J. Cell. Mol. Med. Vol 14, No 7, 2010 pp. 1998-2005

Cell-penetrating peptide TAT-mediated delivery of acidic FGF to retina and protection against ischemia–reperfusion injury in rats

Yi Wang [a, c, #], Haihuan Lin [a, #], Shaoqiang Lin [a], Jia Qu [b], Jian Xiao [a], Yadong Huang [d], Yechen Xiao [e], Xiaobing Fu [f], Yongguang Yang [c], Xiaokun Li [a, *]

[a] Key Laboratory of Biotechnology Pharmaceutical Engineering of Zhejiang Province, School of Pharmaceutical Science, Wenzhou Medical College, Wenzhou, PR China
[b] The Eye Hospital of Wenzhou Medical College, Wenzhou Medical College, Wenzhou, PR China
[c] Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
[d] National Engineering Research Center for Gene Medicine, Jinan University, Guangzhou, PR China
[e] Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, Changchun, PR China
[f] Wound Healing and Cell Biology Laboratory Institute of Basic Medical Science, PLA General Hospital, Postgraduate Medical College, Beijing, PR China

Received: November 21, 2008; Accepted: April 30, 2009

Abstract

The development of non-invasive ocular drug delivery systems is of practical importance in the treatment of retinal disease. In this study, we evaluated the efficacy of transactivator of transcription protein transduction domain (TAT-PTD, $TAT_{49–57}$) as a vehicle to deliver acidic FGF (aFGF) to retina in rats. TAT-conjugated aFGF-His (TAT-aFGF-His) exhibited efficient penetration into the retina following topical administration to the ocular surface. Immunochemical staining with anti-His revealed that TAT-aFGF-His proteins were readily found in the retina (mainly in the ganglion cell layer) at 30 min. and remained detectable for at least 8 hrs after administration. In contrast, His^+ proteins were undetectable in the retina after topical administration of aFGF-His, indicating that aFGF-His cannot penetrate the ocular barrier. Furthermore, TAT-aFGF-His, but not aFGF-His, mediated significant protection against retinal ischemia–reperfusion (IR) injury. After IR injury, retina from TAT-aFGF-His-treated rats showed better-maintained inner retinal layer structure, reduced apoptosis of retinal ganglion cells and improved retinal function compared to those treated with aFGF-His or PBS. These results indicate that conjugation of TAT to aFGF-His can markedly improve the ability of aFGF-His to penetrate the ocular barrier without impairing its biological function. Thus, $TAT_{49–57}$ provides a potential vehicle for efficient drug delivery in the treatment of retinal disease.

Keywords: cell-penetrating peptide • fibroblast growth factor • ischemia and reperfusion injury • retina • transactivator of transcription peptide

Introduction

The retina is the most complex ocular tissue with its ability to process visual information and transmit the information through the optic nerve to the visual cortex. The retina is protected by the ocular barriers, including the corneal, aqueous humour and blood retinal barriers, and only limited materials can penetrate these barriers from outside of the eye or blood circulation. Although the ocular barriers provide a stable, close microenvironment for retinal function, these barriers also make it challenging to deliver potential therapeutic drugs to the retina. Currently, the most widely used delivery method for retinal drugs is sub-conjunctival injection, which is not only a difficult and harmful procedure, but also not practical as a chronic therapy. Thus, the development of non-invasive drug delivery systems would greatly benefit retinal disease treatment.

Cell-penetrating peptides (CPPs) have been reported as vehicles for the intracellular delivery of macromolecules, including oligonucleotides [1], siRNA [2], peptides and proteins [3], nanoparticles [4],

[#]These authors contributed equally to this work.
*Correspondence to: Xiaokun LI
School of Pharmaceutical Science,
University-town, Wenzhou,
Zhejiang, PR China
E-mail: xiaokunli@163.net

doi:10.1111/j.1582-4934.2009.00786.x

iron beads [4], plasmid [5] and liposomes [6]. HIV transactivator of transcription (TAT) peptide is one of the CPPs that has been broadly investigated. TAT has been shown to mediate delivery of biological agents both *in vitro* and *in vivo*. Its ability to translocate into live cells and cross biological barriers (*e.g.* blood-brain barrier) that makes it a potential novel vehicle for clinical drug delivery [7, 8].

