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1. Introduction

Apoptosis is a biological process of immense importance and is physiologically essential for normal development and tissue homeostasis in adults to regulate the balance between proliferation and cell death; therefore, dysregulation of apoptosis can lead to disease. Apoptosis is known or suspected to contribute to acute diseases, such as stroke, heart attack and liver failure, through inducing massive cell death in tissues or organs, and to certain slow-progressing diseases, such as Parkinson's disease. As apoptosis machinery is equipped in almost all cells of our body, it is an attractive target of therapeutic intervention. It is known that a wide range of stimuli, regardless of intra-cellular or extra-cellular stimuli, can induce apoptosis, which is regulated in different ways from cell to cell, and is carried out in a stimulus- and cell type-dependent manner. The common components of the apoptosis mechanism are finally activated in the execution of apoptosis.

For successful systemic drug delivery, it is important to pass through difficult barriers, such as the cellular membrane or specialized cellular barriers, for example, the blood–brain barrier. After several observations that HIV-1 Tat protein [1,2] and homeodomain protein Antennapedia [3,4] can enter cells, many peptide sequences, the protein transduction domain (PTD) or cell-penetrating peptide (CPP), have been identified to be responsible for membrane translocation. The finding that systemically administered TAT-fused β-galactosidase is delivered into the brain [5] has encouraged studies in delivering therapeutic proteins/peptides. This article is intended to review studies on systemic delivery of anti-cell death proteins/peptides intervening in apoptosis

regulation and therapeutic enzymes serving to replace the function of inactive enzymes, to mitigate various diseases in animal models.

2. Regulation of apoptosis; molecular mechanisms and components

2.1. Apoptosis signaling pathways

Various stimuli from outside or inside of a cell can induce the cell to undergo apoptosis through extrinsic or intrinsic apoptosis pathways, respectively. Extrinsic death signals, such as Fas ligand and tumor necrosis factor α, are mediated by ligation of their cognate cell-surface receptors. On the other hand, intrinsic death signals, such as DNA damage, oxidative stress, treatment with cytotoxic drugs, deficiency of survival factors, and contradictory cell cycle signaling, are communicated through mitochondria. It is stressed that death signals of different origin finally lead to a common proteolytic execution, activation of caspases.

In extrinsic apoptosis pathways, ligation of pro-death cytokines to their cognate receptors promotes the formation of the death-inducing signaling complex (DISC) consisting of the death receptors, adaptor proteins (such as FADD, TRADD, and Daxx), and initiator caspases, procaspase-8 and -10, resulting in activation of initiator caspases [6]. Activated caspase-8 leads to the activation of effector caspases including procaspase-3. Meanwhile, intrinsic apoptosis pathways involve procaspase-9 as an initiator caspase. Cytochrome *c* (cyt. *c*) released from mitochondria to the cytosol forms the apoptosome by interacting with Apaf-1 in the presence of dATP or ATP, where the apoptosome recruits and activates procaspase-9 [7]. Activated caspase-9 results in activation of effector caspases, including procaspase-3.

There is crosstalk between extrinsic and intrinsic apoptosis pathways [8]. In some cells, activation of caspase-8 by DISC is not sufficiently strong to directly activate effector caspases. In this case, a Bcl-2 family member, Bid, mediates the death signal from DISC to mitochondria. Activated caspase-8 cleaves Bid to generate its truncated form, tBid, which in turn translocates to mitochondria to activate the intrinsic apoptosis pathway through the release of cyt. *c*.

The mitogen-activated protein kinase (MAPK) cascade has also been reported to participate in apoptosis regulation [9,10]. Activation of signal-regulating kinase (ASK1) initiates the MAPK cascade, leading to activation of stress kinases, c-Jun N-terminal kinase (JNK) and p38 MAPK [11]. ASK1 is one of the MAPKKKs and is ubiquitously expressed in cells. Various stimuli, including ROS, endoplasmic reticulum stress, TNFα, lipopolysaccharide, and Ca^{2+} influx, can activate ASK1. The mechanisms by which activation of JNK and p38 MAPK lead to apoptosis are poorly understood. It is implied that activated stress kinases may, directly [12] or indirectly [13], affect mitochondria to promote the mitochondria-dependent death pathway.

2.2. Mitochondria

2.2.1. Mitochondrial apoptogenic factors

Mitochondria are currently attracting much attention as the etiology of a wide range of human diseases with accompanying unwanted cell death, such as heart failure, diabetes, obesity, stroke, neurodegenerative diseases, and aging, in which disturbances of mitochondrial Ca^{2+} homeostasis, energy metabolism and/or redox metabolism are commonly observed, leading to apoptosis [14]. Mitochondria can give rise to lethal signals for apoptosis and may further switch between the apoptotic and necrotic cell death pathways [15]. It is now widely realized that the mitochondrion is the powerhouse of disease [16]; it is, therefore, important to develop therapies to intervene in the mitochondrial regulation of apoptosis to prevent unwanted cell death.

Mitochondria are double-membrane organelles, where most cellular respiration takes place through the electron transport system to produce ATP. It is a striking finding that cyt. *c*, a component of the electron transport system, can induce apoptosis when released from mitochondria into the cytosol [17]. Thereafter, other apoptogenic factors, Smac/DIABLO, Omi/HTRA2, apoptosis-inducing factor (AIF), EndoG, and Arts were found to be sequestered in mitochondria [18,19]. Smac/DIABLO and Omi/HTRA2 can interact with endogenous inhibitor of apoptosis proteins (IAPs) to prevent IAPs from inhibiting activated caspases. In addition, Omi/HTRA2 seems to be a serine protease, whose function is independent of caspases. AIF shares similarity with bacterial, plant, and fungal oxidoreductases. In response to apoptotic stimuli, AIF translocates to the nucleus and participates in peripheral chromatin condensation and high-molecular weight (50kb) DNA fragmentation in a caspase-independent manner, although AIF has no intrinsic nuclease activity. The oxidoreductase activity of AIF is not involved in its apoptogenic function. EndoG is a non-specific nuclease. During apoptosis, EndoG also translocates to the nucleus to degrade nuclear DNA in a caspase-independent manner. Arts is a septin-like mitochondrial protein and was recently shown to bind an inhibitor of apoptosis protein, XIAP, like Smac/DIABLO and Omi/HTRA2, when released to cytosol [20].

