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LABORATORY STUDY

Retinal degeneration is delayed by tissue factor pathway inhibitor-2 in RCS rats and a sodium-iodate-induced model in rabbits

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Abstract

Purpose **To investigate the *in vivo* effects of tissue factor pathway inhibitor 2 (TFPI-2), which stimulates proliferation of retinal pigment epithelial cells, but not the proliferation of fibroblast and vascular endothelial cells *in vitro*, on retinal degeneration using a sodium-iodate (SI)-induced model in rabbits and Royal Collage of Surgeons (RCS) rats.**

Methods **79 μg of recombinant TFPI-2 (rTFPI-2) or vehicle alone was injected intravitreously to 18 eyes of 12 pigmented rabbits a day after 20 mg/kg of SI was intravenously administered. Retinal function was assessed 4, 7, 14, and 21 days after the injection by analysing amplitudes of the c-wave of a bright flash electroretinogram. Additionally, 10 μg of rTFPI-2 or vehicle alone was injected intravitreously to 11 eyes of RCS rats at both 3 and 4 weeks old, then the retina was examined histologically at 5 weeks old.**

Results **The rTFPI-2-treated eyes in rabbits showed a significantly less decrease in the relative amplitude of the c-wave than control eyes on days 4 and 7. The thickness of the outer nuclear layer was significantly thicker and the vacuole in the photoreceptor layer was less frequently observed in the rTFPI-2-treated RCS rats than the controls.**

Conclusions **Intravitreal injection of TFPI-2 rescues SI-induced retinal degeneration in rabbits and naturally occurring retinal degeneration in RCS rats at least partly. These results may suggest that this compound can be utilized in the treatment of retinal degeneration.**

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Keywords: tissue factor pathway inhibitor-2 (TFPI-2); retinal pigment epithelium; retinal degeneration; sodium iodate; rabbit; Royal Collage of Surgeons (RCS) rat

Introduction

Retinal degeneration is a pathological process characterized by a progressive loss of visual function. Using linkage analyses, as well as candidate gene approach, previous investigators have identified more than 100 causative genes or their loci (RetNet: http://www. sph. uth. tmc. edu/Retnet/). Regardless of the diversity of the causative genes, the pathway that ultimately leads to the cell death in the retina is shared. Previous studies have demonstrated that the retinal degeneration in an animal model is partly rescued by several growth factors, including basic fibroblast growth factor (bFGF), presumably by inhibiting the apoptotic process.[1,2] However, these factors stimulate proliferation of fibroblast[3] and vascular endothelial cells,[4] and are involved in detrimental processes such as proliferative vitreoretinopathy[5–8] and preretinal or subretinal neovascularization,[9–12] suggesting that other compounds without the adverse effect of fibroblast or endothelial growth are necessary to achieve the establishment of pharmaceutical therapy for the retinal degeneration.

Tissue factor pathway inhibitor 2 (TFPI-2) is a multifunctional glycosylated protein also known as placental protein 5 or matrix serine protease inhibitor. TFPI-2 is thought to be involved in the coagulation cascade,[13] as well as the proliferation of smooth muscle cells[14] and inhibition of matrix metalloproteinase,[15] although the mechanism of action is still unclear. It was recently reported that TFPI-2 has unique functional properties to stimulate the growth of RPE cells without the growth stimulatory functions on fibroblasts and vascular endothelial cells.[16] In some groups of retinal degeneration, the RPE is affected primarily, and the degeneration in the neural retina is the secondary process.[17,18] So it is possible that TFPI-2 can ameliorate the progressive loss of visual function due to the loss or dysfunction of the RPE. In this study, we investigated the effects of TFPI-2 on the RPE function after the retinal degeneration was induced by sodium iodate(SI) in Dutch rabbits, using the electrophysiological analysis. In addition, we investigated the effects of TFPI-2 on the retinal degeneration in Royal College of Surgeons (RCS) rats carrying the mutation mertk gene[19] and losing the proper phagocytotic function of RPE.[20]

Materials and methods

Plasmid construction

Full-length human TFPI-2 was amplified by polymerase chain reaction from a placental cDNA library and ligated between *Eco*RI and *Kpn*I sites of mammalian expression vector pSRα (pSRαRPE27). The sequences of the primers used are available on request from the authors. The sequence of PCR product was confirmed by sequencing analysis.