In this study, we investigated the potential of TAT to deliver human acidic fibroblast growth factor (aFGF$_{19–154}$) from the eyeball surface to the retina in rats. aFGF (also known as FGF-1) is one of the most powerful and broad-spectrum mitogens in the FGF family. aFGF regulates the development and morphogenesis of the ectoderm- and mesoderm-derived cells, wound healing, haematopoiesis, angiogenesis, inflammatory processes as well as tumorigenesis [9–16]. aFGF has also been reported to mediate potent protection against ischemia–reperfusion (IR) injury in the brain and heart tissues [17, 18]. Our results showed that TAT-aFGF-His, but not aFGF-His, can efficiently penetrate the ocular barriers and mediate strong protection against retinal IR injury.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 250–300 g (approximately 18 weeks of age) were obtained from the Animal Center of Wenzhou Medical College (Wenzhou, China). Animals were housed at a constant room temperature with a 12:12 hr light/dark cyclic, and fed a standard rodent diet and water. Protocols involving the use of animals were approved by the Wenzhou Medical College Animal Policy and Welfare Committee.

Ischemia–reperfusion model

Rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml/kg). Topical anaesthesia was performed by instilling 0.5% proxymetacaine HCl into the cornea for 5 min., and then the pupils were maximally dilated by topical 0.5% tropicamide and 0.5% phenylephrine HCl. Eyes were then moistened with Ofloxacin (Jiangsu Kwin Pharma Co., Ltd., Jiangsu, PR China) at 10-min. intervals throughout the procedure. The anterior chambers of both the left and right eyes were cannulated with a 27G needle connected to a saline reservoir. Retinal ischemia was induced in the right eye by raising the intraocular pressure (IOP) to 110 mmHg for 60 min. by keeping the saline reservoir at 150 cm above the right eye, and the left eye was set as the sham procedure control without increasing the IOP, as previously described [19]. Retinal ischemia was confirmed by the loss of the red reflex in retinal arteries and the paleness of the bulbar conjunctiva using an ophthalmoscope. After an ischemic period of 60 min., the needle was removed from the anterior chamber, and the reperfusion was confirmed by seeing the recovery of the red reflex in retina arteries by ophthalmoscopy.

Preparation and administration of fusion protein aFGF-His and TAT-aFGF-His

Fusion protein aFGF-His and TAT-aFGF-His were generated in our laboratory, as previously described [20]. Briefly, TAT-aFGF-His cDNA was amplified from the pET-aFGF$_{19–154}$ plasmid by PCR with the forward primer F1 (5′-GGAATTCCATATGCGCAAAAAACGTCGTCAGCGTCGCCGTGCTAACTACAAG-3′) and reverse primer R (5′-GCAGATCTTTAGTGATGATGATGATGATGATCAGAAGAAACTGGCAA-3′). The sequences of TAT and His were included in the primers F1 and R, respectively (underline). The amplified fragment was then ligated into pET3c vector. Another expression vector pET-aFGF-His was generated *via* the same procedure as shown above using the forward primer F2 without the sequence of TAT (5′-GGAATTCCATATGGCTAACTACAAGAAGCCAAAGTTG-3′) and reverse primer R. The two expression vectors were cloned into *E. coli* BL21 (DE3). Recombinant aFGF-His and TAT-aFGF-His were dissolved in PBS. A volume of 2 μg aFGF-His or TAT-aFGF-His in 40 μl solution (50 μg/ml) was topically administered on the surface of the eyes of rats under anaesthesia while control eyes were treated similarly with PBS. In the experiments measuring peptide penetration through the ocular tissues (Fig. 1), aFGF-His, TAT-aFGF-His or the same volume of PBS were administered once and eyes were harvested at different time-points after the treatment. For IR injury protection experiments, aFGF-His, TAT-aFGF-His or the same volume of PBS were administered three times on days 0, 2 and 4 with respect to IR injury.

Immunohistochemistry

Tissue penetration of aFGF-His and TAT-aFGF-His was assessed by immunohistology using a rabbit anti-His polyclonal antibody (QED Bioscience, Inc., San Diego, CA, USA). Briefly, rats were killed in a CO_2 chamber, and both eyes were removed, fixed in 4% formalin at room temperature overnight, processed and embedded in paraffin. Paraffin-embedded tissues were sectioned (4 μm thick) along the vertical meridian of the eye through the optic nerve head. Only sections containing the whole retina with visible portions of the optic nerve head were used for immunohistological analysis. Tissue sections were primarily stained with rabbit anti-His polyclonal antibody (1:1000), and the signal was detected by biotinylated anti-rabbit IgG (Ana Spec Corporate, San Jose, CA, USA) and a streptavidin complex kit (Vector Laboratories, Burlingame, CA, USA).