2.2.2. Ca^{2+} and mitochondrial permeability transition

Mitochondria are important for intra-cellular Ca^{2+} homeostasis as well as energy production and cell death. It is suggested that the physiological significance of mitochondrial Ca^{2+} uptake is to control the rate of oxidative phosphorylation, to modulate cytosolic Ca^{2+} transients, and to induce the mitochondrial permeability transition (MPT) [21]. MPT is a sudden increase in inner membrane permeability to solutes of molecular mass less than around 1500Da, resulting in mitochondrial swelling, loss of mitochondrial membrane potential ($\Delta\Psi m$), and Ca^{2+} release. It is generally assumed that MPT is due to the opening of a putative channel(s), which is referred to as the mitochondrial permeability transition pore (PT pore). The exact molecular composition of the PT pore has not been completely elucidated, although there is general agreement that the PT pore is a supramolecular complex localized at contact sites of the inner and outer membranes, and that the core of the complex consists of the voltage-dependent anion channel (VDAC) (a major protein of the mitochondrial outer membrane, also called porin), the adenine nucleotide translocator (ANT) (inner membrane), and the *cis–trans* peptidyl-prolyl isomerase Cyclophilin D (CypD) (matrix) [22]. Other mitochondrial proteins, such as VDAC-associated hexokinase (cytoplasm),

the peripheral benzodiazepine receptor (outer membrane), and creatine kinase (inter-membrane space), seem to participate in the complex [22]. Dysregulation of the opening and closing of PT pores is attracting renewed interest, because many studies have proposed that apoptogenic factors are released from mitochondria via PT pores in apoptotic conditions [23], although other mechanisms, such as mitochondrial apoptosis-inducing channel (MAC) and Bax/Bak-mediated channel, are also proposed [24,25]. In normal physiological conditions, the PT pore is regulated by adenine nucleotide pools, matrix pH, $\Delta\Psi m$ and the redox state. In some pathological conditions, including Ca^{2+} overload [26,27], oxidative stress such as reactive oxygen species (ROS) [26,28], chemotherapy [29] and ischemia/reperfusion injury [30,31], the PT pore is forced to open, releasing apoptogenic factors [18,23]. It is predicted that prolonged opening of the PT pore can cause ATP depletion. Marked loss of ATP leads to necrosis instead of apoptosis, because apoptosis depends on energy [15,32]. Very recently, mammalian VDACs have been reported to be dispensable for cell death by both MPT and pro-apoptotic Bcl-2 family members that will be mentioned below [33]. Mammals have 3 *Vdac* genes. The authors concluded that none of the three Vdac isoforms disrupts MPT function and provides no protection from necrotic or apoptotic cell death, suggesting that alternative proteins and/or mechanisms must be involved in mitochondrial-dependent cell death through PT pores [33].

2.3. Bcl-2 family

2.3.1. Heterodimerization of anti-apoptotic and pro-apoptotic family members

The Bcl-2 protein family plays a central role in apoptotic regulation. The family is structurally defined by the presence of conserved regions known as four Bcl-2 homology domains, BH1 to BH4, and consists of more than 25 family members [34]. Although they share homology domains, the family is functionally divided into two groups. Anti-apoptotic members, such as Bcl-2 and Bcl-x_L, inhibit apoptosis, whereas pro-apoptotic members, such as Bax, Bak and Bid, accelerate or induce apoptosis. Interestingly, they can interact with each other to form homodimers and heterodimers, via the BH domains [34]. The formation of a heterodimer between anti-apoptotic and pro-apoptotic members of the family is expected to abolish their partners' activity, which causes difficulty in evaluating the original functions of family members. The ratio between anti-apoptotic and pro-apoptotic members of the Bcl-2 family is suggested to determine survival or death following an apoptotic stimulus [35].

2.3.2. Bcl-2 and Bcl-x_L

Bcl-2 resides in mitochondria, endoplasmic reticulum (ER) and the nuclear envelop [36]. Bcl-x_L is localized in mitochondria as well as the nuclear envelope and cytosol [37,38]. It is worth mentioning that Bcl-2 and Bcl-x_L can protect cells from necrosis as well as apoptosis [39–41]. Bcl-2 and Bcl-x_L inhibit $\Delta\Psi m$ disruption induced by various apoptotic or necrotic death stimuli, including anti-cancer drugs, respiration inhibitors and calcium ionophore [42,43]. In addition, Bcl-2 and Bcl-x_L prevent the release of apoptogenic factors, cyt. *c* [17,44], Smac/DIABLO [45], Omi/HTRA2 [46], AIF [47], and EndoG [48] from mitochondria. Bcl-2 family members seem to regulate the PT pore by interacting with VDAC, whereas Bcl-x_L closes VDAC, and the pro-apoptotic members, Bax and Bak, accelerate the opening of VDAC [49]. It is proposed that the BH4 domain of Bcl-2 and Bcl-x_L is essential to inhibit VDAC activity and apoptotic $\Delta\Psi m$ loss, although the BH4 domain is not important for binding [50]. The binding region of Bcl-x_L seems to include helices 4 to 7, but not the BH4 domain [51]. A recent report that VDAC is not essential to induce MPT and release of cyt. *c* from mitochondria [33] may force reconsideration of the physiological and pathological significance of the interaction between VDAC and Bcl-2 family members.

2.3.3. Three-dimensional structure of Bcl-x_L and ion channel activity

The three-dimensional structure of Bcl-x_L was first revealed by X-ray crystallography and NMR spectroscopy [52,53]. The striking feature of the tertiary structure of the protein is that Bcl-x_L resembles the membrane insertion (that is, pore- or channel-forming) domain of bacterial toxins, diphtheria toxin, insecticidal δ-endotoxin, and colicins A, E1 and Ia [52], although there is no significant sequence similarity among them. By analogy, it was suggested that Bcl-2 family proteins may form pores [52]. This hypothesis was proven by subsequent studies; Bcl-x_L, Bcl-2, Bax and Bid can form ion-conducting channels in synthetic lipid membranes [54,55]. The tertiary structure of Bcl-x_L determined with a higher resolution revealed that the putative pore-forming domain, helix α5-loop-helix α6, is stabilized by nine intramolecular hydrogen bonds [53]. Although several mutagenesis experiments have been performed to clarify the relationship between channel activity and the apoptosis-regulating function [56–59], it is still unclear how the channel activity detected *in vitro* is associated with apoptosis regulation *in vivo*. It is also unknown how the channel activity is associated with their opposing functions in apoptosis regulation.

An unexpected role of Bcl-2 and Bcl-x_L has emerged from current studies. Bcl-2 and Bcl-x_L can interact with inositol 1, 4, 5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) [60]. $InsP_3Rs$ are ligand-gated calcium channels of the ER, where $InsP_3$ induces the channels to open, resulting in the release of calcium from the ER lumen into the cytosol. Although there are conflicting results regarding the effect of Bcl-2 proteins on $InsP_3R$-mediated calcium release [60,61], it seems that Bcl-2 proteins control ER calcium dynamics. As ER is located upstream of mitochondria in intracellular calcium signaling pathways, it is important to understand Bcl-2-associated ER calcium regulation and to reveal how this regulation is linked to mitochondrial calcium loading and/or associated with apoptosis regulation.

3. Protein transduction

In 1999, Schwarze et al. showed that large proteins (up to 120kDa) are easily delivered into cells of most tissues, when fused with the TAT peptide. TAT peptide consists of 11 amino acid residues, which is the protein transduction domain (PTD) of HIV Tat protein [5]. A great feature of TAT is to deliver cargo proteins

into the brain [5]. The blood–brain barrier blocks all molecules except those that cross cell membranes by means of lipid solubility and those that are allowed in by specific transport systems. This has caused many difficulties in brain drug delivery. Recently, we have shown that TAT can cross another barrier, the blood–labyrinth (inner ear) barrier, which appears to be less tight than the blood–brain barrier [62].

