厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

MRI-ASL 法による MELAS 脳卒中用発作における発作前間歇期の脳血流の評価

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研究要旨　MELAS 患者 3 名の脳卒中様発作において，MRI での ASL 灌流画像を用いて，発作前間歇期に局所脳血流が増加していることを明らかにした．ASL 画像は，脳卒中様発作の予測に有用である可能性が示された．

A．研究目的

mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes（MELAS）は，繰り返す脳卒中様発作が特徴であり，発作はしばしば重篤な神経学的症状を呈する．しかし，脳卒中様発作発現の機序はいまだ不明であり，その発現予測も困難である．今回我々は，3 例の MELAS 患者に対して，MRI にて造影剤を使わず灌流画像が得られる arterial spin labeling（ASL）画像を経時的に撮影し，脳卒中様発作病変となった部位の，発作前の間歇期における局所脳血流を後方視的に検討した．

B．研究方法

ミトコンドリア遺伝子 A3243G 変異を有し，脳卒中様発作を発症した MELAS 患者 3 例(男性 2 例，女性 1 例，平均 36.3 歳）に対し，ASL 法を含めた頭部 MRI を，発作前間歇期および脳卒中様発作発現時に撮影した．発作の症状発現時に，拡散強調画像（DWI）や FLAIR 画像などの通常撮影法にて高信号が新たに出現した部位を発作病変と定義し，その発作前の間歇期に撮影していた ASL 画像における同部位の局所脳血流を検討した．なお，ASL 撮像を行うことについては，福井大学医学部附属病院 IRB の承認と患者の同意を得ている．C．研究結果

3 例とも，発作の 3-7 ヶ月前の間歇期に撮影された ASL 画像にて，局所的な脳血流の増加が認められたが，DWI および FLAIR 画像では同部位の信号変化は認められなかった．うち 1 例では，同部位から得られた MR spectroscopy（MRS）での乳酸ピークはわずかであり，同部位はこの時点では発作病変ではないと考えられた．発作発現時には，同部位は 3 例とも，DWI および FLAIR 画像で高信号域となり，急性の発作病変として描出された．

D．考察

脳卒中様発作では，病変出現の数ヶ月前より，局所脳血流が増加していることが明らかとなり，発作前段階における血流増加が発作発現に関与している可能性が示された．すなわち，MELAS 患者においては，間歇期での MRI 通常画像や MRS で異常が認められなくとも，ASL 画像で血流増加が認められる部位は，近い将来に脳卒中様発作病変となる可能性が高い．

E．結論

ASL 法は，通常の MRI 撮影に加えて簡便に脳血流を評価でき，MELAS 患者における脳卒中様発作の発現予測に有用であると考えられた．

F．健康危険情報

とくになし．

G．研究発表

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Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 主な刊行物・別刷

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BRAIN RESEARCH

Research Report

Mild hypothermia enhanced the protective effect of protein therapy with transductive anti-death FNK protein using a rat focal transient cerebral ischemia model

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ABSTRACT

We previously reported that the protein transduction domain fused FNK (PTD-FNK) protein, which was derived from anti-apoptotic Bcl-xL protein and thereby gained higher anti-cell death activity, has a strong neuroprotective effect on rat focal brain ischemia models. The aim of this study was to investigate the effect of PTD-FNK protein and hypothermia combined therapy on cerebral infarction. Male SD rats were subjected to 120 min middle cerebral artery occlusion (MCAO) with intraluminal thread. Rats were divided into 4 groups: 1) 37 °C vehicle administration (37V); 2) 37 °C PTD-FNK administration (37F); 3) 35 °C vehicle administration (35V); and 4) 35 °C PTD-FNK administration (35F). PTD-FNK protein was intravenously administered 60 min after the induction of MCAO. Hypothermia (35 °C) was applied during 120 min MCAO. Rats were sacrificed 24 h later; infarct volumes were measured, and Bax, Bcl-2, TUNEL and caspase-12 immunostaining was evaluated. There was significant infarct volume reduction in 37F, 35V, and 35F groups compared to 37V. There was also a significant difference between 37F and 35F. This suggests that hypothermia enhanced the effect of PTD-FNK. Similar results were found in neurological symptoms. Caspase-12 and TUNEL staining showed a significant difference between 37F and 35F; however, Bax and Bcl-2 staining failed to show a difference. In this study we showed an additive protective effect of hypothermia on PTD-FNK treatment, and immunohistological results showed that the protective mechanisms might involve the inhibition of apoptotic pathways through caspase-12, but not through Bcl-2.

1. Introduction

Many pharmacological agents have been shown to have neuroprotective abilities in animal experimental studies; however, most of them failed to show effectiveness in clinical trials. Therefore, exploring new therapeutic strategies is still an important issue.

Proteins of the Bcl-2 family have pro- and anti-apoptotic abilities and their role in the sequence of neuronal death is shown to be important. Bax promote apoptosis, whereas Bcl-

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2 and Bcl-xL block the translocation of cytochrome c and thereby prevent neurons from apoptosis; therefore, a potential neuroprotectant will be an anti-apoptotic protein such as Bcl-2 or Bcl-xL. Introducing proteins into brain cells by intravenous injection is known to be difficult because of the function of the blood–brain barrier. Protein transduction domain (PTD) from HIV Tat protein has been proposed to deliver therapeutic proteins directly into ischemic brain tissue by intravenous injection (Katsura et al., 2008; Wadia and Dowdy, 2002).

FNK protein is artificially derived from anti-apoptotic protein Bcl-xL by substituting three amino acids and thereby gains higher anti-cell death activity (Asoh et al., 2000). FNK protein was fused with PTD (protein transduction domain) of HIV/Tat protein to be able to pass through cell membranes. It was shown to be transduced into neuronal cells rapidly (Katsura et al., 2008).

PTD-FNK protein has been shown to have cytoprotective effects in various cells and various conditions (Arakawa et al., 2007; Asoh et al., 2005; Chen et al., 2007). PTD-FNK protected human neuroblastoma cells and rat neocortical neurons against staurosporine-induced apoptosis and glutamate-induced excitotoxicity (necrosis). It was also shown to have anti-necrotic and anti-apoptotic activity in cultured neuronal cells (Asoh et al., 2002).

We have previously shown that PTD-FNK had a strong neuroprotective effect using 90-min middle cerebral artery occlusion (MCAO) and reperfusion model (Katsura et al., 2008). The cortical infarct volume decreased to about 50% in that study.

On the other hand, hypothermia is known to have strong neuroprotective effects via many mechanisms (such as decreasing the metabolic rate, reducing glutamate release, reducing reactive oxygen species, preventing blood–brain barrier breakdown, modifying the gene expression of inflammation, apoptotic proteins etc.) (Yenari et al., 2008). However, clinical application of hypothermia to cerebral ischemia had been difficult because of several severe side effects. Therefore, we chose the very mild hypothermia of 35 °C which may have much less side effects even in clinical application. We have published several studies in which very mild hypothermia enhanced the protective abilities, and elongated the therapeutic time windows of several drugs (Nito et al., 2003, 2004).

Our aim of the present study was to clarify whether combination therapy with PTD-FNK and very mild hypothermia is more effective than monotherapy, and to study the mechanisms of protection.

2. Results

2.1. *Effect on physiological parameters*

Table 1 shows physiological parameters, measured before, during and after focal ischemia. Although $PaCO_2$ 10 min after reperfusion in normothermic groups increased significantly compared to individual values before ischemia, there was no significant difference between vehicle-treated groups and PTD-FNK-treated groups. By two-factor ANOVA, there was no significant difference between normothermic and hypothermic parameters at individual time points.

Table 1 – Physiological parameters before, during and after ischemia.