Histological evaluation of retina injury following ischemia reperfusion

The ischemic (right eyes) and non-ischemic eyes (left eyes) were enucleated and cut into half by coronal section through the ora serrata. The vitreous was removed, and the posterior half of the eye was immersed in 4% paraformaldehyde for 24 hrs, and then sectioned, stained with haematoxylin and eosin. Samples were analysed blandly for the cell morphology in the inner nuclear layer (INL) and ganglion cell layer (GCL), and the number of ganglion cells in GCL was counted in five vision fields selected through each 100-μm retina length across the retina starting from the optic disc. Retinal ganglion cells (RGCs) in GCL, which are regular in shape and arrangement with large nuclei, were identified and counted based on the morphology. We normalized the results by dividing the data of the experimental eye (right eye) by those of the control eye (left eye) of the same rat.

Scotopic electroretinogram (ERG) recordings

ERGs were elicited from the right eyes simultaneously using a Ganzfeld bowl at 1, 3 and 7 days after IR. Rats were dark-adapted for 2 hrs and prepared under dim red light. Topical anaesthesia was performed by dropping

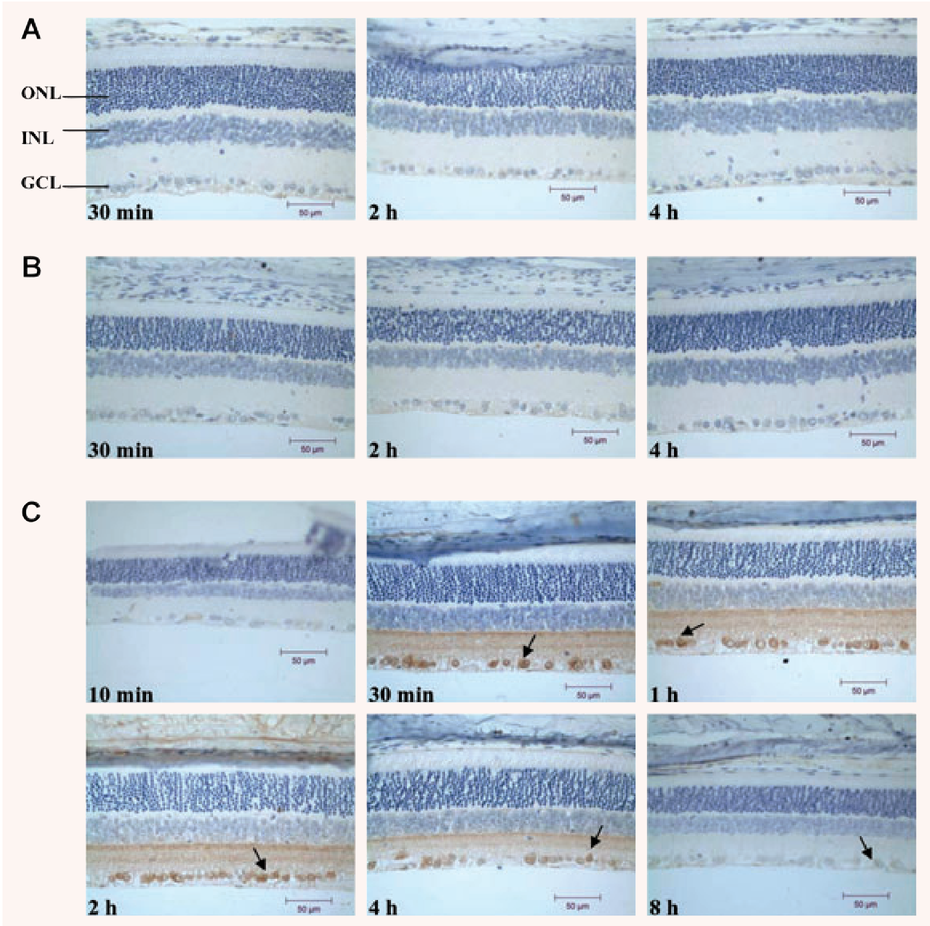

Fig. 1 TAT-aFGF-His, but no aFGF-His, fusion proteins show penetration into the retina after topical administration onto the eye surface. Eyes were harvested 10 min., 30 min., 1, 2, 4 and 8 hrs after topical treatment with PBS (**A**), aFGF-His (**B**) or TAT-aFGF-His (**C**), and tissue distribution of fusion proteins was examined by immunohistochemistry using anti-His. Arrows indicate representative His^+ cells (brown colour). Four animals per group were analysed at each time-point, and the representative pictures are shown. Because histological findings in eyes from rats treated with PBS and aFGF-His appeared similar at all time-points, representative data from three time-points are presented. The scale bars denote 50 μm.