Until now, many PTDs have been identified from viral (such as HIV/Tat and HSV-VP-22) and cellular proteins (such as Antennapedia, histones and Kaposi fibroblast growth factor), bacterial toxins (such as diphtheria toxin and anthrax toxin) or antibacterial peptides (such as protegrins) [63–66]. PTD peptides are also referred to as cell-penetrating peptides (CPPs). Based on the cationic feature of TAT, synthetic PTDs such as $(Arg)_9$ oligomer have been designed [63–66]. Numerous proteins have been successfully introduced into cells using various PTD peptides [63]. It is strongly suggested that endocytosis is involved in the cellular uptake of PTD-fused proteins, while some studies support that PTD-fused proteins directly translocate across cellular membranes [63–66]. Characterization of the intra-cellular mechanism of the delivery of protein conjugates with TAT has shown that uptake occurs through lipid raft-dependent endocytosis involving either macropinocytosis or a caveolae pathway [65], although a full-length Tat protein is reported to enter T cells essentially like diphtheria toxin, using clathrin-mediated mechanisms [67]. Cells possibly exhibit distinct uptake mechanisms for different PTDs [66]. Internalization of PTDs is not dependent on specific cell-surface receptors, indicating that PTD cargo can be introduced into any type of cell. In addition, denaturation by urea enhances the ability of TAT-fusion proteins to transduce into cells [68], whereas this denaturation step causes difficulties in TAT-mediated transduction of metal-containing enzymes, such as Cu/Zn superoxide dismutase, as discussed in a later section. Cell-surface heparan sulfate proteoglycans likely participate in the internalization of cation-rich PTDs such as TAT and (Arg)-oligomer [63]. Interestingly, it has been reported that the chirality of cationic PTDs is not required for internalization, where D-isomers of TAT and (Arg)-oligomer effectively enter cells [63]. In addition, the mechanisms of endosomal release of transduced proteins into the cytosol remain to be addressed. Fischer et al. recently suggested that cationic PTDs use the retrograde transport system, where they indicated a sequence similarity of Tat and Antp peptides to a highly conserved arginine-rich motif of 8–10 amino acids in the A sub-units of several toxins, including Shiga toxin, which are reported to be transported by means of retrograde transport [69].

These PTDs have been used to deliver biologically active proteins/peptides to intervene in cell death signaling pathways. The target sites and therapeutic proteins/peptides to treat various animal disease models, which will be discussed in the following sections, are described in Fig. 1 and summarized in Table 1.

4. Delivery of anti-cell death proteins and therapeutic enzymes

4.1. Anti-apoptotic Bcl-x_L protein

4.1.1. Ischemia/reperfusion injury of the brain

Bcl-x_L and its derivative, FNK, both of which consist of 233 amino acid residues, were the first proteins to be delivered as therapy for neurodegenerative diseases, including brain ischemic injury, using experimental animal models; FNK is described in a later section. Bcl-x_L, unlike Bcl-2, has been shown to be expressed in both embryonic and adult neurons,

Fig. 1. Schematic illustration of key elements and therapeutic targets involved in cell death signaling pathways described in the text.

Table 1
Summary of the therapeutic proteins/peptides used in disease animal models

Cargo	Biological activity	PTD	Animal model	Injection	Effect	Reference
Full-length proteins						
Bcl-x_L	Anti-apoptotic protein	TAT	Brain ischemia	Intra-venous injection	Decrease in infarction, the number of caspase-3-reactive and DNA-fragmented cells	71
			Brain ischemia	Intra-peritoneal injection	Decrease in infarction	72
			Brain ischemia	Intra-peritoneal injection	Decrease in cerebral tissue loss, and inhibition of AIF and activation of caspases	73
			Optic nerve axotomy	Intra-vitreal injection	Protection of retinal ganglion cells and cerebellar granule neurons	74
			Autoimmune encephalomyelitis	Intra-vitreal injection	Protection of retinal ganglion cells	75
			Islet transplantation	*In vitro* transduction	No adverse effect on insulin secretion	76
			Islet	*In vitro* transduction	Protection against IL-1β toxicity and decrease in spontaneous apoptosis	115
FNK	Genetically engineered Bcl-x_L (a gain-of-function phenotype) Caspase inhibitor	TAT	Brain ischemia	Intra-peritoneal injection	Protection against delayed neuronal cell death	77
			Liver ischemia	Intra-peritoneal injection	Protection of hepatic cells from apoptosis	78
			Isolated heart ischemia	Intra-myocardial injection	Decrease in myocardial infarction, and improvement in recovery of myocardial function	79
			Liver injuries induced by CCl_4, ethanol and dexamethasone	Intra-peritoneal injection	Protection of the liver from zonal necrosis, and decrease in serum ALT and AST	41
			Hearing loss induced by aminoglycoside	Intra-peritoneal injection	Protection of the sensory hair cells (inner ear) and attenuation of hearing loss	62
			Cartilage	*In vitro* transduction	Protection of chondrocytes from anti-Fas and NO	82
			Bone marrow transplantation	*In vitro* transduction	Increase in efficiency of donor cells into ischemic region	84
			Cryopreservation	*In vitro* transduction	Protection against cell death caused by freezing and thawing (neurons, cartilage, and bone marrow cells)	88
XIAP	Caspase inhibitor	TAT	Brain ischemia	Topical application	Decrease in infarction and improvement of learning/memory deficits	93
GDNF	Neurotrophic factor	TAT	Brain ischemia	Intra-venous injection	Decrease in infarction and improvement of neurological deficits	95
			Optic nerve axotomy	Intra-vitreous injection	Protection of retinal ganglion cells	96
			Parkinson's disease (MPTP)	Intra-peritoneal injection	No protection of neurons in the substantia nigra pars compacta	97
Tyrosine hydroxylase (TH)	Enzyme involved in dopamine synthesis	TAT	Parkinson's disease (6-OHDA)	Intra-venous injection	Attenuation of apomorphine-induced rotation	99
Cu/Zn-SOD	Anti-oxidant Enzyme	TAT	Streptozotocin-induced diabetes	Intra-peritoneal injection	Protection of pancreatic β cells	101
			Brain ischemia	Intra-peritoneal injection	Prevention of neuronal cell death in the hippocampus	102

		PEP-1	Brain ischemia	Intra-peritoneal injection	Prevention of neuronal cell death in the hippocampus	103
			Parkinson's disease (paraquat)	Intra-peritoneal injection	Decrease in loss of neurons in the substantia nigra	105
Purine nucleoside phosphorylase (PNP)	Enzyme involved in purine degradation	TAT	Immunodeficiency	Intra-peritoneal injection (twice weekly)	Preventing abnormalities in thymus and T lymphocyte number and functions for 24 weeks, even in the presence of antibodies raised against injected TAT-PNP	107
Peptides						
BH4 domain	Derived from anti-apoptotic proteins, Bcl-x_L and Bcl-2	TAT	X-ray irradiation	Intra-peritoneal injection	Decrease in apoptosis of the small intestine	110
					Inhibition of splenic T lymphocytes and B cells	117
			Fulminant hepatitis (Fas)	Intra-peritoneal injection	Suppression of fulminant hepatitis	110
			Isolated heart ischemia	Perfusion	Decrease in activation of caspase-3 and TUNEL-positive cells, and prevention of mitochondrial dysfunction. Improvement of cardiac function.	110, 111
					Decrease in infarction.	112
			Islet	*In vitro* transduction	Protection against IL-β toxicity and decrease in spontaneous apoptosis	115
			Sepsis	Infusion and intra-peritoneal injection	Decrease in lymphocyte apoptosis	116
BIR2 and/or BIR3/RING domains	Derived from XIAP	TAT	Brain ischemia	Intra-venous injection	Decrease in infarction	93
		Antp HD	Brain ischemia	Intra-peritoneal injection	Improvement of neurological deficits	118
			Epilepsy (kainate)	Intra-peritoneal injection	Protection against kainate-induced cell death in the CA3 sub-field	119
c-Jun binding domain (JBD), D, L-forms	Derived from JIP-1/IB1	TAT	Brain ischemia	Intra-ventricular administration or intra-peritoneal injection	Decrease in infarction and improvement of behavioral deficits. Long therapeutic window	123
			Brain injury (malonate)	Intra-striatal injection	Suppression of coagulation necrosis and spongy degeneration	126
			Heart ischemia	Intra-peritoneal injection	Decrease in infarction	127
			Hearing loss induced by aminoglycoside or acoustic trauma	Topical application	Prevention of the hair-cell death (inner ear) and hearing loss	128, 129
			Brain ischemia	Cerebral ventricular injection	Inhibition of JNK3 activation, cyt. *c* release and caspase-3 activation. Increase in the survival of CA1 pyramidal cells.	124
GluR6-9c	Derived from the C-terminus of kainate receptor sub-unit GluR6	TAT	Brain ischemia	Cerebral ventricular injection	Inhibition of neurodegeneration of the hippocampal CA1 region.	134
			Epilepsy (kainate)	Intra-cerebroventricular injection	Decrease in neuronal loss in hippocampal CA1 and CA3 sub-fields.	135
NEMO-binding domain (NBD)	Derived from IKK-β	PTD-5	Islet transplantation	Infusion into the common bile duct	Increase in islet viability during isolation procedure	139