		Before ischemia	During ischemia	After ischemia
Normothermia (37 °C)				
MABP	Vehicle (n=5)	98.0±10.4	108.0±5.7	94.0±13.9
(mmHg)	FNK(n=5)	94.0±8.9	109.0±18.5	96.0±21.6
Blood glucose	Vehicle (n=5)	102.4±21.1	89.4±20.3	90.4±21.8
(mg/dl)	FNK (n=5)	97.4±12.5	84.2±10.1	91.8±14.5
pH	Vehicle (n=5)	7.42±0.03	7.40±0.02	7.38±0.02
	FNK (n=5)	7.41±0.02	7.39±0.02	7.37±0.03
$PaCO_2$	Vehicle (n=5)	39.8±2.6	43.0±1.4	45.6±0.9*
(mmHg)	FNK (n=5)	42.4±2.3	44.6±3.7	48.6±4.2*
PaO_2 (mmHg)	Vehicle (n=5)	99.8±10.4	95.0±11.2	92.2±2.6
	FNK (n=5)	100.0±9.2	96.2±10.6	97.8±7.2
Hypothermia (35 °C)				
MABP	Vehicle (n=6)	103.3±12.1	117.5±12.9	107.5±14.8
(mmHg)	FNK (n=8)	101.3±7.4	121.3±7.4	104.4±9.4
Blood glucose	Vehicle (n=6)	98.7±15.2	82.0±5.7	86.3±6.8
(mg/dl)	FNK (n=8)	101.8±13.6	87.6±11.3	89.5±15.9
pH	Vehicle (n=6)	7.40±0.03	7.40±0.02	7.38±0.02
	FNK (n=8)	7.40±0.02	7.39±0.02	7.37±0.02
$PaCO_2$	Vehicle (n=6)	41.5±2.3	45.5±2.7	44.8±2.9
(mmHg)	FNK (n=8)	42.1±2.3	46.0±1.9	46.4±2.6
PaO_2 (mmHg)	Vehicle (n=6)	110.2±6.5	102.5±12.1	104.3±5.1
	FNK (n=8)	114.9±8.4	106.8±4.7	106.4±5.0

* $p<0.05$ compared to before ischemia.

2.2. *Effect of combination therapy on infarct volume*

Two-factor ANOVA showed a significant influence of PTD-FNK treatment or temperature change on infarct volume (n=5–8 for each group). Total infarct volume of 37F, 35V, or 35F was significantly reduced compared to that of 37V ($p<0.01$, each), as shown in Fig. 2. Hypothermia and PTD-FNK combination therapy showed significant additional reduction of total infarct volume compared to the hypothermia-alone group. Although there was a similar tendency in cortex infarct volume, there was no significant difference between 35V and 35F. Striatum infarct volume was decreased only in 35F, not 37V or 37F.

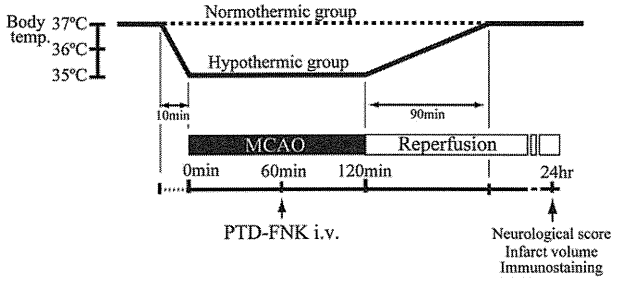

Fig. 1 – Experimental protocol including time course of temperature change, injection of PTD-FNK, evaluation of infarct volume, neurological score and immunostaining.

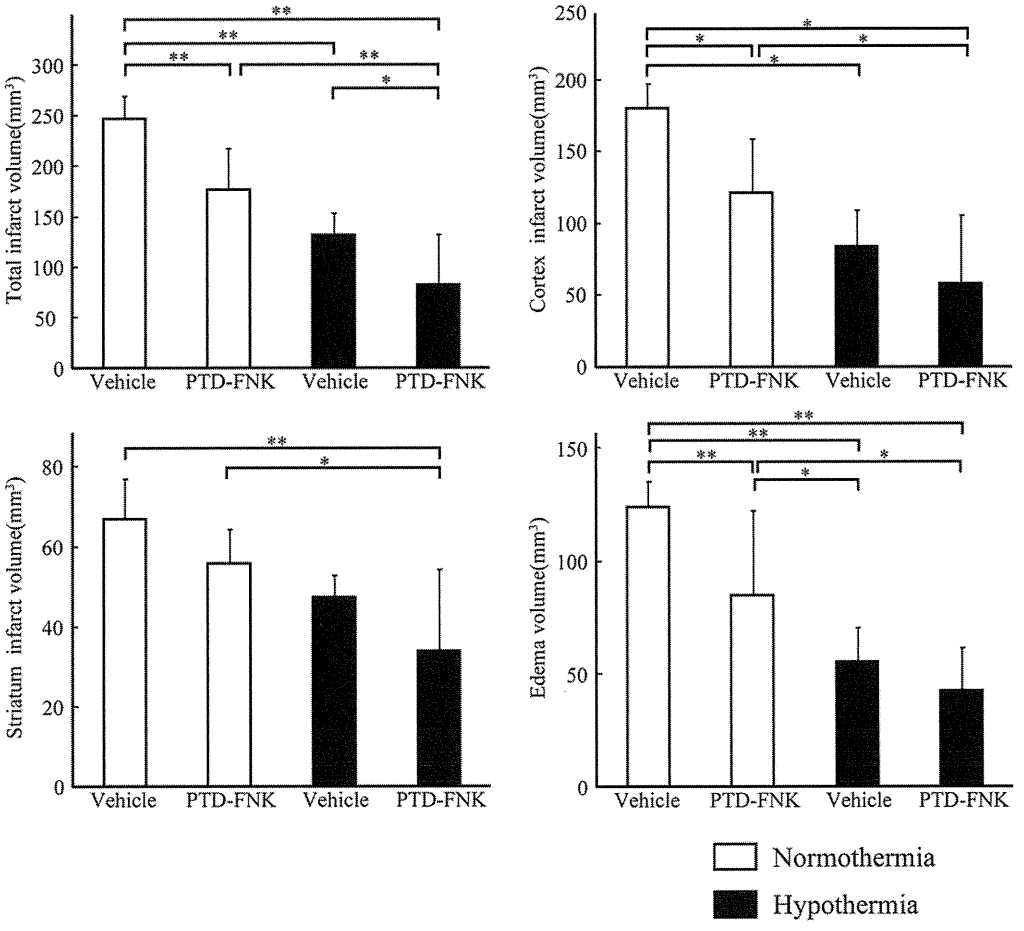

Fig. 2 – Effect of combination therapy on total, cortical and striatal infarct volume, and edema volume. Normothermic PTD-FNK showed significant reduction of total and cortical infarct volume and edema volume. PTD-FNK in the hypothermia group showed additional reduction of total, cortical, and striatum infarct volume and edema volume, compared to normothermic PTD-FNK group. *: p<0.05, **: p<0.01.

2.3. *Edema volume*

Two-factor ANOVA showed a significant influence of PTD-FNK treatment or temperature change on edema volume. Total edema volume was significantly reduced in 37F, 35V, and 35F compared to 37V ($p<0.01$). Edema volume of 35F was significantly decreased compared to 37F.

2.4. *Effect of treatment on neurological symptoms*

The neurological scores of 35F showed significant differences compared to those of 37V (Fig. 3). Combination therapy might have shown further improvement than hypothermia-alone therapy.

2.5. *TUNEL staining and immunohistological staining*

To elucidate the protective mechanism of hypothermia and PTD-FNK, we examined TUNEL, Bax , Bcl-2 and caspase-12 staining using other animals (n=4 for each group).

Bax scores significantly decreased in 35F against 37V, although there was no significant difference between 37V and 37F, or 35V. Combination therapy showed synergetic effects and made the protective effect significant (Fig. 4).

There was no significant difference in Bcl-2 scores in hypothermic- or PTD-FNK-treated groups against 37V (Fig. 4).