Transfection and purification of recombinant TFPI-2

Recombinant TFPI-2 was generated as previously described in detail.[16] Briefly, CHO cells were transfected with pSRαRPE27 together with pAd DHFR plasmid vector by lipofection using lipofectin (GIBCO BRL, Rockville, MD, USA). The cells were grown and selected in αMEM supplemented with 10% foetal calf serum (FCS) in the absence of folic acid and followed by methotrexate selection. The selected cells were grown in αMEM supplemented with 10% FCS in a 150 cm^2 flask. At confluency, the medium was replaced with serum-free medium and was changed every other day nine times. The supernatant was applied to S-Sepharose FF column. Following sample application, the column was washed with 20 mM sodium citrate and 0.2 M NaCl, and was eluted with 20 mM sodium citrate containing 0.4 M NaCl. The eluent was added with trifluoroacetic acid (TFA) at a final concentration of 0.1%, and injected to a Resouce RPC column using high-performance liquid chromatography. The column was eluted with a gradient formed from 0.1% TFA in 0–70% acetonitrile at a flow rate of 1%/1 ml/1 min. The recombinant TFPI-2 (rTFPI-2) fraction was diluted with 40 mM phosphate buffer (pH 7.2) and applied to SP-Sepharose FF column. The column was eluted with 20 mM phosphate buffer containing 0.45 M NaCl. The resultant purity of recombinant TFPI-2 was 97% by sodium dodecylsulphate-polyacrylamide gel electrophoresis analysis.

Animals

In all, 12 pigmented Dutch male rabbits were obtained from Kitayama Labes (Nagano, Japan). Before the experiment, the rabbits were kept in 12 h light: 12 h dark cyclic lighting in our animal rooms for longer than 1 week. Six RCS rats were obtained from CLEA Japan (Shizuoka, Japan). All procedures conformed to the ARVO Resolution on the Use of Animals in Research.

Sodium iodate-induced model for retinal degeneration in rabbits

The retinal degeneration model in rabbits was created by the intravenous injection of SI as described elsewhere.[21] Briefly, the rabbits received intravenous injection of 20 mg/kg SI (Kanto Kagaku, Tokyo, Japan) diluted in saline at a concentration of 20 mg/ml. One day after the SI injection, the rabbits were anaesthetized with a combination of 30 mg/kg of ketamine hydrochloride (Ketalar®; 50 mg/ml, Sankyo, Tokyo, Japan) and 5 mg/kg xylazine hydrochloride (Celactal®; 20 mg/ml, Bayer, Tokyo, Japan); the left eyes received intravitreal injection of 79 μg of rTFPI-2 in 0.1 ml of phosphate-buffered saline (PBS) and the right eyes received PBS alone as controls. Nine rabbits were used for electroretinogram (ERG) study, and three rabbits for histological examination.

ERG was performed in a standard fashion just before and 4, 7, 14, and 21 days after the SI was injected. Nine rabbits were dark-adapted for 1.5 h and anaesthetized with ketamine and xylazine as described above. The pupils were maximally dilated with drops of 10% phenylephrine and 1% tropicamide (Mydrin P; Santen Pharmaceutical, Osaka, Japan). Then bright flash ERG was recorded using an Ag–AgCl contact-lens electrode (WLS-20; Tomey, Nagoya, Japan) placed on the cornea, which was lubricated with one drop of 1.5% hydroxyethylcellulose (Scopisol®15, Senju Pharmaceutical, Tokyo, Japan). A reference electrode was placed on the ear lobe, and a ground electrode was placed on the thigh. The stimulus light (1000 lux, 5.5 s) was emitted by light emission diode (LED) in the contact-

lens electrode (WLS-20), and the response was amplified using a direct-current amplifier (AD-641G, Nihon Kohden, Tokyo, Japan) with a bandpass to 10 kHz, and displayed on an oscilloscope (VC-11, Nihon Kohden, Tokyo, Japan). The amplitude of the c-wave was measured from the prestimulus baseline to the positive peak of the c-wave.