0.5% proxymetacaine HCl onto the cornea after anaesthetization with 10% chloral hydrate, and the pupils were maximally dilated by 0.5% tropicamide and 0.5% phenylephrine HCl as described above. The recording electrodes were placed in the brim of the sclera, the reference electrodes were cannulated into visor subcutaneously, and a stainless steel sheet under the animals was used as the ground electrode. Each stimulus (the optical power is 0 cds/m^2, and the bandpass was filtered from 1 to 300 Hz) was given to every responder once with a duration of 250 microseconds (ms). The intensities of a-wave, b-wave and Ops-wave (oscillatory potentials) were recorded by RETI2 scan vision electrophysiology detection machine (Roland Consult, Brandenburg, Germany).

TUNEL staining and apoptotic analysis

TUNEL staining was performed on paraffin-embedded sections using the *in situ* cell death detection kit (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, PR China) according to the manufacture's protocol. In brief, the sections were dewaxed, rehydrated, and incubated in a 20 μg/ml proteinase K working solution for 20 min. at 37°C. The slides were rinsed with PBS (3 min., three times), dried, and incubated in 20 μl TUNEL reaction mixture for 1 hr at 37°C. The reaction was terminated by rinsing the samples with PBS (3 min., three times), followed by adding 20 μl Converter-POD solution on samples for 30 min. at 37°C. After rinsing with PBS (3 min., three times), 50 μl DAB reaction solution was add to samples for 45 sec. at 37°C and sections were re-stained with haematoxylin for 10 sec. After washed, 1% ammonia rinsed and re-hydrated by using different concentration of alcohol, the sections were sealed and detected by a light microscope. $TUNEL^+$ (apoptotic) cells were quantified by counting brown-coloured cells in 10 fields (6000 μm^2 per field) in GCL along the optic disc, and data are presented as numbers (mean ± S.D.s) of positive cells per mm^2.

Statistic analysis

Data were analysed using SPSS V15.0 for one-way ANOVA, Dunnett T3. A P-value <0.05 was considered to be significant.

Results

Penetrating peptide TAT-mediated delivery of aFGF to retina after topical administration onto the eye surface

To determine the ability of TAT to penetrate the ocular barriers and deliver aFGF to the retina, we compared tissue penetration of