Two-factor ANOVA showed a significant influence of PTD-FNK treatment or temperature change on TUNEL staining. The

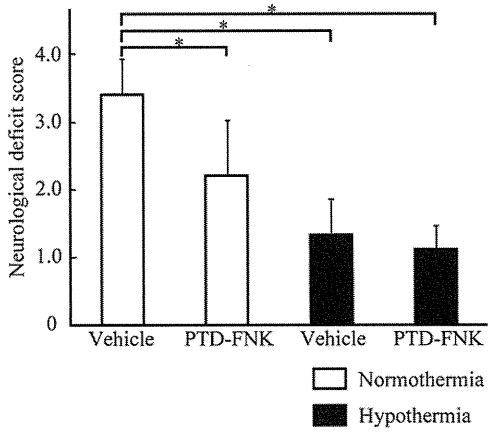

Fig. 3 – Effect of combination therapy on neurological deficit score. Hypothermic PTD-FNK showed significant reduction compared to normothermic vehicle. *: p<0.05.

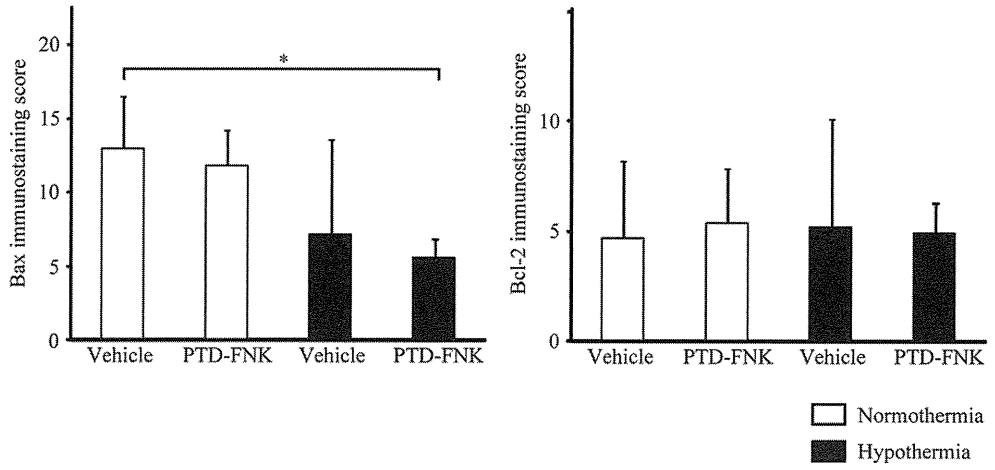

Fig. 4 – Effect of combination therapy on Bax and Bcl-2 immunostaining. Hypothermic PTD-FNK showed significant reduction compared to normothermic vehicle; however, hypothermia failed to show significant changes on Bax or Bcl-2 immunostaining. *: $p<0.05$.

number of TUNEL-positive cells was decreased in 35F compared to 37V or 37F. Hypothermia added additional protective effects to PTD-FNK therapy (Fig. 5).

Two-factor ANOVA showed the significant influence of PTD-FNK treatment and temperature change on caspase-12 staining. The number of caspase-12-positive cells was decreased in 35F compared to 37V or 37F. Hypothermia contributed to the additional reduction of caspase-12-positive cells for PTD-FNK therapy (Fig. 6).

3. Discussion

In this study, we found significant protective effects of combination therapy between PTD-FNK and hypothermia. PTD-FNK treatment and hypothermia showed an additive relationship and showed a more protective effect than each single treatment. We previously showed that PTD-FNK was more protective when combined with FK506 treatments (Katsura et al., 2008), and in this study we showed that mild hypothermia enhanced the protective effects of PTD-FNK treatment.

Compared with studies using wild-type Bcl-xL, which is reported to be cytoprotective (Reed, 1997), PTD-FNK treatment in this study was more effective at a very low dose, which was one hundred and forty-fourth of the dose of PTD-Bcl-xL used by Cao et al. (2002), or one-sixteenth of the dose of PTD-Bcl-xL used by Kilic et al. (2002). A lower dose is reasonably expected to reduce the possibilities of side effects from the protein transduction peptide. Although we used a more severe ischemic model, using 120-min occlusion (90-min occlusion in the previous study (Katsura et al., 2008)) in this study, PTD-FNK significantly reduced infarct volume and edema

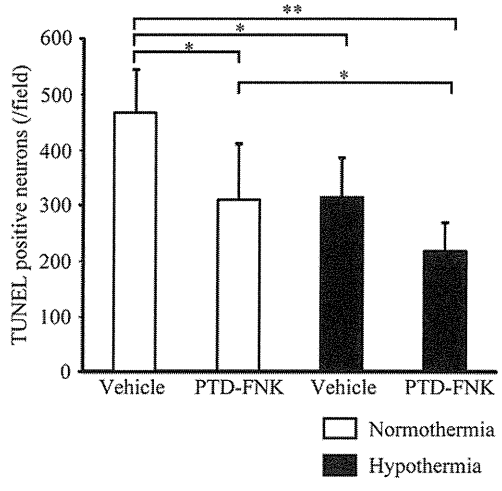

Fig. 5 – Effect of combination therapy on TUNEL staining. Hypothermic PTD-FNK showed significant reduction of TUNEL-positive neurons compared to normothermic PTD-FNK. *: $p<0.05$, **: $p<0.01$.

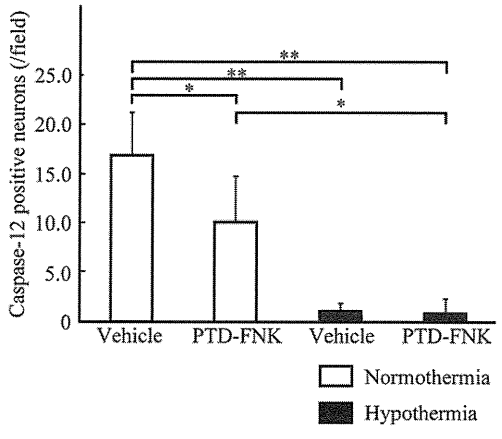

Fig. 6 – Effect of combination therapy on caspase-12 staining. Normothermic PTD-FNK showed significant reduction compared to normothermic vehicle. Hypothermic PTD-FNK showed significant reduction compared to normothermic PTD-FNK. *: $p<0.05$, **: $p<0.01$.

volume (Fig. 2), although the improvement of neurological scores only showed a tendency (Fig. 3). Thus, PTD-FNK treatment was shown to be protective against cerebral ischemia, as shown in the previous study (Katsura et al., 2008). The protective mechanisms of PTD-FNK treatment may be mainly related to the inhibition of apoptosis (Fig. 5); however, Bcl-2 and Bax staining were not affected (Fig. 4). These results may indicate that another apoptotic pathway, unrelated to Bcl-2 and Bax, is involved in the protection of PTD-FNK. Combined with previous results that PTD-FNK was thought to reduce intracellular calcium concentration through the inhibition of calcium release from the endoplasmic reticulum (Katsura et al., 2008), we thought that PTD-FNK may inhibit the apoptotic pathway through the endoplasmic reticulum and caspase-12 activation (Breckenridge et al., 2003); thus, we showed the reduction of the number of caspase-12-positive cells in the PTD-FNK-treated group (Fig. 6).

3.1. *PTD-FNK treatment combined with mild hypothermia*

Very mild hypothermia of 35 °C reportedly attenuates neuronal injury in an animal model of cerebral ischemia (Minamisawa et al., 1990). In particular, the effect of very mild hypothermia had an additional protective effect as well as elongation of the therapeutic time window of FK506 treatment (Nito et al., 2004). Several studies have shown the beneficial effects of mild to moderate hypothermia on ischemic brain injury following either global (Katsura et al., 1992) or focal (Nito et al., 2004) cerebral ischemia. A large number of protective mechanisms of hypothermia have been identified, such as reduction of the metabolic rate and energy depletion, decreased excitatory transmitter release, suppression of free radicals, and reduced vascular permeability, blood–brain barrier disruption and brain edema (Colbourne et al., 1997; Dietrich et al., 1996). Recently, it was reported that hypothermia reduced mRNA coding for caspase-12 in the peri-infarcted region (Florian et al., 2008), which is compatible with our result (Fig. 6). In addition, experimental studies have suggested that pharmacotherapy combined with hypothermia is more effective than monotherapy (Coimbra et al., 1996; Dietrich et al., 1995; Nito et al., 2004). Under such conditions, hypothermia may increase the protective effects of drugs or expand the therapeutic window. Similar to previous studies, the present results showed the additive effect of hypothermia on PTD-FNK treatment.