and apparently regulates apoptosis in the adult central nervous system [70], which is one reason to use the protein to treat neurodegenerative diseases.

Kilic et al. showed that intra-venous administration of TAT-Bcl-x_L significantly reduced infarct volume and neurological deficits in the middle cerebral artery occlusion (MCAO) model in mice, when measured at 24 h after reperfusion [71]. TAT-Bcl-x_L was delivered to the brain across the blood–brain barrier in 3 to 4 h to diminish the number of caspase-3-reactive and DNA-fragmented cells caused by the ischemic insult, resulting in an increase in the number of viable neurons in the striatum at 72 h after reperfusion. Using the same model, Cao et al. also showed that intra-peritoneally administered TAT-Bcl-x_L was delivered to the brain within 1 to 2 h and decreased cerebral infarction, when measured at 72 h after ischemia [72]. TAT-Bcl-x_L prevented staurosporine-induced apoptosis of cultured cortical neurons by inhibiting the release of cyt. *c*. Importantly, both studies demonstrated that post-administration of TAT-Bcl-x_L still exhibited neuroprotective activity, suggesting that TAT-Bcl-x_L might be clinically applied to treat strokes. Recently, Yin et al. have reported that intra-peritoneally injection of TAT-Bcl-x_L attenuated neonatal brain damage, including cerebral tissue loss and forelimb use asymmetry, following hypoxic ischemia [73]. They demonstrated that TAT-Bcl-x_L inhibited nuclear translocation of AIF as well as the activation of caspase-3 and -9.

4.1.2. Retrograde neurodegeneration

Bähr's group investigated whether TAT-Bcl-x_L can inhibit retrograde neuronal apoptosis induced by axotomy [74]. TAT-Bcl-x_L prevented low-potassium-induced cell death of cultured cerebellar cells. TAT-Bcl-x_L injected into the vitreous space of the eye was transduced into the retinal ganglion cell (RGC) layer to protect RGCs and cerebellar granule neurons from cell death caused by optic nerve lesion. In the following study, his group demonstrated that intra-vitreal injection of TAT-Bcl-x_L increased RGC survival during acute autoimmune optic neuritis, which reflects neurodegenerative aspects of multiple sclerosis [75]. In the animal model, experimental autoimmune encephalomyelitis was induced by immunization of rats with myelin oligodendrocyte glycoprotein, resulting in inflammation of the optic nerve and consecutive apoptosis of RGCs.

4.1.3. Islets for transplantation

In addition to the application of TAT-Bcl-x_L to neurodegenerative diseases, Embury et al. reported that TAT-Bcl-x_L transduces a pancreatic β-cell line to protect cells from TNF-α-induced cell death [76]. TAT-Bcl-x_L did not affect insulin secretion capabilities of islets after transplantation. In their study, they also showed that TAT-PEA-15 exhibits protection against TNF-α, where PEA-15 is a 15-kDa, death effector domain-containing protein and inhibits DISC formation induced by ligation of the death ligand and its cognate death receptor. Apparently, TAT-Bcl-x_L increased the viability of pancreatic β-cells treated with TNF-α, compared with TAT-PEA-15, although the two proteins inhibited the activation of caspase-3 to the same extent. TAT-Bcl-x_L transduction seems to be useful for islet transplantation.

4.2. FNK, genetically engineered Bcl-x_L with a gain-of-function phenotype

4.2.1. Construction of super anti-apoptotic protein FNK

The refined structure of Bcl-x_L determined by X-ray crystallography enabled us to design a more powerful anti-apoptotic protein by remodeling the structure. As mentioned above, the putative pore-forming domain of Bcl-x_L is stabilized by nine intra-molecular hydrogen bonds [53]. Three amino acid substitutions (Y22F/Q26 N/R165K) to destroy three out of nine hydrogen bonds were expected to make the domain more flexible to be inserted into a membrane and to enhance cytoprotective activity. As expected, the resultant mutant Bcl-x_L, named FNK (originally referred to as Bcl-xFNK), exhibited marked cytoprotective activity against various death stimuli including oxidative stress, a calcium ionophore and heat treatment, compared with Bcl-x_L, when overexpressed in cells [59].

4.2.2. Ischemia/reperfusion injuries of the brain, liver and heart

To evaluate the efficacy of FNK in preventing cell death to reduce injury severity, we generated TAT-FNK by fusion FNK with the TAT of HIV/Tat [77]. In *in vitro* experiments, TAT-FNK was rapidly transduced into neuroblastoma cells to localize to mitochondria within 1 h and decayed with a half-span of approximately 2 h [77]. TAT-FNK protected against glutamate-induced excitotoxicity in primary cultured cortical neurons at concentrations as low as 0.3 pM. The protective effect of TAT-FNK against staurosporine- or glutamate-induced cell death was stronger than that of TAT-Bcl-x_L, where TAT-FNK increased the number of surviving cells 3 to 4-fold, compared with TAT-Bcl-x_L. When TAT-FNK was pre-injected into the abdominal cavity of gerbils, the protein prevented delayed neuronal death in the hippocampus caused by transient global ischemia [77]. Recently, we have shown that TAT-FNK protects hepatic cells and isolated hearts from apoptosis and myocardial infarction caused by ischemia/reperfusion, respectively [78,79]. In hepatic ischemia/reperfusion injury, TAT-FNK (0.3 mg/kg) reduced the caspase-3/caspase-3-like activity by around 80% and the vacuolized area (cytoplasmic degeneration) by half, compared with vehicle treatment, whereas there was no significant difference between the vehicle and TAT-Bcl-x_L (0.3 mg/kg) groups [78].