In addition, for the purpose of histological examination, three rabbits were killed 4 days after the SI was injected. The eyes were immediately enucleated, and fixed in 4% glutalaldehyde for 1 h, followed by 10% formalin. They were then transferred into 70% ethanol and processed for paraffin embedding. Once embedded, 4.0 μm sections of tissue were prepared for staining with haematoxylin and eosin (H&E).

Effect of TFPI-2 on the retinal degeneration in RCS rats

In all, 11 eyes of six RCS rats were used. General anaesthesia was induced with an intraperitoneal injection (1000 μl/kg) of a mixture (5:1 in volume) of ketamine hydrochloride (Ketalar®) and xylazine hydrochloride (Celactal®), and then 10 μg of rTFPI-2 in 10 μl of PBS ($n=5$) or PBS alone as controls ($n=6$) was injected into the vitreous cavity similar to the previous studies.[1] All eyes had two of injections of either the drug or the vehicle alone at 3 and 4 weeks old. At 5 weeks old, the rats were killed by an overdose of diethyl ether, and the eyes were immediately enucleated, and subjected for histological analysis.

After the eyes were enucleated, they were fixed in 4% glutalaldehyde for 1 h, followed by 10% formalin. They were then transferred into 70% ethanol and processed for paraffin embedding. Once embedded, 4.0 μm sections of tissue were prepared for staining with H&E. In light-microscopic examination, the vertical thickness of the outer nuclear layer (ONL) in the sensory retina was measured. Three measurements were performed in each specimen, and the mean value was used for analysis.

Statistical analysis

For the comparison of the c-wave amplitude between the rTFPI-2-treated eyes and the control, a paired *t*-test was used. For the comparison of the ONL thickness in RCS rat between the rTFPI-2-treated eyes and the control, Mann–Whitney U test was used. A *P*-value less than 0.05 was considered significant.

Results

Effect of rTFPI-2 on RPE damage by SI

In order to investigate the effects of TFPI-2 on the retinal degeneration, we first used SI-induced retinal degeneration in Dutch rabbits, and the effect of TFPI-2 on the RPE was investigated by the analysis of c-wave in ERG (Figure 1a). After 4, 7, 14, and 21 days, the mean amplitude of the c-wave from the control eyes was reduced to 39, 47, 69, and 75%, respectively, of the value before the SI- injection, essentially similar to the previous studies.[21–23] In contrast, the mean amplitude of the c-wave from the rTFPI-2-treated eyes was 84, 83, 88, and 95% after 4, 7, 14, and 21 days, respectively. There was a significant difference in the relative amplitude of the c-wave between the control and the rTFPI-2-treated eyes after 4, 7, and 14 days, suggesting accelerated recovery of the RPE function or protection of RPE from apoptosis.

Effect of rTFPI-2 on the retinal degeneration in RCS rats

Next, to investigate the effect of rTFPI-2 on the retinal investigation in RCS rats, we injected TFPI-2 into the vitreous cavity similar to the previous studies.[1] At the age of 5 weeks, the thickness of the ONL was significantly thicker in the rTFPI-2-treated rats compared with the control (Figure 2a). We also found that the vacuole in the photoreceptor layer was less frequently