The present study showed that PTD-FNK significantly reduced infarct volume after 2 h of MCAO, and PTD-FNK together with hypothermia significantly reduced infarct volume compared to normothermic PTD-FNK treatment. A significant reduction compared to normothermic PTD-FNK treatment was observed for cortical and striatal infarct volume, as well as edema volume. The neurological deficit score showed a similar tendency. Hypothermic PTD-FNK showed a significant reduction of neurological deficit scores against the normothermic vehicle using the Shirley-Williams non-parametric multiple comparison method. Hypothermic PTD-FNK showed significant reduction of Bax immunostaining against the normothermic vehicle; however, Bcl-2 immunostaining scores were not influenced among the groups. Although hypothermia showed a tendency to reduce the Bax staining score, PTD-FNK- or hypothermia-alone treatment did not influence Bax or Bcl-2 staining. Although some papers have reported that mild hypothermia showed a significant increase of Bcl-2 immunostaining and reduction of Bax staining, those reports involved hypothermia of around 33 °C or below (Yenari et al., 2002; Zhang et al., 2010), not as mild as in this study. As shown in the previous study (Nito et al., 2004), hypothermia of 35 °C alone did not show significant protective effects; therefore, it seems compatible with previous data that hypothermia alone did not show a significant effect on Bax or Bcl-2 staining. As discussed above, the protective effect of PTD-FNK may be not mainly related to Bax or Bcl-2, because PTD-FNK treatment did not influence Bax or Bcl-2 immunostaining significantly. Only Bax immunostaining was significantly reduced with hypothermic PTD-FNK treatment, whereas PTD-FNK treatment showed significantly reduced caspase-12 immunostaining scores. From these results, we suggest that the protective effect of PTD-FNK involved inhibition of the anti-apoptotic pathway through caspase-12 activation. In addition, hypothermic PTD-FNK treatment showed significant reduction of Bax immunostaining scores, TUNEL-positive neurons, and caspase-12-positive neurons. From these results, we speculate that hypothermia additional to PTD-FNK treatment showed additional protective effects through enhanced inhibition of Bax, which is localized in the ER membrane, leading to inhibition of the increase of cytosolic calcium concentration, which may also reduce the activity of Bax in the mitochondrial membrane. Bax is a member of the Bcl-2 family proteins, which can promote the apoptosis by forming oligomers onto the mitochondrial outer membrane and creating a channel for the release of cytochrome C and other apoptotic substances (Eskes et al., 2000). Study also suggested that Bax can promote the release of cytochrome C from voltage dependent anion channel (VDAC) (Vander Heiden et al., 1997). Bax translocation onto the mitochondrial membrane becomes one of the important indicators for the onset of mitochondria-mediated apoptosis which is thought to be one of the major pathway to cell death (Abe et al., 1995). Thus, the results suggest that it may inhibit cytochrome C release from mitochondria and activation of calpain, which cleaves porcaspase-12 to produce active caspase-12, leading to cell death (Florian et al., 2008; Katsura et al., 2008). Therefore, hypothermia and PTD-FNK treatment might have reduced ER stress and mitochondrial stress simultaneously.

Brain temperature reduction is a promising method of reducing neuronal injury following cerebral ischemia; however, the clinical application of hypothermia is known to have severe adverse side effects, such as myocardial arrhythmia, respiratory insufficiency, blood hypercoagulability, and hemodynamic instability (Schubert, 1995; Schwab et al., 1998). Although these high risks and complexity have limited clinical application, mild hypothermia may not involve such complications; therefore, mild hypothermia of 35 °C was chosen in the present study. Mild hypothermia did not affect any physiological variables in the present study.

In conclusion, mild hypothermia of 35 °C enhanced the protective effect of PTD-FNK by reducing TUNEL staining, and Bax and caspase-12 immunostaining. The present combined therapy is an effective and safe strategy for neuronal protection against cerebral ischemia.

4. Experimental procedures

4.1. Animal model

Male Sprague–Dawley rats (250–300 g) were anesthetized with halothane (5% for induction and 1% for maintenance) in nitrous oxide/oxygen (70%/30%, v/v) under spontaneous breathing. The tail artery was cannulated to monitor mean blood pressure and blood sampling. The tail vein was cannulated for drug administration. Focal ischemia was produced by intraluminal occlusion of the left middle cerebral artery (MCA) with a nylon monofilament with a rounded tip and a distal silicon rubber cylinder. The animals underwent middle cerebral artery (MCA) occlusion for 120 min and were then reperfused.

Animals were randomly divided into the following four groups: (1) vehicle-treated normothermic group (37V); (2) PTD-FNK-treated normothermic group (37F); (3) vehicle-treated hypothermic group (35V); and (4) PTD-FNK-treated hypothermic group (35F). Temporal muscle and rectal temperature were maintained during ischemia at 37±0.5 °C in the normothermic groups and 35±0.5 °C in the hypothermic groups using a heating lamp or ice packs. Fig. 1 shows the experimental protocols.

Animals were intravenously administered 0.0625 mg/kg PTD-FNK protein in the PTD-FNK-treated groups, and vehicle in the vehicle groups 60 min after the initiation of ischemia. After recovering from anesthesia, the animals were maintained in an air-conditioned room at 20 °C.

4.2. Infarct and edema volume analysis

At 24 h after MCA occlusion, the brain was removed and sliced into six coronal sections (2 mm thick). All six sections were stained with 3% of 2,3,5-triphenyltetrazorium chloride (TTC). After taking digital images of all sections, they were analyzed to evaluate infarct volume. The border between infarct and non-infarct areas in each section was outlined using Image J software (NIH). The area of infarction was estimated by subtracting the non-infarct area in the ipsilateral hemisphere from that on the contralateral side in a blinded fashion. The volume of infarction was calculated by integration of the lesioned areas (Swanson et al., 1990). The increase in brain volume caused by edema was measured by subtracting the area in the non-infarct contralateral hemisphere from that in the ipsilateral hemisphere. Edema volume was also calculated by integration.

4.3. Score of neurological deficits

After 24 h of reperfusion, neurological symptoms in each rat were scored on a scale of 0–5 as follows (Murakami et al., 1998): 0, no neurological deficit; 1, failure to fully extend the right forepaw; 2, circling to the right; 3, falling to the right; 4, unable to walk; and 5, dead.

4.4. TUNEL staining and immunohistochemistry

Animals were transcardially perfusion-fixed with 4% paraformaldehyde 24 h after reperfusion (n=4, each), and 20-μm-thick coronal frozen sections at the level of the anterior commissure (bregma +0.70 mm) were cut on a cryostat. TUNEL staining was carried out using an in situ cell death detection kit (Roche Diagnostics, Mannheim, German) according to the manufacturer's instructions. Immunohistochemistry staining was carried out as follows. Frozen sections were blocked with 3% normal goat serum in PBS and then incubated in anti bcl-2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:10, anti-bax rabbit polyclonal antibody (Calbiochem, Darmstadt, Germany) at a dilution of 1:40 at 4 °C overnight, or anti-caspase-12 (Santa Cruz Biotechnology) at a dilution of 1:50 at 4 °C. The sections were incubated in Alexa Fluor 488(495/519) goat anti-rabbit IgG (Molecular Probes) at a dilution of 1:400 at room temperature for 1 h. The sections were mounted in VECTASHIELD Mounting medium with DAPI (360/460) (Vector Laboratories, CA, USA). Each procedure was followed by several rinses in PBS. Blank staining was carried out in the same way as above, except for eliminating the primary antibody.