4.2.3. Liver necrosis induced by carbon tetrachloride

Bcl-x_L and Bcl-2 seem to prevent some forms of necrosis, for example, necrosis caused by hypoxia [39,40]. Carbon tetrachloride (CCl_4) is widely accepted to cause necrosis and is one of the most typical agents for studying the pathogenesis of liver injury. TAT-FNK was shown to be markedly more potent in protecting hepatoma cells from necrotic death induced by CCl_4 than TAT-Bcl-x_L, where the cell death accompanied the appearance of a necrotic fragment of poly(ADP-ribose) polymerase-1 and no activation of caspase-3 [41]. TAT-FNK (3 nM) increased the number of surviving cells by 370%, compared to 130% for TAT-Bcl-x_L (3 nM). PTD-FNK suppressed the CCl_4-

induced decrease in intra-cellular ATP levels and mitochondrial membrane potential that is required for maintaining mitochondrial functions. Systemic administration of TAT-FNK, but not TAT-Bcl-x_L, prevented zonal necrosis and an elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in acute and chronic liver injuries caused by CCl_4 as well as ethanol and dexamethasone [41]. In the acute injury model, TAT-FNK (0.3 mg/kg) reduced serum AST and ALT levels to 30% of those of the vehicle-treatment group, but TAT-Bcl-x_L (0.3 mg/kg) did not induce any significant reduction. TAT-FNK clearly increased the apoptotic population among cells treated with CCl_4, strongly suggesting that TAT-FNK forces the cells in a necrotic pathway into an apoptotic pathway, probably due to its strong activity in preserving mitochondrial functions as mentioned above. This result supports the hypothesis that necrosis and typical apoptosis are two extremes of a spectrum of death programs varying with the strength of the death stimulus [15].

4.2.4. Delivery to the inner ear and the cartilage

Dowdy's group showed that TAT-β-galactosidase can be delivered to the liver, kidney, spleen, lung, heart muscle, bowel, blood cells and brain [5,80]. TAT-FNK was also delivered into the brain [77] and liver [41], when systemically administered into animals; however, two organs/tissues remain unknown. First, can TAT-fusion proteins be delivered into the labyrinth (the inner ear)? The labyrinth is separated from the circulation by the blood–labyrinthine barrier to maintain the ionic characteristics of cochlear fluids and to inhibit drug delivery to the inner ear. Secondly, can TAT-fusion proteins be delivered to chondrocytes in cartilage? Chondrocytes are embedded in the extra-cellular matrix, which is rich in chondroitin sulfate carrying numerous sulfate residues with negative charges. Electrostatic interactions between chondroitin sulfate (negative charge) and the TAT peptide (positive charge) may disturb the delivery of TAT-fusion proteins into chondrocytes.

For the inner ear, we investigated whether TAT-FNK can prevent aminoglycoside ototoxicity. Aminoglycoside antibiotics, such as gentamicins, are highly effective against severe bacterial infections, but cause serious ototoxic and nephrotoxic side effects. Aminoglycoside ototoxicity is induced by damaging the sensory hair cells, in which, following excess generation of ROS, an intrinsic apoptotic pathway is considered as the major pathway for aminoglycoside ototoxicity [81]. Using anti-myc-tag antibody, intra-peritoneally injected TAT-myc-FNK (myc-tagged TAT-FNK) was immunohistochemically shown to be delivered to guinea pig cochlear tissue including the hair cells within 3 h [62]. Systemically administered TAT-FNK significantly attenuated auditory brainstem response threshold shifts and apoptotic cell death of the hair cells induced by an ototoxic combination of an aminoglycoside, kanamycin, and a loop diuretic, ethacrynic acid, which potentiates the ototoxicity of aminoglycosides. This study indicated that TAT-mediated protein transduction technology can be used to deliver therapeutic protein(s) across the blood–labyrinthine barrier into cells in the cochlear organs of Corti and can be an alternative to gene therapy in inner ear research.

For cartilage, we investigated the penetration of TAT-FNK into human articular cartilage slices *in vitro* [82]. After cartilage slices (2 mm width × 7 mm length × full thickness) were incubated with TAT-FNK or TAT-Bcl-x_L in culture medium for 6 h, both proteins were immunohistochemically detected to the same extent in chondrocytes located 200 μm inside from the cartilage surface, indicating that the TAT peptide has the ability to deliver passenger proteins into chondrocytes by penetrating the extra-cellular matrix of cartilage. TAT-FNK was shown to protect chondrocytes from cell death induced by anti-Fas and nitrogen oxide, more than TAT-Bcl-x_L. The amount of chondrocyte cell death in the TAT-FNK group decreased up to one fourth of that in the TAT-Bcl-x_L group. It is well known that cartilage is one of the tissues most sensitive to endotoxin. We paid close attention to removing endotoxin, since TAT-FNK and TAT-Bcl-x_L were overproduced and prepared from *Escherichia coli*. It was noted that the final purified preparations of the fusion proteins contained less than 10 EU/ml endotoxin.

4.2.5. Bone marrow transplantation

Bone marrow transplantation has attracted great interest in regenerative medicine. One of the fundamental issues to be resolved is the significantly low survival rate of injected cells post-transplantation [83]. This massive elimination of donor cells is thought to be caused by multiple factors, where a lack of nutrient supply in the ischemic region contributes to massive loss. We examined whether TAT-FNK improves the transplantation efficiency of bone marrow mononuclear cells (BM-MNC) [84]. When transduced with TAT-FNK *in vitro*, more BM-MNCs survived in the absence of serum and retained the potential to differentiate into endothelial progenitor cells. TAT-FNK was incorporated into almost all BM-MNCs, regardless of CD34-positive or -negative cells. Importantly, when BM-MNCs were transplanted into ischemic areas of a male rat ischemic hindlimb model, cells pre-treated with PTD-FNK were incorporated into blood vessels with higher efficiency than untreated BM-MNCs, indicating that TAT-FNK improves the transplantation efficiency of BM-MNCs into ischemic regions.

4.2.6. Cryopreservation

Cryopreservation technology is important for long-term storage of biological tissues or cells, and provides a great advantage for transplantation. It is generally accepted that intra-cellular ice-formation is lethal and usually solutes, such as dimethyl sulfoxide and hydroxyethyl starch, are added as a cryoprotectant to inhibit the formation and/or growth of ice crystals [85]. Loss of membrane integrity is known to be caused by cellular freezing, but cell death caused by freezing and thawing is not yet well characterized. Recently, it has been shown that apoptosis is involved in the cell death of lymphocytes and CD34-positive cells in cryopreserved cord blood [86,87]. TAT-FNK protected isolated BM-MNCs, as well as chondrocytes in isolated articular cartilage-bone sections and cultured neocortical neurons, from freeze–thaw damage [88]. TAT-FNK allowed CD34-positive cells among BM-MNCs to survive more efficiently from two successive freeze–thaw cycles. TAT-FNK may be used as a new type of cryoprotectant for the cryopreservation of tissue/cells, especially cord blood.

4.3. Caspase inhibitor, X-linked inhibitor of apoptosis protein (XIAP)

4.3.1. The structure of XIAP

XIAP, a 62-kDa protein, is a member of the IAP family. XIAP contains three baculovirus IAP repeat (BIR) domains and a COOH terminal really-interesting-new-gene (RING) zinc-finger motif. During apoptosis, XIAP is cleaved into two fragments, the N-terminal fragment, containing BIR1 and 2 domains (BIR1-2), and the C-terminal fragment, containing BIR3 and RING domains (BIR3-RING). BIR1-2 is a specific inhibitor of caspase-3 and 7, whereas BIR3-RING is specific for caspase-9 [89]. In addition, the RING domain has ubiquitin-protein ligase (E3) activity required for the degradation of caspase-3 [90] and Smac [91], and activation of NF-κB [89]. Viral-mediated overexpression of XIAP exhibited neuroprotection against ischemia [92].