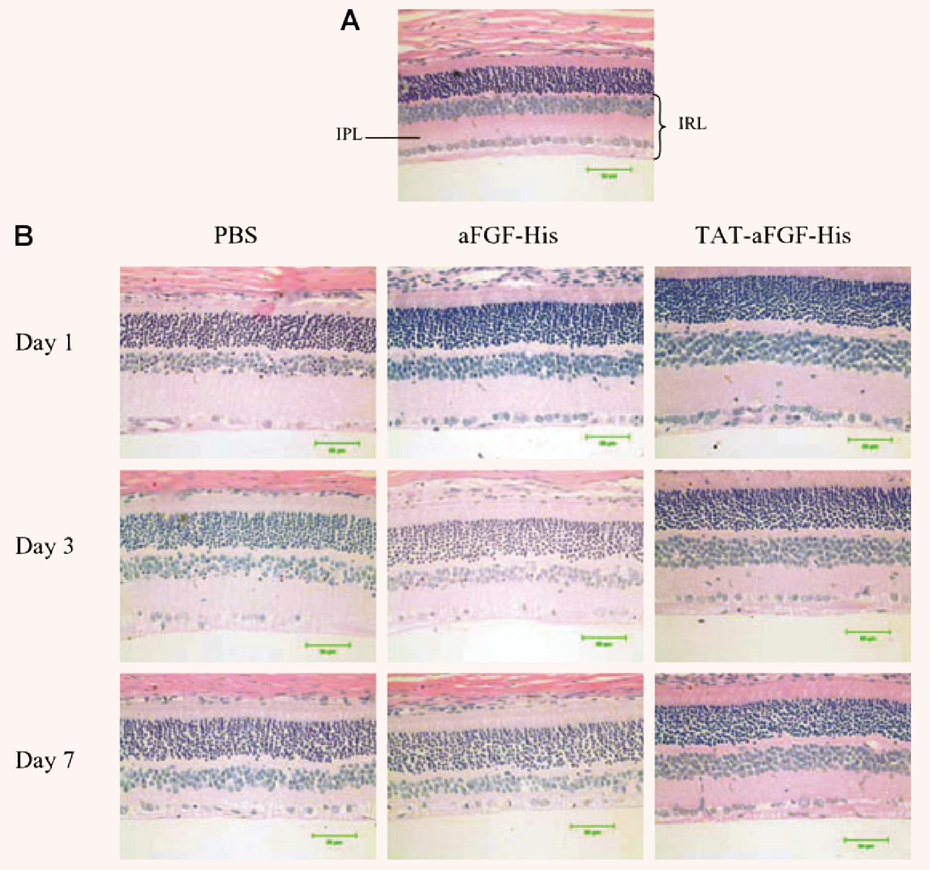

Fig. 2 Histological changes in the retina after IR. Rats were treated with PBS, 2 μg aFGF-His or TAT-aFGF-His on days 0, 2 and 4 after retinal IR. Eyes were harvested at days 1, 3 and 7 after IR, retina sections were prepared and stained with haematoxylin and eosin. Representative retinas from a sham procedure eye (**A**) and experimental eyes at days 1, 3 and 7 after reperfusion (B) are shown. Five animals per group were analysed at each time-point, and the representative pictures are shown. The scale bars denote 50 μm.

aFGF-His and TAT-aFGF-His in the eyes of rats after topical administration. Eyes were removed at various time-points after topical administration on the eye surface of 2 μg aFGF-His or TAT-aFGF-His, or the same volume of PBS (as controls), and tissue distribution of fusion proteins was examined by immunohistochemistry. Similar to PBS controls (Fig. 1A), no His^+ cells (brown colour) were found in the retina from aFGF-His-treated rats by 4 hrs (Fig. 1B). However, His^+ cells were readily detected in the retina from TAT-aFGF-His-treated rats by 30 min. after administration (Fig. 1C). TAT-aFGF-His proteins were found mainly in RGCs in the GCL. The levels of TAT-aFGF-His proteins, which were determined by the number and staining intensity of His^+ cells, peaked around 30 min. to 1 hr, and gradually declined thereafter but were still detectable at 8 hrs after administration. These data indicate that TAT-aFGF-His, but not aFGF-His, has the ability to penetrate the ocular barriers.

Topical administration of TAT-aFGF-His eye drops protects against retinal ischemia–reperfusion injury

It has been reported that injection of aFGF through jugular vein reduces retinal IR injury in rats [19]. To determine whether TAT-aFGF

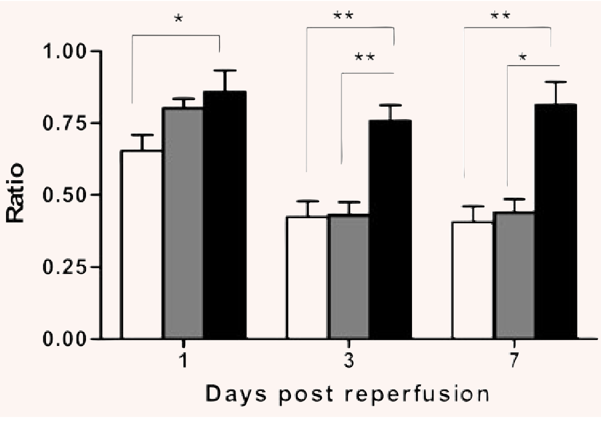

Fig. 3 TAT-aFGF-His, but no aFGF-His protects against IR-induced reduction in ganglion cells in the GCL. Rats were treated with PBS (□), 2 μg aFGF-His (■) or TAT-aFGF-His (■) on days 0, 2 and 4 after retinal IR. Eyes were harvested at days 1, 3 and 7 after IR, and retina samples were prepared and stained with haematoxylin and eosin (five rats per group were analysed at each time-point). The number of ganglion cells in the GCL was counted in five vision fields of 100-μm length across the retina. Data are presented as the ratio (mean ± S.D.s) of the experimental eyes to the sham-operated control eyes. $*P < 0.05$, $**P < 0.01$