The number of TUNEL-positive cells and caspase-12 cells was counted, and signals for Bax and Bcl-2 in the peri-infarct area were semi-quantified in 5 randomly selected microscopic fields (0.12 mm^2, each) under ×200 magnification in a blinded fashion (n=4, each). A four-point rating scale was used for semi-quantified analysis as follows: 0, none; 1, trace; 2, weak; 3, moderate; and 4, strong (Ay et al., 2001).

4.5. Construction and preparation of PTD-FNK

PTD-FNK was produced in our laboratory as described in our previous report (Katsura et al., 2008). Briefly, an oligonucleotide encoding MGYGRKKRRQRRRG (TAT protein transduction domain of 11 amino acids is underlined) was ligated to the 5′ end of FNK protein (see Asoh et al., 2000) coding sequence by PCR to construct PTD-FNK. The ligated DNA fragment was inserted between the Nco I and Hind III sites in pPROEX1 expression vector (Invitrogen Life Technologies). The integrity of the construct was confirmed by sequencing. The resultant plasmid was introduced into an *Escherichia coli* cell DH5αMCR, followed by IPTG induction. PTD-FNK protein was solubilized from inclusion bodies with 7 M urea and 2% SDS in the presence of 0.1 mM DTT, and then subjected to SDS-PAGE for further purification. After the gel was briefly incubated in 1 M KCl, a band corresponding to PTD-FNK was cut out. PTD-FNK was electrophoretically extracted from the gel slice in extraction buffer (25 mM Tris, 0.2 M glycine, and 0.1% SDS; used as vehicle) to inject into animals. The extraction buffer was used as the vehicle.

4.6. Statistics

Two factor-factorial ANOVA was used for comparisons in physiological parameters, infarct volume, edema volume, number of TUNEL-positive cells and number of caspase-12 positive cells followed by Student–Newman–Keul's post hoc test. Multicomparisons of non-parametric neurological scores and immunohistological scores were calculated with the Shirley–Williams test. Data are expressed as the means±S.D., and statistical significance was set at $P<0.05$.

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ORIGINAL ARTICLE

Topical application of the antiapoptotic TAT-FNK protein prevents aminoglycoside-induced ototoxicity

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We previously demonstrated that an artificial protein, TAT-FNK, has antiapoptotic effects against cochlear hair cell (HC) damage caused by ototoxic agents when applied systemically. To examine the feasibility of topical protein therapy for inner ear disorders, we investigated whether gelatin sponge soaked with TAT-FNK and placed on the guinea pig round window membrane (RWM) could deliver the protein to the cochlea and attenuate aminoglycoside (AG)-induced cochlear damage *in vivo*. First, we found that the immunoreactivity of TAT-myc-FNK was distributed throughout the cochlea. The immunoreactivity was observed from 1–24 h after application. When Tat-FNK was applied 1 h before ototoxic insult (a combination of kanamycin sulfate and ethacrynic acid), auditory brainstem response threshold shifts and the extent of HC death were significantly attenuated. When cochlear organotypic cultures prepared from P5 rats were treated with kanamycin, TAT-FNK significantly reduced the extent of caspase-9 activation and HC death. These findings indicate that TAT-FNK topically applied on the RWM can enter the cochlea by diffusion and effectively prevent AG-induced apoptosis of cochlear HCs by suppressing the mitochondrial caspase-9 pathway.

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Keywords: protein therapy; apoptosis; cochlea; aminoglycoside; topical application

INTRODUCTION

Apoptosis is involved in cochlear sensory hair cell (HC) death caused by a variety of insults, which include acoustic trauma, loss of trophic factor support, ischemia–reperfusion, and exposure to ototoxic agents such as aminoglycoside (AG) antibiotics and the anti-neoplastic agent cisplatin.[1–3] Protecting cells from apoptosis by controlling the balance of pro- and antiapoptotic proteins by techniques such as gene therapy is considered a good strategy for protection of HCs from ototoxic insults. Overexpression of Bcl-2 proteins by delivery of the *Bcl-2* gene into HCs has been reported to prevent the degeneration of HCs exposed to AG or cisplatin.[4] Injection of the *Bcl-x_L* gene into mice cochlea also prevents HC degeneration induced by kanamycin.[5] However, such gene transfer application cannot control the amount or exposure time of the target protein to achieve optimal prevention of cell death. In addition, gene transfer technology cannot avoid the possibility of detrimental insertion of transgenes. Therefore, injection of the target protein could be an alternative method. For example, several proteins such as granulocyte-colony stimulating factor[6] have already been used in clinics. Such protein therapy, however, is not always applicable for treatment of inner ear disorders because the blood–labyrinth barrier may inhibit the delivery of high-molecular-weight proteins into the cochlea. This problem may be solved by using the protein transduction domain technology. When fused with a protein transduction domain such as the TAT domain of the HIV/Tat (transcription-transactivating) protein, a variety of high-molecular-weight proteins have been successfully introduced into cells both *in vitro* and *in vivo*.[7,8]

We first constructed a powerful artificial antiapoptotic protein, FNK (originally designated Bcl-xFNK by Asoh *et al.*[9]), which has three amino-acid substitutions, Tyr-22 to Phe(F), Gln-26 to Asn(N) and Arg-165 to Lys(K), to strengthen the cytoprotective activity of Bcl-x_L. We then demonstrated that fusion of FNK with TAT enabled FNK to penetrate highly negatively charged chondrocytes[9,10] and the blood–brain barrier,[11] and that TAT-FNK showed an antiapoptotic effect in a model of brain and hepatic ischemia.[11,12] When injected intraperitoneally into guinea pigs *in vivo*, we observed that TAT-FNK was distributed widely in the cochlea and that it reduced the expression of cleaved poly-(ADP-ribose)-polymerase (PARP), auditory brainstem response (ABR) threshold shifts, and HC loss induced by a combination of ethacrynic acid (EA) and kanamycin sulfate (KM), *in vivo*.[13]

Another potential drug delivery system for treatment of cochlear disorders is topical drug application into the middle ear space. Compared with systemic injections, such local delivery is beneficial because it requires significantly lower amounts of drug and reduces systemic side effects. A major side effect after long-term administration of an antiapoptotic drug is a possibility of carcinogenesis. Overexpression of Bcl-x_L, the original protein of FNK, is reported to have the potential to cause tetraploidization, which would result in neoplasia.[14,15] Schuknecht[16] has developed a topical drug application technique for inner ear disorders: injection of streptomycin into the middle ear space of patients with Ménière's disease. Intra-tympanic dexamethasone injections have also been performed as primary treatment for sudden sensorineural hearing loss.[17] Intra-tympanic drug application has also been used in animal studies to examine the effects on inner ear function or disorders. It is quite difficult, however, to achieve the delivery of high-molecular-weight proteins into the inner ear because these proteins cannot pass through the round window membrane (RWM), which is the main route into the inner ear.

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In the current study, we examined whether the TAT fusion technique could make transtympanic protein therapy applicable for inner ear disorders. We investigated whether TAT-FNK applied topically on the RWM could be successfully delivered into the cochlea, and protect cochlear HCs from an ototoxic combination of KM and EA. We also investigated whether TAT-FNK could prevent HC death caused by KM by suppression of the mitochondrial caspase-9 pathway.

