTGAAA-3′) were designed to include the conserved sequences for human, mouse (Discovery System; Celera, Gaithersburg, MD), and rat to amplify the open reading frame of porcine optineurin mRNA using a commercial system (Superscript One-Step RT-PCR System; Invitrogen-Gibco), according to the manufacturer's protocol.

The PCR products were cloned into a TA cloning vector (pDrive; Qiagen, Valencia, CA) using a PCR Cloning Kit (Qiagen), and the inserts were sequenced using a fluorescent dideoxynucleotide automated sequencer (CEQ2000XL DNA Analysis System; Beckman-Coulter, Fullerton, CA). The missing 3′ and 5′ ends of the cDNAs were amplified using the 3′ and 5′ rapid amplification of cDNA ends (RACE) method (Marathon cDNA Amplification Kit; BD Biosciences-Clontech, Palo Alto, CA). The full-length cDNA sequence of porcine optineurin can be obtained from GenBank under accession number AF513722 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Cloning of Porcine Myocilin cDNA

The same mRNA used for optineurin cDNA cloning was used for myocilin cDNA amplification. The sense primer, 5′-ATGCCAGCTS(G/C)TCCAGCTGCT-3′, and antisense primer, 5′-GACCATGTTGAAGTTGTCCCA-3′, were designed to include the conserved sequence of human, mouse, rat, and bovine myocilin and to amplify the open reading frame of porcine myocilin mRNA, using the RT-PCR system (Superscript One-Step RT-PCR System; Invitrogen-Gibco). The PCR products were cloned into a TA cloning vector (TA Cloning Kit; Invitrogen, San Diego, CA), and the inserts were sequenced. The missing 3′ and 5′ ends of the cDNA were amplified using the RACE method (Marathon cDNA Amplification Kit; BD Biosciences-Clontech). The full-length cDNA sequence of porcine myocilin can be obtained from GenBank under accession number AF350447.

Sequence Analysis of Porcine Optineurin and Myocilin

Amino acid sequences of both optineurin and myocilin were analyzed for domain structure and potential protein modification sites. The PROSITE scanning tool[23] (http://www.nhri.org.tw/prosite/; provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland) was used to scan the optineurin protein sequence for the occurrence of patterns and profiles stored in the PROSITE database. Potential glycosylation and phosphorylation sites were predicted by the program developed by Hansen et al.,[24] and Blom et al.,[25] respectively. Sequence homology was determined by a sequence-analysis program (Omiga 2.0; Accelrys, San Diego, CA).

Stress Experiments for Optineurin and Myocilin

All stress experiments were performed using fourth-passage TMCs and astrocytes from three different porcine eyes. For the DEX treatment, DEX stock solution (50 mM DEX/dimethyl sulfoxide) was added to cultured TMCs and astrocytes at a final concentration of 500 nM. The culture medium was replaced every 3 days and maintained for 2 weeks. For control, cultured cells were treated with dimethyl sulfoxide alone.

To examine the effect of hypoxia, both types of cultured cells were incubated in 7.0% O_2 and 5% CO_2, in a multiple gas incubator (model 9200; Wakenyaku, Kyoto, Japan) for 12, 24, 48, or 72 hours. Control cells were incubated for the same times in 5% CO_2 and 95% air in a standard CO_2 incubator.

To examine the effects of hydrostatic pressure, we exposed both types of cultured cells to a hydrostatic pressure of 33 mm Hg above atmospheric pressure for 12, 24, 48, or 72 hours in a CO_2 incubator, using the system illustrated in Figure 1. The culture flasks were filled with the medium and capped with a silicon stopper to prevent leakage. The height of the reservoir containing the medium was adjusted to control the pressure in the flask. For gas exchange, the medium was circulated with a peristaltic pump (Eyela, Tokyo, Japan), and the pressure was monitored with a pressure gauge (model PG-208; Copal Electronics, Tokyo, Japan). Control cells were exposed to hydrostatic pressure of 3 mm Hg above atmospheric pressure for 12, 24, 48, and 72 hours.

To examine the effects of mechanical stretching, cultured porcine TMCs were transferred onto a 10-cm^2 collagen-coated silicon chamber (S.Tec, Osaka, Japan). The silicon chamber had a 100-μm-thick transparent bottom, and the side walls were 1.5-mm thick to prevent narrowing at the bottom center. The silicon chamber was then attached to a stretching apparatus for a 10% linear stretch for 24 hours in a standard CO_2 incubator. Control cells were plated onto a collagen-coated silicon chamber without the stretching for the same amount of time.

Optineurin and Myocilin Transcript Analysis

Total RNA was isolated from cultured cells exposed to stimuli or stresses (RNAzol B; Tel-Test, Friendswood, TX). The total RNA was reverse transcribed (Superscript First Strand Synthesis System for RT-PCR; Invitrogen-Gibco) according to the manufacturer's protocol. Real-time quantitative PCR was performed to determine the optineurin, myocilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript with a sequence-detection system (GeneAmp 5700; Applied Biosystems, Inc. [ABI], Foster City, CA). PCR reactions were performed in 50 μL of reaction mixture containing 25 μL master PCR mix (SYBR Green PCR Master Mix; ABI), 5 pM primer pairs, and 1 μL cDNA samples. To measure myocilin transcript, 4 μL cDNA samples was used because of lower expression. The 18S ribosomal RNA gene was used as