4.3.2. Brain ischemia

Guégan et al. constructed three fusion proteins, where a full length of XIAP, the C-terminal fragment BIR3-RING, and the BIR2 domain were fused with the TAT peptide, and tested their efficacy after permanent distal occlusion of the middle cerebral artery (dMCAO) in mice [93]. PTD(TAT)-XIAP, unlike other TAT-fusion proteins mentioned above, failed to accumulate in brain structures and to reduce infarct volume after intra-venous delivery; however, when applied topically, PTD(TAT)-XIAP properly transduced cortical cells to reduce the infarct volume and functional deficits caused by dMCAO. Shorter constructs efficiently targeted the lesion after intra-venous delivery to reduce infarct volumes. It was noted that PTD(TAT)-BIR3/RING was still efficient when administered 3 h after dMCAO. All fusion proteins retained their caspase inhibitory activity. In addition, PTD(TAT)-XIAP treatment resulted in the activation of NF-κB and AP1 transcription factors involved in neuronal survival. The authors discussed that higher amounts of intra-venous PTD(TAT)-XIAP might have resulted in detectable brain penetration, because both PTD(TAT)-BIR2 and PTD (TAT)-BIR3/RING were able to cross the blood–brain barrier.

4.4. Glial cell-line-derived neurotrophic factor (GDNF)

4.4.1. GDNF as a neuroprotectant

GDNF is a member of the transforming growth factor-β superfamily, and was originally purified as a trophic factor for embryonic midbrain dopamine neurons. As GDNF was later found to protect dopamine neurons in animal models of Parkinson's disease, to support motor neuron survival and axon regeneration after peripheral nerve injury, and to reduce ischemic brain injury, GDNF has attracted attention as a potential therapeutic agent for the treatment of certain neurological diseases [94]. GDNF functions on the cell surface by binding to the cognate receptor to activate intra-cellular signaling pathways; therefore, it is required to deliver GDNF to the cell surface of targeted neurons (not inside the neurons), but GDNF cannot pass through the blood–brain barrier, like other neurotrophic factors.

4.4.2. Brain ischemia

Kilic et al. generated GDNF (around 40 kDa) fused with the TAT peptide [95]. Intra-venously injected TAT-GDNF was delivered to the brain within 3 to 4 h. In a mouse MCAO model, post-injection of TAT-GDNF significantly reduced the number of caspase-3-immunoreactive and DNA-fragmented cells and increased the number of viable neurons in the striatum. Pre-injection of TAT-GDNF greatly reduced the infarct volume and improved neurological deficits, and post-injection of TAT-GDNF did to a much lesser extent. The same group also showed that intra-vitreous injection of TAT-GDNF reduced the number of activated caspase-3-positive cells and increased the number of surviving RGCs after optic nerve axotomy [96].

4.4.3. Parkinson's disease model

TAT-GDNF was recently applied to a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease [97]. Intra-peritoneally injected fusion protein did indeed reach the dopaminergic neurons; however, TAT-GDNF did not provide neuroprotection of dopaminergic neurons, as revealed by immunohistochemistry and counting the number of tyrosine-hydroxylase-immunoreactive neurons in the substantia nigra pars compacta. The authors discussed that the amount that actually reached that target site might have been insufficient to provide neuroprotection and the application of higher systemically given doses might be necessary to achieve therapeutic effects. In this study, mice were injected with 5 nmol of TAT-GDNF and the dose limit was determined by the solubility of TAT-GDNF.

4.5. Tyrosine hydroxylase (TH) and Parkinson's disease model

TH is a monooxygenase with a molecular weight of 55 kDa and a rate-limiting enzyme to catalyze the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) in the biosynthesis of dopamine. Parkinson's disease (PD) is characterized by a progressive loss of dopamine (DA)-producing neurons in the midbrain, which results in a decline of DA innervations present in the forebrain, in particular, the striatum. Oral administration of L-DOPA provides very good symptomatic relief, but causes severe side effects. To provide continuous and stable synthesis of DA in the brain, viral vector-mediated direct gene transfer, including the TH gene, is currently being explored [98].

Wu et al. constructed a fusion protein of TH with TAT to deliver TH into the brain of the unilateral 6-hydroxydopamine (6-OHDA)-lesioned PD model rats [99]. Intra-venously injected TAT-TH was shown to penetrate the striatum and midbrain, peaked at 5 to 8 h and then declined gradually. Importantly, a single injection of TAT-TH significantly attenuated apomorphine-induced rotations during 17 days of observation. It remains unknown how long a single injection of TAT-TH is effective. For the treatment of PD, TAT-TH would need to be injected repeatedly over a long period to provide stable synthesis of DA. The generation of antibodies raised against injected TAT-TH should be taken into consideration, and will be discussed in a later section.

4.6. Cu/Zn-superoxide dismutase (SOD); a native or denatured form for transduction

4.6.1. Anti-oxidant enzyme, SOD

SOD is an important anti-oxidant enzyme, capable of scavenging the ROS superoxide anion. Three isoforms of SOD are known in mammals. Intra-cellular Cu/Zn-containing SOD, in which the copper ion is essential for enzyme activity, is located predominantly in the cytoplasm and nucleus of cells. Mn-containing SOD is found predominantly in the mitochondria. The third isoenzyme of SOD is extra-cellular SOD. ROS are inevitably generated in various normal cellular processes and adequately removed by anti-oxidant enzymes. Accumulation of ROS has been implicated in macromolecular damage, cell death, aging and pathological processes of various diseases.

4.6.2. TAT-SOD; a denatured form with lower activity for delivery to the pancreas and brain

Choi's group constructed a fusion protein of Cu/Zn-SOD (17 kDa) with TAT and enhanced the transduction potential of TAT-SOD by regaining copper ions lost during urea denaturation [100]. The copper ion may be required to make the protein structure more favorable for transduction as well as enzyme activity. They applied TAT-SOD to streptozotocin-induced diabetic mice, since ROS are considered an important mediator in pancreatic β cell destruction, thereby triggering the development of insulin-dependent diabetes mellitus [101]. ROS, nitric oxide-induced cell death, lipid peroxidation, and the DNA fragmentation of insulin-producing cells were found to be significantly reduced by the intra-cellular dismutation activities of SOD, when cells were pre-treated with TAT-SOD. Multiple intra-peritoneal injections of TAT-SOD significantly increased SOD activity in the pancreas to enhance the tolerance of pancreatic β cells to streptozotocin-induced oxidative stress, as revealed by blood glucose levels and the number of insulin-positive cells in the pancreas. In another study, TAT-SOD doubled the SOD activity in the brain and prevented neuronal cell death in the hippocampus caused by transient forebrain ischemia, when intra-peritoneally injected into gerbils [102].