RESULTS

Transduction of TAT-myc-FNK into cochlear tissue

Immunohistochemical staining using an anti-myc-tag antibody revealed that TAT-myc-FNK was detectable in the cochlea from 1 to 24 h after the application onto the RWM. There was a statistically significant difference between the groups as determined by one-way analysis of variance (ANOVA) ($F_{6,203} = 41.239$, $P < 0.01$). Scheffe's *post hoc* test revealed that the labeling indices (LIs) at 1, 3, 6, 12 and 24 h were significantly higher than that of the control ($P < 0.01$). The LIs gradually increased from 1 to 6 h, but no differences were observed among the LIs at 1, 3 and 6 h. Beginning 12 h after the application, the LIs gradually decreased. The LIs at 6 and 12 h, 6 and 24 h, and 3 and 24 h were significantly different ($P < 0.01$). No significant difference was observed between the LI of the control and that at 48 h (2a). High-power views of the organ of Corti (OC) and the spiral ganglion revealed that many spots consisting of TAT-myc-FNK were localized within the cells outside their nuclei (Figures 1b and c). The basal turn tended to show higher immunoreactivity than the upper turns, but there was no statistically significant difference between the cochlear turns at 1 and 6 h. Two-way ANOVA was conducted to examine the cochlear turns and the time course. There were no interactions between the two factors ($F_{2,174} = 0.051$, $P = 0.950$). There was also no statistical difference in the main effect of the cochlear turns ($F_{2,174} = 1.033$, $P = 0.358$; Figure 2b). In addition to the OC, the spiral ganglion cells (SGCs), the stria vascularis (SV) and spiral ligament (SL) also appeared to show greater immunoreactivity than the control 6 h after the application of TAT-myc-FNK onto the RWM. Because the background immunoreactivity in the control sections varied between the organs, the normalized LIs, that is, the ratio of the LIs in each organ to those of the control, of these organs were compared by one-way ANOVA. There was a statistically significant difference between the groups ($F_{3,116} = 39.257$, $P < 0.01$). Scheffe's *post hoc* test revealed that immunoreactivity was strongest in the cells in the OC, followed by that in the SGCs. The normalized LI of the OC was significantly greater than that of the SGCs, the SV and the SL ($P < 0.01$). The normalized LI of the SGCs was also significantly higher than that of the SV and the SL ($P < 0.01$; Figure 2c). Specific immunoreactivity to myc was not observed in any control ears that were administered only myc-FNK (that is, without TAT) or in the ears of animals administered TAT-myc-FNK in the contralateral ear (Figure 1d).

Protective effects of TAT-FNK against ABR threshold shifts induced by ototoxic insults

The baseline ABR thresholds measured before ototoxic insult were statistically not different at all tested frequencies among animals (data not shown). Neither experimental nor drug control animals showed any signs of systemic illness, such as diarrhea or hair loss, until euthanasia. For drug control animals, a gelatin sponge soaked with TAT-FNK was placed on the RWM, but a combination of KM and EA was not given. These animals showed no ABR threshold shifts at any tested frequency (data not shown), indicating that topical application of TAT-FNK on the RWM is not harmful to cochlear function.

ABR threshold shifts 14 days after the ototoxic insult in the experimental animals are shown in Figure 3 ($n = 8$ each). Two-way ANOVA was conducted to examine the effect of the TAT-FNK administration and the frequency on the ABR threshold shifts. There was no significant interaction between TAT-FNK administration and hearing frequency ($F_{2,42} = 0.042$, $P = 0.959$). There was a main effect of TAT-FNK administration ($F_{1,42} = 27.355$, $P < 0.01$) but no significant difference in frequency ($F_{2,42} = 0.833$, $P = 442$),

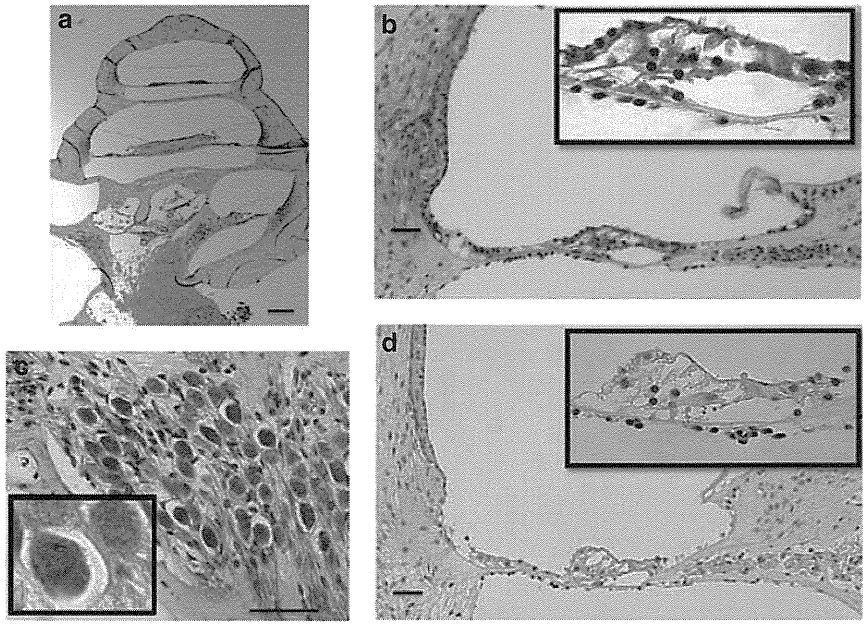

Figure 1. Transduction of TAT-myc-FNK protein into guinea pig cochlea. (**a**–**c**) The anti-myc-tag antibody was used to stain TAT-myc-FNK. Entire cochlea (**a**). An enlarged image of the OC (**b**) and the spiral ganglion (**c**). The insets in panels **b** and **c** are high-power views of the cells in the OC and SGCs, respectively. (**d**) An image of the contralateral ear, with a high-power view of OC in the insets. Scale bar: 200 μm, panel **a**; 40 μm, **b**–**d**.

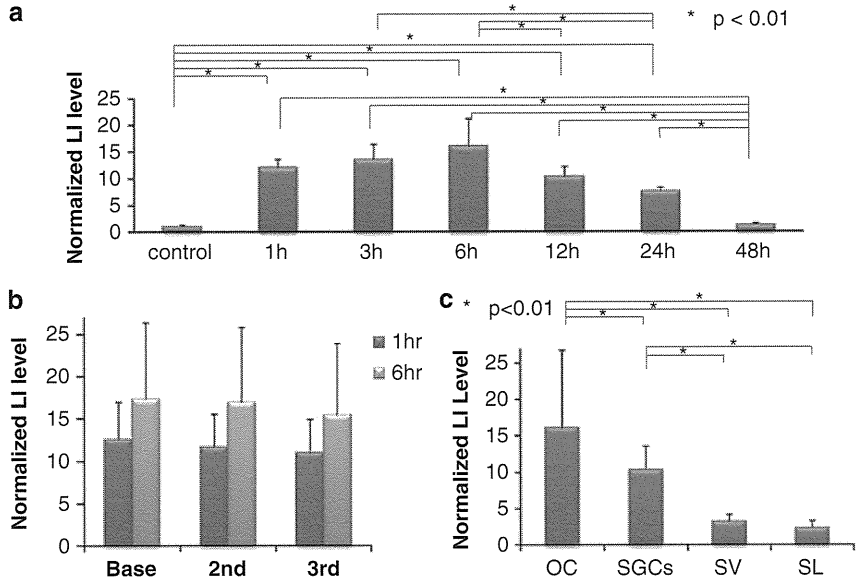

Figure 2. LIs for TAT-myc-FNK immunostaining of the inner ear. The time course of the normalized LIs level for immunostaining of the OC is shown (**a**). Error bar: s.d. $*P<0.01$. (**b**) Normalized LIs for each turns at 1 and 6 h after application of TAT-myc-TNK. (**c**) Normalized LIs at 6 h for OC, SGCs, the SV and the SL. Error bar: mean (s.d.). $*P<0.01$.