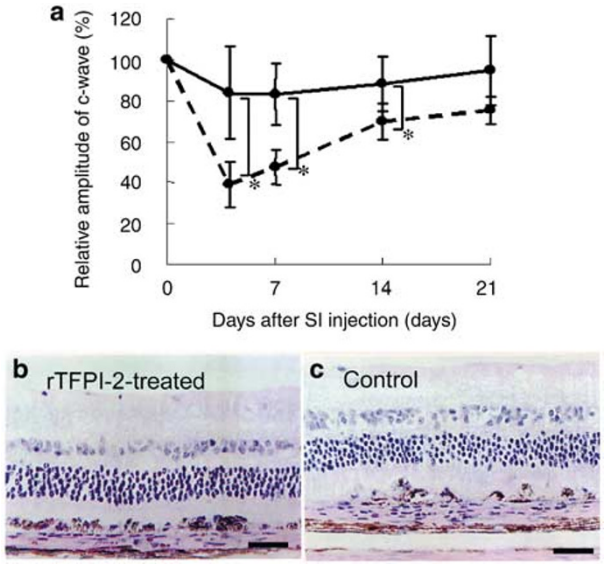

Figure 1 Effect of intravitreal rTFPI-2 injection on the RPE function of SI-induced rabbits. (a) The amplitudes of the c-waves in the rTFPI-2-treated eyes (solid line) and the control eyes (dashed line) 4, 7, 14, and 21 days after SI injection. All values are expressed as that before the injection of SI is 100%. Each data represents a mean value, and error bars are standard errors of the mean. The rTFPI-2-treated eyes showed a significantly less decrease in the c-wave amplitude after 4, 7, and 14 days than the control eyes (paired *t*-test;*: $P=0.007$, 0.001, and 0.017 after 4, 7, and 14 days, respectively). Representative sections from rTFPI-2-treated (b) and control (c) eyes 4 days after the SI injection. Note that there seems more RPE cells in the rTFPI-2-treated eye than the control eye. Scale bars, 25 μm.

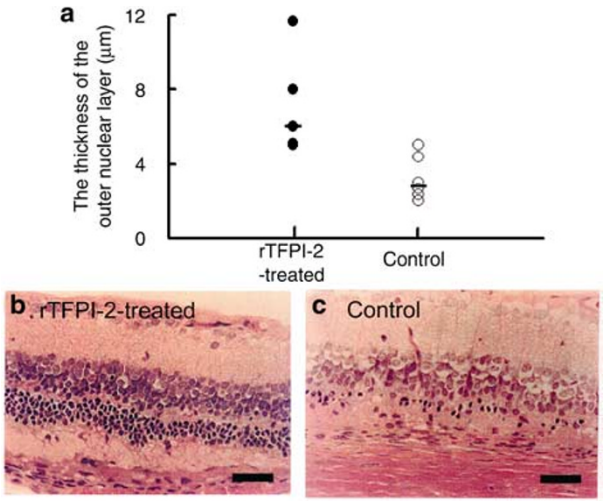

Figure 2 Effects of rTFPI-2 on the retinal degeneration in RCS rats. (a) Distribution of the ONL thickness in the rTFPI-2-treated eyes (filled circle) and the control eyes (empty circle) of RCS rats at 5weeks old. Bars are median values. Quantitative analysis revealed that the ONL thickness in the rTFPI-2-treated eyes was significantly more than the control eyes (Mann–Whitney U test; $P = 0.011$). Representative sections from rTFPI-2-treated (b) and control (c) eyes. Note that the vacuole in the photoreceptor layer was less frequently observed in the rTFPI-2-treated rats. Scale bars, 10 μm.

observed in the TFPI-2-treated rats (compare Figure 2b and c).