4.6.3. PEP-1-SOD; a native form with higher activity for delivery to the brain

The same group generated another construct, a fusion protein of SOD with a short amphipathic peptide carrier, PEP-1, of 21 residues [103]. PEP-1 was originally developed for efficient delivery of a variety of peptides and proteins into cells without the need for prior chemical covalent coupling or denaturation steps [104]. Pep-1 consists of three domains: a hydrophobic tryptophan-rich motif, required for efficient targeting to the cell membrane and for forming hydrophobic interactions with cargo proteins, a hydrophilic lysine-rich domain derived from the nuclear localization sequence of SV40 large T antigen, required for intra-cellular delivery and solubility of the peptide vector, and a linker domain, separating the two domains. PEP-1-SOD transduced cultured astrocyte cells to significantly increase SOD activity and prevented neuronal cell death in the hippocampus caused by transient forebrain ischemia, when intra-peritoneally injected into gerbils. In the following study [105], systemically administered PEP-1-SOD was shown to be delivered to the brain to increase SOD activity. Multiple injections of PEP-1-SOD greatly prevented the loss of TH-immunoreactive and Nissl-stained neurons in the substantia nigra in a paraquat-induced PD mouse model. Interestingly, PEP-1-SOD also markedly increased the expression of a potent anti-apoptotic intra-cellular chaperone, HSP70, in the substantia nigra. HSP70 is shown to be induced by various stresses, including oxidative stress, and to increase resistance against ROS.

In these studies, native PEP-1-SOD, but not denatured, was prepared and used for transduction. Transduced PEP-1-SOD enzyme activity was three to four times higher than that of TAT-SOD [103]. The authors stressed that PEP-1-SOD does not require refolding after transduction, whereas denatured TAT-SOD is needed for refolding after transduction. The overall mechanism of internalization of PEP-1/cargo complex (not fusion proteins) has not been fully understood; however, it is proposed that the complex interacts with the cell membrane and penetrates the membrane, associated with conformational changes which induce membrane structure perturbation, resulting in release of the complex into the cytoplasm with partial "de-caging" of the cargo [106]. It was demonstrated that "de-caging" occurs rapidly in the cytoplasm, a few minutes after cellular uptake, and that PEP-1 localizes in the nucleus. It is totally unknown whether the internalization mechanism of PEP-1-fused proteins is the same as that of PEP-1/cargo complex. In addition, sub-cellular localization of PEP-1-fused proteins will need to be verified, because the two domains of PEP-1 may disturb the localization of the fused cargo protein into the right sub-cellular compartment to perform its function.

4.7. Purine nucleoside phosphorylase (PNP): delivery and sheltering from neutralization

Toro and Grunebaum reported an interesting study [107]. They injected TAT-PNP into PNP-deficient mice to monitor the enzyme activity of transduced TAT-PNP and antibodies raised against TAT-PNP for a period of 24 weeks.

PNP, like adenosine deaminase, is a ubiquitous enzyme essential for purine degradation and salvage. Defective PNP activity causes severe T cell immunodeficiency. In PNP-deficient mice, deoxyguanosine accumulates in the mitochondria of thymocytes, and is converted to deoxy-dGTP to interfere with DNA synthesis and repair, resulting in T cell depletion by apoptosis [108]. When TAT-PNP was intra-peritoneally injected into $PNP^{-/-}$ mice, TAT-PNP was rapidly delivered into various tissues, including the brain, maintained enzyme activity in the tissues over 8 h and restored uric acid production, which is an end product of purine degradation in mice, over 24 h TAT-PNP was injected twice weekly for 24 weeks. Uric acid production was restored during this period and prevented abnormalities in the thymus and T lymphocyte number and functions, and early death of $PNP^{-/-}$ mice with no apparent side effects, although antibodies to TAT-PNP were detected after 2 weeks and reached maximum levels after 7 to 8 weeks. An interesting issue is how

transduced TAT-PNP still exerted a biological effect in mice which acquired immunity against TAT-PNP. The enzyme activity of TAT-PNP was neutralized with anti-serum *in vitro*, but, importantly, when TAT-PNP was incubated with anti-serum in the presence of PNP-deficient cells, activity was detected within the cells, clearly indicating that the fusion protein entered the cells, despite the presence of neutralizing antibodies. It was noted that the antibodies raised against injected TAT-PNP were directed against PNP but not against TAT. The authors discussed that rapid inter-cellular delivery by TAT partially protected PNP from neutralization. It is also possible that the TAT domain of the TAT-PNP/antibody complex still has the ability to deliver TAT-PNP into cells, since Mie showed that proteins captured by antibodies were delivered into living cells using an antibody/PTD-fused protein A complex [109]. This study threw light on the further use of PTD to treat diseases that require frequent administrations of therapeutic proteins.

5. Delivery of anti-cell death peptides

5.1. BH4 domain of Bcl-2 and Bcl-x_L

5.1.1. X-ray and heart ischemic injury

The BH4 domain consisting of 16 amino acid residues is located in the N-terminal region of Bcl-x_L and is shared by anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-w. Shimizu et al. showed that the BH4 domain is essential for the regulation of MPT to inhibit apoptosis [50]. In this study, HeLa cells were treated with VP-16 (etoposide) to induce apoptosis in the presence or absence of TAT-BH4 (BH4 domain derived from Bcl-x_L) peptide. TAT-BH4 effectively inhibited the apoptosis [50]. In the following study, they showed that pre-treatment of TAT-BH4 for 30 min inhibited X-ray-induced apoptosis of PC12 cells but not tunicamycin-induced apoptosis [110]. They also showed that intra-peritoneal injection of TAT-BH4 peptide greatly inhibited X-ray (20 Gy)-induced apoptosis in the small intestine of mice and partially suppressed Fas-induced fulminant hepatitis [110]. In addition, pre-perfusion of TAT-BH4 markedly suppressed heart failure after ischemia-reperfusion injury in Langendorff-perfused hearts, probably preventing mitochondrial dysfunction [110,111]. Chen et al. also reported that pre-perfusion of TAT-BH4 peptide reduced the infarct size and release of creatine kinase caused by ischemia/reperfusion injury using Langendorff-perfused hearts [112]. In *in vitro* experiments, TAT-BH4 (BH4 domain derived from Bcl-2) protected endothelial cells from apoptosis induced by serum starvation and H_2O_2 by reducing caspase-3 activity [113] and from Aβ-induced apoptosis by activating the Akt/eNOS pathway [114].

5.1.2. Comparison between TAT-BH4 and TAT-Bcl-x_L

Some studies investigated and compared cytoprotective activity of TAT-BH4 and TAT-Bcl-x_L. Klein et al. reported that human and monkey islets cultured with IL-1β in the presence of TAT-BH4 or TAT-Bcl-x_L exhibited normal insulin secretion profiles to glucose challenge, whereas un-transduced islets cultured with IL-1β showed a non-physiological response [115]. TAT-BH4 and TAT-Bcl-x_L inhibited the activation of caspase 6 associated with spontaneous isolated islet apoptosis to the same extent. TAT-BH4 and TAT-Bcl-x_L protected NIT-1 insulinoma cells from cell death induced by staurosporine and serum deprivation, although TAT-BH4 seemed to prevent staurosporine-induced cell death more effectively and, conversely, serum deprivation-induced cell death less effectively. It was suggested that TAT-BH4 and TAT-Bcl-x_L could be an asset in the improvement of islet viability for transplantation [76,115]. Hotchkiss et al. applied TAT-BH4 and TAT-Bcl-x_L to sepsis, in which apoptosis is a key pathogenic mechanism [116]. They showed that TAT-BH4 and TAT-Bcl-x_L prevented *E. coli*-induced human lymphocyte apoptosis *ex vivo* to the same extent and that administration of TAT-BH4 via infusion pumps with an additional intra-peritoneal injection markedly decreased lymphocyte apoptosis in an *in vivo* mouse model of sepsis. Concerns about social safety from nuclear terror attacks, in addition to clinical safety, brought about an investigation of TAT-BH4 and TAT-Bcl-x_L as a radioprotectant. McConnell et al. showed that TAT-BH4 and, seemingly to a lesser extent, TAT-Bcl-x_L, protected isolated human peripheral blood mononuclear cells against apoptosis following exposure to 15 Gy radiation [117]. Importantly, *in vivo* administration of TAT-BH4 after irradiation, as well as before irradiation, inhibited apoptosis of splenic T lymphocytes and B cells [117].