Table 1 Number of apoptotic cells in the ganglion cell layer

Group (*n*)	Time after reperfusion (days)		
	1	3	7
PBS (5)	795.0 ± 145.6	668.3 ± 87.3	664.3 ± 115.0
aFGF$_{19–154}$-His (5)	599.3 ± 12.7	615.0 ± 112.6	640.0 ± 89.8
TAT-aFGF$_{19–154}$-His (5)	451.7 ± 75.6	331.7 ± 46.9$^{**,\#}$	303.3 ± 24.0$^{*,\#\#}$

Shown are numbers (mean ± S.D.s) of apoptotic cells/mm^2 in the GCL.
$^{*}P < 0.05$, $^{**}P < 0.01$, compared with PBS-treated group.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with aFGF$_{19–154}$-His-treated group.

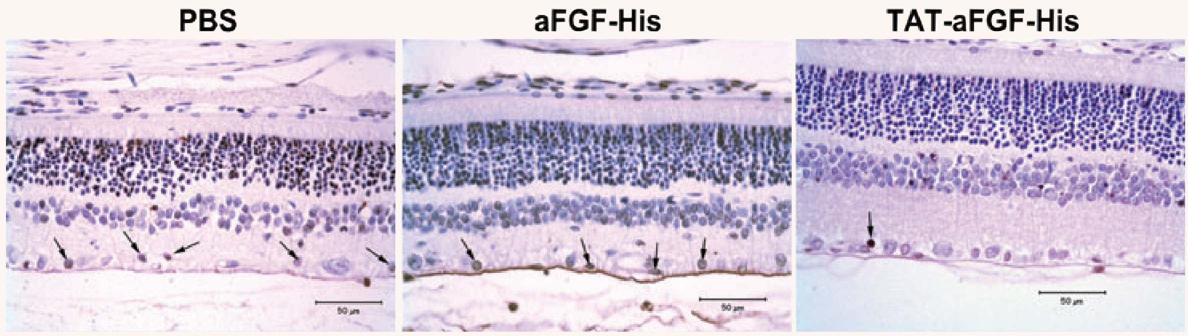

Fig. 4 Protection against IR-induced RGC apoptosis by TAT-aFGF-His treatment. Eye samples were harvested from the indicated groups at days 1, 3 and 7 after IR, and apoptotic cells in GCL were determined by TUNEL staining. Five animals per group were analysed at each time-point, and the representative pictures from day 3 samples are shown (detailed results are presented in Table 1). Arrows indicate TUNEL$^+$ (apoptotic) cells (brown colour). The scale bars denote 50 μm.

fusion proteins remain biologically functional, we assessed the therapeutic effect of TAT-aFGF-His in a rat retinal IR injury model. Compared to sham-operated eyes (Fig. 2A), IR resulted in severe injury in the PBS control group (Figs 2 and 3): on day 1, the retina appeared dropsical (especially the inner plexiform layer), and showed reduced RGCs in the GCL, interrupted cellular distribution in the INL, and an increased number of cells with pyknotic nuclei in the INL; on day 3, the condition of oedema was subsidized, but the number of RGCs kept decreasing; on day 7, most RGCs in the GCL exhibited pyknotic nuclei. Similar pathological changes were seen in the retina following IR in the aFGF-His-treated group (Figs 2B and 3), which is consistent with the inability of aFGF-His to penetrate through the ocular barriers to the retina (Fig. 1B). However, significant protection against IR injury was seen in the TAT-aFGF-His treated-group. Retinas from TAT-aFGF-His treated eyes showed markedly less severe pathological changes, including better maintained histological structure (Fig. 2B) and a lower reduction in RGC numbers (Fig. 3), compared to those treated with PBS or aFGF-His. Furthermore, TUNEL staining revealed that TAT-aFGF-His treatment also reduced IR-induced RGC apoptosis. As shown in Table 1 and Fig. 4, the numbers of apoptotic cells in the GCL were significantly less in TAT-aFGF-His-treated group than those in PBS- and aFGF-His treated groups at days 3 and 7 after IR.