Discussion

It was previously demonstrated that the c-wave in the ERG originates from the change in potassium concentration at the apical membrane of the RPE.[21,24,25] SI degenerates the RPE and breaks down the integrity of the apical RPE cell membrane, leading to diminish or decrease the amplitude of the c-wave[21,23] immediately after SI is injected. Subsequently, the RPE regenerates and the integrity of the RPE is recovered 1–2 weeks after the injection of SI.[26–28] Similar to previous studies,[21] we analysed the function of RPE by the amplitudes of the c-wave in the ERG and confirmed that the functional loss of RPE was highly reproducible. Our results demonstrated that the RPE function is relatively spared in SI-induced damage in rabbits after the intravitreal administration of rTFPI-2. This effect could be attributed either to the trophic or the proliferative effect of TFPI-2 on RPE cells. There seemed more RPE cells in the rTFPI-2-treated eyes 4 days after the SI injection (compare Figure 1b and c), supporting the fact that TFPI-2 stimulated RPE growth. However, the patchy degeneration of the RPE and the migration of RPE cells into the neural retina precluded the quantitative analysis of RPE proliferation. Further studies are necessary to elucidate the mechanism underlying our observation in Dutch rabbits. Our results also demonstrated that photoreceptor cell loss is rescued by the administration of rTFPI-2 in RCS rats. Different from the SI-induced degeneration in rabbits, the retinal degeneration in RCS rat results from impairment of phagocytosis of photoreceptor outer segment, and not from the decrease in the number of the RPE cells.[17] Thus, even if TFPI-2 stimulated the growth of the RPE cells, it is unlikely that the resultant increase in the number of the functionally abnormal RPE cells could contribute to the decreased degeneration of the neural retina. Thus, our results imply that TFPI-2 ameliorates the retinal degeneration, not by the growth stimulatory effect but by the trophic effect, at least in RCS rats. Since previous investigators have demonstrated that several growth factors can function to prevent retinal cell apoptosis,[29] it is possible that TFPI-2 possesses anti apoptotic properties. Growth factors such as basic FGF can partially rescue retinal degeneration; however, the growth stimulatory effects on other cell types are problematic.[7,8,10–12,30] Although further studies are necessary, in the light of our observations, we believe TFPI-2 can be utilized without such adverse effects in the treatment of retinal degeneration.

References

1 Faktorovich EG, Steingerg RH, Yasumura D, Matthes MT, LaVail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature* 1990; **347**: 83–86.
2 Unoki K, LaVail MM. Protection of the rat retina from ischemic injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. *Invest Ophthalmol Vis Sci* 1994; **35**: 907–915.
3 Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino Jr MA, Shepard HM. Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. *Science* 1985; **230**: 943–945.
4 Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA *et al*. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* 1986; **233**: 545–548.
5 Sivalingam A, Kenney J, Brown GC, Benson WE, Donoso L. Basic fibroblast growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. *Arch Ophthalmol* 1990; **108**: 869–872.
6 Cassidy L, Barry P, Shaw C, Duffy J, Kennedy S. Platelet derived growth factor and fibroblast growth factor basic levels in the vitreous of patients with vitreoretinal disorders. *Br J Ophthalmol* 1998; **82**: 181–185.
7 Hueber A, Wiedemann P, Esser P, Heimann K. Basic fibroblast growth factor mRNA, bFGF peptide and FGF receptor in epiretinal membranes of intraocular proliferative disorders (PVR and PDR). *Int Ophthalmol* 1996-97; **20**: 345–350.
8 Limb GA, Little BC, Meager A, Ogilvie JA, Wolstencroft RA, Franks WA, Chignell AH, Dumonde DC. Cytokines in proliferative vetreoretinopathy. *Eye* 1991; **5**: 686–693.