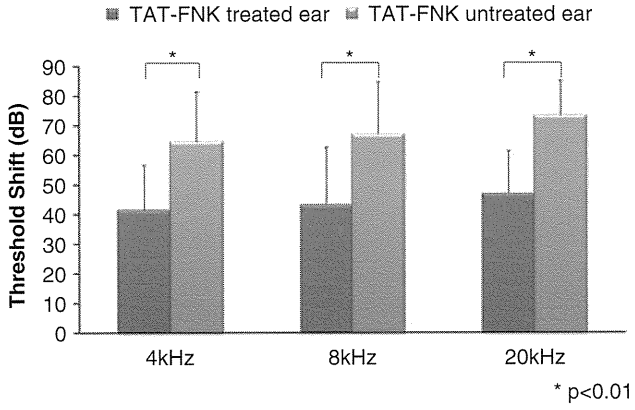

Figure 3. ABR threshold shifts at each tested frequency in both TAT-FNK treated and untreated ears. Pure tone 4, 8 and 20-kHz ABR threshold shifts before ototoxic insult and 14 days after the insult are shown. The dark gray bars indicate the values for the TAT-FNK-treated ears. The light gray bars indicate the values for the TAT-FNK-untreated ears. Error bar: s.d. $*P<0.01$.

indicating that the ABR threshold shifts were significantly smaller at all the tested frequencies in the TAT-FNK-treated ears than in the untreated contralateral ears. This result suggests that the TAT-FNK treatment significantly attenuated the ABR threshold shifts induced by the ototoxic agents.

HC protective effects of TAT-FNK *in vivo*

Figures 4a and b show the average cytocochleograms in the TAT-FNK-treated and contralateral untreated ears, respectively, which were produced by plotting the average percentage of HC loss in every segment between 5 and 16 mm from the apex that was averaged across all subjects ($n=6$ each). Segments measuring under 5 mm and over 16 mm were excluded because the extent of HC damage could not be quantified owing to damage in some samples during surface preparation. The frequency map was added in the *x*-axis according to the data of Tsuji and Liberman.[18]

The ototoxic agents induced losses of $91.7\pm7.0\%$ of the outer HCs (OHCs) and $13.8\pm5.9\%$ of the inner HCs (IHCs) in the TAT-FNK-untreated ears, whereas the losses of the OHCs and the IHCs in the treated ears were reduced to $64.0\pm29.6\%$ and $8.3\pm3.5\%$, respectively (Figure 4c). Two-way ANOVA was conducted to examine the effect of the TAT-FNK administration and the type of HC on HC loss. There was no significant interaction between TAT-FNK administration and type of HC ($F_{1,20}=3.055$, $P=0.096$). There were main effects of TAT-FNK administration ($F_{1,20}=6.869$, $P=0.016$) and type of HCs ($F_{1,20}=110.657$, $P<0.01$), indicating that the TAT-FNK treatment significantly attenuated the HC damage induced by KM and EA. Drug control animals administered only TAT-FNK showed minimal HC loss throughout the cochlea.

In vitro effect of TAT-FNK on protection of HCs and caspase-9 activation

Figure 5c shows an intact, untreated cochlear explant that was double-labeled with rhodamine-conjugated phalloidin (red) and activated caspase-9 (green). The stereocilia bundles on the three rows of OHCs and one row of IHCs have normal morphology and negligible green staining. Figure 5a shows a cochlear explant treated with KM for 10 h. HCs are missing and caspase-9 labeling is present in the HC regions. These results indicate that KM treatment caused an increase in caspase-9 activation, leading to apoptosis of the HCs by a mitochondria-mediated pathway. Addition of TAT-FNK to the explants greatly suppressed caspase-9 activation (Figure 5b). The number of HCs with activated caspase-9 in the explants treated only with KM was 20.6 ± 5.2 per 0.2-mm length, whereas the number was reduced to 7.6 ± 3.2 per 0.2-mm length in the explants treated with KM and TAT-FNK (Figure 5d, $n=4$ each). The number of HCs with activated caspase-9 in explants treated with KM and FNK (without TAT) was 16.7 ± 3.2. There was a statistically significant difference between the groups as determined by one-way ANOVA ($F_{3,12}=31.337$, $P<0.01$). Scheffe's *post hoc* test revealed that there were significant differences between the KM with TAT-FNK-treated explants, and the KM-treated explants or the KM with FNK-treated explants ($P<0.01$). There was no statistically significant difference between the KM-treated explants and the KM with FNK-treated explants

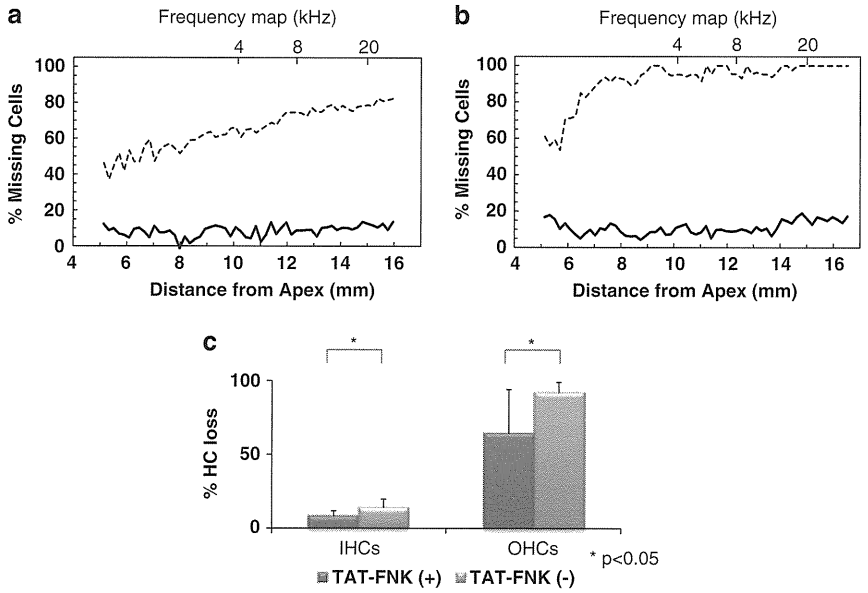

Figure 4. Average cytocochleograms and average missing HCs for each experimental group 2 weeks after exposure to EA and KM. TAT-FNK-untreated ear (**a**). TAT-FNK-treated ear (**b**). The solid line represents the percentage of missing IHCs and the dashed line represents the percentages of missing OHCs. (**c**) The mean number of missing IHCs and OHCs from 5 to 16 mm. Error bar: s.d. $^{*}P<0.05$.

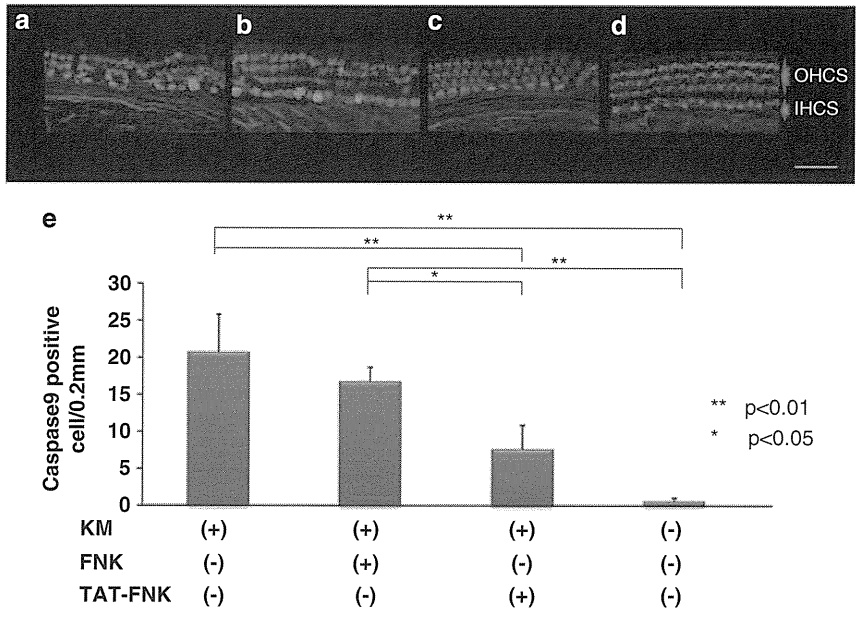

Figure 5. Fluorescence micrographs of caspase-9 activation and HC morphology. Rhodamine phalloidin (red) was used to stain the cell morphology and the fluorescent caspase substrate fam-LEHD-fmk (green) was used to stain caspase-9. Scale bar = 30 μm. (**a**) TAT-FNK-untreated group. (**b**) FNK-treated group. (**c**) TAT-FNK-treated group. (**d**) Control group. (**e**) Mean number of caspase-9-positive HCs (IHCs + OHCs) present in 0.2-mm length of the cochlea. Error bar: s.d. $^{**}P<0.01$; $^{*}P<0.05$.