Retinal function was further determined by ERGs. ERG measurements reflect the physiological condition of the whole retina. At day 1 after IR, a significant decrease ($P < 0.05$) in the amplitudes of ERG a-wave, b-wave and Ops was seen in all groups (Fig. 5 and Table 2). However, TAT-aFGF-His-treated group showed accelerated recovery compared to those treated with PBS or aFGF-His. Both PBS- and aFGF-His-treated groups showed continued decreases in ERG a-wave, b-wave and Ops between day 1 and day 3, whereas all these ERG measurements began increasing 3 days after IR. It has been reported that acute retinal ischemia results in a predominant loss of the b-wave with relative preservation of the a-wave [21]. Thus, suppression of the b-wave of the ERG has been taken as an electrophysiological indicator for reduced retinal blood flow in human beings [22] and in experimental animals [23]. ERG b-wave amplitudes in the TAT-aFGF-His-treated group at days 3 and 7 were significantly higher than those in BPS- and aFGF-His-treated groups (Fig. 5 and Table 2).

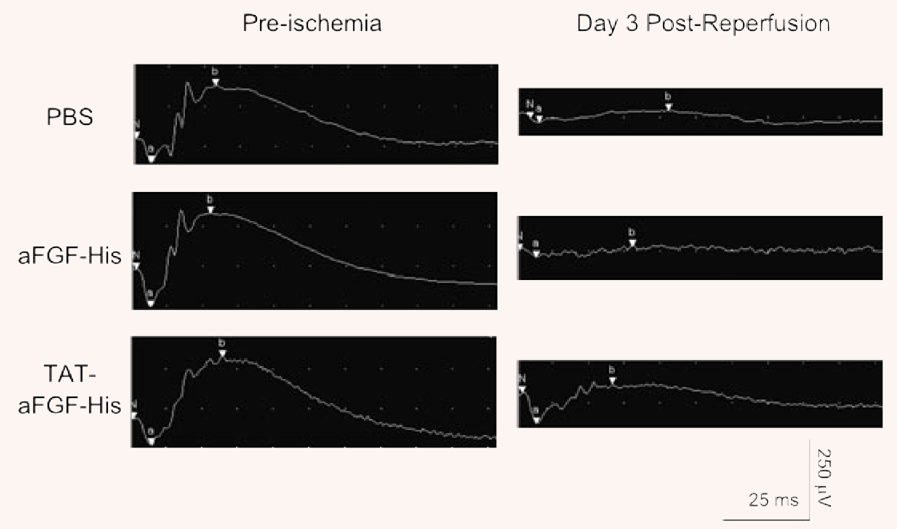

Fig. 5 Comparison of electroretinograms between PBS-, aFGF-His- and TAT-aFGF-His-treated rats. ERGs were elicited from the right eyes simultaneously using a Ganzfeld bowl prior to ischemia, and at days 1, 3 and 7 after IR, and the intensities of a-wave, b-wave and Ops-wave (oscillatory potentials) were recorded. Shown are representative electroretinograms of pre-ischemia and 3 days after reperfusion from the indicated groups (detailed data are presented in Table 2).

Table 2 Electroretinogram amplitude changes

Group (*n*)	Pre-ischemia	Time after reperfusion (days)		
		1	3	7
a-wave				
PBS (5)	182.9 ± 49.20	39.6 ± 19.32	25.6 ± 7.40	170.4 ± 12.93
aFGF-His (5)	195.5 ± 55.22	101.4 ± 56.55	57.0 ± 20.58	164.0 ± 27.97
TAT-aFGF-His (5)	189.8 ± 44.41	66.4 ± 28.86	135.0 ± 34.39**#	161.2 ± 34.02
b-wave				
PBS (5)	482.7 ± 109.61	59.2 ± 8.79	62.0 ± 15.41	145.2 ± 22.51
aFGF-His (5)	501.8 ± 87.83	121.6 ± 63.43	58.8 ± 18.01	176.8 ± 48.30
TAT-aFGF-His (5)	465.7 ± 98.28	118.0 ± 42.10	199.2 ± 73.20*#	287.4 ± 71.24*#
Ops				
PBS (5)	172.1 ± 47.08	29.0 ± 19.54	25.0 ± 12.33	83.6 ± 12.38
aFGF-His (5)	165.5 ± 44.78	45.4 ± 16.10	28.2 ± 6.26	76.0 ± 14.92
TAT-aFGF-His (5)	171.3 ± 42.37	41.8 ± 15.16	70.2 ± 17.25*	111.8 ± 16.10

Data shown are microvolts (mean ± S.D.s) for each group at the indicated time-points.
*$P < 0.05$, **$P < 0.01$, compared with PBS-treated group.
#$P < 0.05$, ##$P < 0.01$, compared with aFGF-His-treated group.