9 D'Amore PA. Mechanisms of retinal and choroidal neovascularization. *Invest Ophthalmol Vis Sci* 1994; **35**: 3974–3979.
10 Frank RN, Amin RH, Eliott D, Puklin JE, Abrams GW. Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. *Am J Ophthalmol* 1996; **122**: 393–403.
11 Ogata N, Matsushima M, Takeda Y, Tobe T, Takahashi K, Yi X *et al*. Expression of basic fibroblast growth factor mRNA in developing choroidal neovascularization. *Curr Eye Res* 1996; **15**: 1008–1018.
12 Kimura H, Sakamoto T, Hinton DR, Spee C, Ogura Y, Tabata Y *et al*. A new model of subretinal neovascularization in the rabbit. *Invest Ophthalmol Vis Sci* 1995; **36**: 2110–2119.
13 Sprecher CA, Kisiel W, Mathewes S, Foster DC. Molecular cloning, expression, and partial characterization of a second human tissue-factor-pathway inhibitor. *Proc Natl Acad Sci USA* 1994; **91**: 3353–3357.
14 Shinoda E, Yui Y, Hattori R, Tanaka M, Inoue R, Aoyama T *et al.* Tissue factor pathway inhibitor-2 is a novel mitogen for vascular smooth muscle cells. *J Biol Chem* 1999; **274**: 5379–5384.
15 Herman MP, Sukhova GK, Kiesiel W, Foster D, Kehry MR, Libby P *et al.* Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implication for atherosclerosis. *J Clin Invest* 2000; **107**: 1117–1126.
16 Tanaka Y, Utsumi J, Sudo T, Matsui M, Sudo T, Nakamura N *et al.* Purification, molecular cloning and expression of a novel growth-promoting factor for retinal pigment epithelial cells, REF-1/TFPI-2. *Invest Ophthalmol Vis Sci* 2004; **45**: 245–252.
17 Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG *et al*. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. *Nat Genet* 2000; **26**: 270–271.
18 Gu SM, Thompson DA, Srikumari CR, Lorenz B, Finckh U, Nicoletti A *et al.* Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. *Nat Genet* 1997; **17**: 194–197.
19 D'Cruz PM, Yasumura D, Weir J, Matthes MT, Abderrahim H, LaVail MM *et al*. Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. *Hum Mol Genet* 2000; **9**: 645–651.
20 Bok D, Hall MO. The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. *J Cell Biol* 1971; **49**: 664–682.
21 Noell WK. Experimentally induced toxic effects on structure and function of visual cells and pigment epithelium. *Am J Ophthalmol* 1953; **36**: 103–116.
22 Lurie M, Marmor MF. Similarities between the c-wave and slow PIII in the rabbit eye. *Invest Ophthalmol Vis Sci* 1980; **19**: 1113–1117.
23 Noell WK. The origin of the electroretinogram. *Am J Ophthalmol* 1954; **38**: 78–93.
24 Steinberg RH, Schmidt R, Brown KT. Intracellular responses to light from cat pigment epithelium: origin of the electroretinogram c wave. *Nature* 1970; **227**: 728–730.
25 Oakley B, Green DG. Correlation of light-induced changes in retinal extracellular potassium concentration with c-wave of the electroretinogram. *J Neurophysiol* 1976; **39**: 1117–1133.
26 Ringvold A, Olsen E, Flage T. Transient breakdown of the retinal pigment epithelium diffusion barrier after sodium iodate: a fluorescein angiographic and morphologic study in the rabbit. *Exp Eye Res* 1981; **33**: 361–369.
27 Korte GE, Reppucci V, Henkind P. RPE destruction causes choriocapillary atrophy. *Invest Ophthalmol Vis Sci* 1984; **25**: 1135–1145.
28 Korte GE, Wanderman MC. Distribution of Na^+ K^+-ATPase in regenerationg retinal pigment epithelium in the rabbit. A study by electron microscopic cytochemistry. *Exp Eye Res* 1993; **56**: 219–229.
29 LaVail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, Steinberg RH. Multiple growth factors, cytokines, and neurotorophins rescue photoreceptors from the damaging effects of constant light. *Proc Natl Acad Sci USA* 1992; **89**: 11249–11253.
30 Karsan A, Yee E, Poirier GG, Zhou P, Craig R, Harlan JM. Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. *Am J Pathol* 1997; **151**: 1775–1784.