($P=0.429$). Therefore, the TAT-FNK treatment significantly reduced the number of HCs entering the caspase-9-dependent apoptotic pathway after KM application.

We counted the number of viable HCs ($n=6$ each) after 12 h of culture. In the control that was not administered any additional agent such as KM, FNK or TAT-FNK, no or only few HCs were lost. When the number of viable HCs ($n=6$ each) was counted after 12 h of culture with KM (that is, in the absence of TAT-FNK), massive losses of the OHCs and the IHCs were induced, as only 25.2 ± 9.1% and 28.5 ± 11.9% of the cells survived, respectively. The TAT-FNK treatment attenuated OHC and IHC damages, as 80.1 ± 14.9% and 74.1 ± 20.6%, respectively, of the cells remained. In the explants treated with KM with FNK, the extent of survival was 27.6 ± 5.9% for the OHCs and 38.3 ± 15.9% for the IHCs. Two-way ANOVA was conducted to examine the effect of the drug administration and the type of HC on HC loss. There was a main effect of drug administration ($F_{3,40}=99.432$, $P<0.01$). There was also a main effect of type of HC ($F_{1,40}=419.899$, $P<0.01$). Finally, there was interaction between drug administration and type of HC ($F_{3,40}=47.846$, $P<0.01$). The simple effects analysis revealed significant differences between the KM with TAT-FNK-treated explants, and the KM-treated explants or the KM with FNK-treated

explants ($P<0.01$), in the OHCs. There was no significant difference between the KM-treated explants and the KM with FNK-treated explants. However, in the IHCs, there were no significant differences between the KM with TAT-FNK-treated explants, and the KM-treated explants or the KM with FNK-treated explants. These results indicate that the TAT-FNK treatment significantly attenuated damage to the OHCs; however, FNK alone did not protect the HCs against KM (Figures 6c and d). Drug control animals administered only TAT-FNK showed minor HC loss.

DISCUSSION

In the present study, we demonstrated that the TAT-fusion technique enabled the macromolecule FNK protein, which was infiltrated into a gelatin sponge and placed on the RWM, to successfully enter the cochlea because it allowed the protein to penetrate through the RWM. TAT-myc-FNK was distributed throughout all turns of the cochlea, but immunoreactivity was not observed in the contralateral ears, suggesting that, when topically applied, the distribution of TAT-FNK may be confined to the applied cochlea.

The LIs of TAT-myc-FNK gradually increased until 6 h, but there were no significant differences in the LIs at 1, 3 and 6 h. Beginning at 12 h, the LIs gradually decreased. At 48 h, the immunoreactivity disappeared. This suggests that TAT-myc-FNK was immediately distributed into the cochlea 1 h after administration onto the RWM and remained in high concentration until 6 h. It gradually decreased beginning at 12 h and disappeared by 48 h. When examining the entire cochlea at 6 h after administration of TAT-myc-FNK, the strongest immunoreactivity was present in the cytoplasm of the supporting cells and the HCs in the OC, followed by the SGCs. Immunoreactivity could also be observed in the SV and SL. This suggests that xTAT-myc-FNK was distributed most prominently in the OC followed by SGCs, and that it also reached the SV and SL at 6 h after administration on the RWM. The duration of FNK expression was much longer compared with when it was administered systemically.[13] A single topical administration of TAT-FNK on the RWM effectively protected cochlear HCs from the combination of KM and EA *in vivo*. These findings imply that, when fused with TAT and soaked in a gelatin sponge macromolecular proteins can be applied on the RWM as an effective and selective therapeutic agent to function in the cochlea. Considering the adverse effects introduced by systemic injection, this technology is feasible as a novel treatment for inner ear disorders. TAT-FNK attenuated KM-induced HC death by suppressing the activation of pro-caspase-9 *in vitro*, suggesting that the antiapoptotic protein FNK has the potential to regulate the mitochondria-related apoptotic pathway in the inner ear.

The RWM is a main gate and barrier for various kinds of substances to enter from the middle ear into the inner ear.[19] The membrane consists of three layers: an outer epithelium facing the middle ear, a core of connective tissue and an inner epithelium facing the inner ear.[20-22,16-18] The structure of the outer epithelium is such that substances can pass from the middle to the inner ear by selective absorption and secretion.[23] The factors influencing permeability through the RWM include the molecular weight and configuration of the protein, its contact time and the concentration of the substances in the middle ear.[23-25] Among these, molecular weight is the most important in determining permeability. Generally, low-molecular-weight compounds, such as antibiotics, corticosteroids and labeled ions, can easily pass through the RWM to enter the inner ear,[26,27] whereas penetration of high-molecular-weight substances, such as proteins and lipids, is limited.[19,27-30] In the current study, myc-FNK, whose molecular weight is about 30 kDa, did not pass through the RWM, suggesting that it is too large to pass through. Many proteins related to apoptosis, such as p53, AKT and super oxide dismutase, have a molecular weight of 30–60 kDa. Thus, when considering the

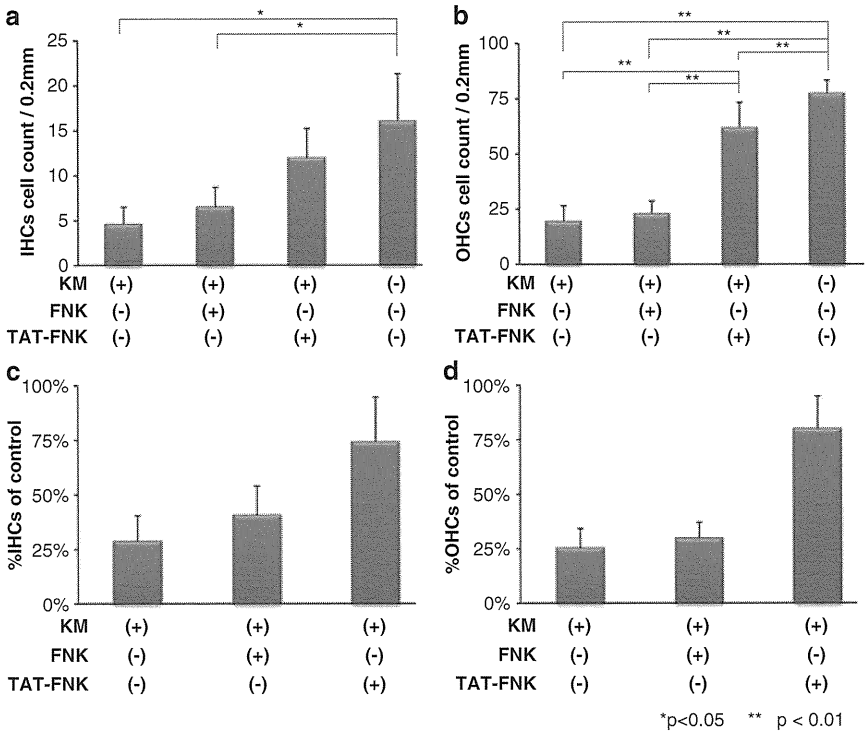

Figure 6. Mean number of surviving HCs of cultured OC after exposure to KM. The mean number of IHCs (**a**) and OHCs (**b**) present in a 0.2-mm length of cochlea taken from four independent parts of the middle cochlear section. Mean percentage of IHCs (**c**) and OHCs (**d**) relative to control cultures. Error bar: s.d. $^{**}P<0.01$; $^{*}P<0.05$.