Discussion

It is well established that TAT-PTD, a peptide derived from the HIV-1 TAT protein, enhances cellular delivery of attached molecules. The mechanisms by which TAT mediates cell entry remains controversial, with evidence for the involvement of both endocytic and non-endocytic pathways [24–26]. Several have reported that cargos anchored with TAT can cross the blood-brain barrier [7, 20, 27]. In the present study, we proved that TAT-PTD ($TAT_{49–57}$) provides a potential vehicle for drug delivery to retina. We observed that TAT-aFGF-His, but not aFGF-His, can be detected in the retina after topical administration to the ocular surface. TAT-aFGF-His proteins were readily found in retina by 30 min., remained detectable for more than 8 hrs after administration. Exogenous aFGF could reduce IR injury in both *in vivo* and *in vitro* models [28–31]. However, in agreement with the inability of aFGF to penetrate the ocular barriers (Fig. 1B), aFGF confers no protection against retinal injury when given topically as eye drops to the ocular surface (Figs 2 and 3, Tables 1 and 2). In contrast, topical administration of TAT-aFGF-His mediated strong protection against retinal IR injury, as shown by both histological and functional analyses. These results indicate that the biological activity of aFGF was well retained by the TAT-aFGF-His fusion protein, and that $TAT_{49–57}$ is a potential vehicle for efficient drug delivery in the treatment of retinal disease. It has been reported that aFGF injection *via* jugular vein can also ameliorate retina ischemic injury in rats [19]. Although topical administration of TAT-aFGF-His provides an attractive means of non-invasive drug delivery, it would be important to further investigate the tissue/blood distribution and therapeutic efficacy in comparison with systemic aFGF administration.

Drug delivery to the eye, a relatively isolated anatomic compartment, remains a challenge. The ocular barriers include corneal, aqueous humour and blood retinal barrier. Previous studies have shown that the intact corneal epithelium is a barrier against TAT fusion protein penetration. Using an *in vitro* corneal culture model, it was found that TAT-β-galactosidase cannot penetrate the corneal epithelial cells deeper than the superficial cells unless the corneal epithelium was damaged [32]. Similar results were observed in our studies, in which TAT-aFGF-His proteins were only detected in the epithelium of the cornea but not in the stroma and deeper endothelium after topical administration on the ocular surface (data not shown). Although further studies are clearly needed to determine how TAT-aFGF-His proteins were delivered to the retina after administration on the ocular surface, these results indicate that they were unlikely delivered by penetration through the cornea.

Because of the lack of an identified cell surface TAT receptor, TAT is thought to target the lipid bilayer component of the cell membrane and therefore, its cell entry is expected to occur in all mammalian cell types. However, although TAT peptide uptake was seen in many cell types [33, 34], the existence of cell-specific mechanisms for cellular entry of TAT peptides has been suggested by the observation that TAT peptides were poorly or non-permeable in well-differentiated epithelial cells [35]. The selective uptake of TAT conjugates by retinal cells was also reported, in which TAT conjugates were predominately uptaken by RGCs and by a subset of inner nuclear layer cells [36, 37]. In our studies, the uptake of TAT-aFGF-His proteins was also detected mainly in RGCs. Because RGC death following ischemic insult is the major cause of a number of vision-threatening diseases, selective delivery of aFGF to RGCs may have the potential to improve the therapeutic outcome.

Acknowledgements

The study was supported by grants from Zhejiang Province Extremely Key Subject Building Funding ‘Pharmacology and Biochemical Pharmaceutics 2008’ and Zhejiang province key Natural Science Foundation (Z205755 to X.L.), and National Natural Scientific Foundation of China (30670541 to Y.H.), The Program of New Century Excellent Talents in University, and Zhejiang Provincial Program for the Cultivation of High-Level Innovative Health Talents (X.L.), The National Basic Research Program of China (973 grant no. 2005CB522603) and by the 5010 Project of Wenzhou Medical College.

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