In vitro studies appeal that TAT-BH4 is as potent as TAT-Bcl-x_L in preventing apoptosis, although it was suggested that TAT-BH4, unlike Bcl-2 and Bcl-x_L, only inhibits some types of apoptosis [110]. Further studies are required to examine how effectively TAT-BH4 mitigates pathology using various experimental animal models of disease/injury. TAT-BH4, regardless of whether D- or L-form, is readily synthesized without endotoxin contamination in large quantities by solid-phase peptide synthesis.

5.2. Caspase inhibitor peptide BIR3/RING derived from XIAP; brain ischemia and seizures induced by kainate

As mentioned above, the C-terminal fragment containing the BIR3 and RING domains (BIR3/RING) of XIAP is a specific inhibitor of caspase-9 [89]. Fan et al. constructed a fusion protein PTD-BIR3/RING, where BIR3/RING was fused with the PTD of Antennapedia homeodomain (Antp HD) [118]. Using a rat transient MCAO model, they showed that the intra-peritoneally injected fusion peptide was delivered into the brain. The fusion peptide decreased the number of TUNEL-positive cells and inhibited the activation of caspase-3 in the ischemic boundary zone to improve ischemia-induced neurological outcomes, but it is unknown to what extent PTD(Antp HD)-BIR3/RING decreased infarct volumes.

Li et al. applied PTD(Antp HD)-BIR3/RING to kainic acid-induced neuronal death [119]. Kainate has been widely used to induce seizures in rodents as a model of human temporal lobe epilepsy. PTD(Antp HD)-BIR3/RING inhibited the activation of caspase-3 and -9, significantly decreased the number of TUNEL-positive cells and increased the number of surviving cells in the ipsilateral CA3 sub-field of the hippocampus at 24 h after KA-induced seizures.

5.3. Peptide inhibitors of c-Jun N-terminal kinase (JNK)

5.3.1. JNK signal signaling pathway as a therapeutic target

Excitotoxicity is a major mechanism of neuronal death in stroke and traumatic brain injury. The overactivation of ionotropic glutamate receptors such as the *N*-methyl-D-aspartate receptor (NMDAR) leads to an excessive influx of Ca^{2+}, triggering cell death. On the other hand, JNK (c-Jun N-terminal kinase) and p38 MAPK, which are downstream targets of ASK1 mentioned above, are activated during brain ischemia [120,121], although the pathways by which NMDA induces the activation of JNK and p38 MAPK have not been well characterized. In mice, disruption of the gene encoding JNK3, which is one member of the JNK family and selectively expressed in the nervous system, caused the mice to be resistant to the excitotoxic glutamate-receptor agonist kainic acid, resulting in a reduction in seizure activity and prevention of hippocampal neuron apoptosis [122]; therefore, the JNK signaling pathway is considered to be a potential target to prevent apoptosis. JNK activation is facilitated by association with c-Jun N-terminal kinase-interacting protein-1 (JIP-1)/islet-brain-1 (IB1), where the c-Jun binding domain (JBD) of JIP-1/IB1 is responsible for the association. One idea for a therapeutic approach is that JBD peptides competitively bind to JNK, resulting in perturbing the association between JNK and JIP-1 required for JNK activation [123,124].

5.3.2. D- and L-forms of JNK inhibitor; brain and heart injuries

Bonny's group designed a JNKI-1 peptide inhibitor, in which a 20-amino acid JNK binding motif is linked to the TAT sequence, and synthesized a D-form of JNKI-1 (D-JNKI-1) as well as an L-form of JNKI-1 (L-JNKI-1), where D-JNKI-1 was expected to be protease-resistant to expand its half-life *in vivo* [125]. D-JNKI-1 selectively inhibited the phosphorylation of c-Jun in a cell-free system, although about 15- to 20-fold less than that of L-JNKI-1 [123,125]. D-JNKI-1 completely prevented IL-1β-induced apoptosis of insulin-secreting βTC-3 cells without affecting the normal insulin response to glucose [125]. They investigated whether D-JNKI-1 mitigates ischemic brain injury [123]. In NMDA-treated cortical neurons in culture, both L-JNKI-1 and D-JNKI-1 inhibited the activation (phosphorylation) of c-Jun, which is a major target of activated JNK, and transcription of the c-Fos gene, which is regulated by the JNK transduction pathway, resulting in protecting neurons against the excitotoxic effect of NMDA. As expected, D-JNKI-1 prolonged the survival of NMDA-treated neurons more than L-JNKI-1. D-JNKI-1 was able to cross the blood–brain barrier and to ameliorate brain ischemic injury. Importantly, D-JNKI-1 significantly decreased the phosphorylation of c-Jun in the ischemic region of the cortex and reduced infarct volumes in a rat model of permanent distal MCAO, even though D-JNKI-1 was intraperitoneally given at 12 h after ischemia [123]. In another group, Asanuma et al. reported that JNK inhibitor I, which has the same sequence as L-JNKI-1 and is commercially available from Calbiochem/Merck, attenuated malonate-induced brain injury, where malonate, a reversible inhibitor of succinate dehydrogenase, causes secondary excitotoxic lesions similar to those of focal ischemia/reperfusion when injected intra-striatally [126].

Using Langendorff-perfused hearts, Milano et al. reported that pre-perfused D-JNKI-1 inhibited JNK activity, reduced mitochondrial cyt. *c* release, caspase-3 activation, and the number of TUNEL-positive apoptotic cells caused by ischemia/reperfusion injury to attenuate ischemic myocardial dysfunction [127]. Intraperitoneal injection of D-JNKI-1 at the onset of ischemia decreased infarct size by half in a rat model of *in vivo* myocardial ischemia/reperfusion injury.

5.3.3. Otoprotection by topical application to the inner ear

Exposure to noise, infections or ototoxic agents such as aminoglycoside and cisplatin sometimes causes the death of sensory hair cells in the cochlea, resulting in hearing loss. Evidence supports that ROS generation in the cochlea may cause apoptosis, probably via the MAPK/JNK signaling pathway. Wang et al. examined the otoprotective efficacy of D-JNKI-1 [128]. Exposure of organ of Corti explants to the aminoglycoside neomycin caused both hair-cell loss and c-Jun phosphorylation in sensory cell nuclei, which were inhibited by treatment with D-JNKI-1. D-JNKI-1 was perfused to the scala tympani of the cochlea of pigmented guinea pigs using an osmotic minipump over a period of 7 days. Two days after start of D-JNKI-1 perfusion, the animals were exposed to neomycin or acoustic trauma. Direct application of D-JNKI-1 into the scala tympani prevented nearly all hair-cell death and permanent hearing loss induced by neomycin ototoxicity, and the local delivery of D-JNKI-1 also prevented acoustic trauma-induced permanent hearing loss in a dose-dependent manner [128]. Subsequent careful analysis of the events occurring after sound trauma demonstrated that JNK activates a mitochondrial cell death pathway (i.e., activation of Bax, release of cyt. *c*, activation of procaspases, and cleavage of fodrin), which was prevented by D-JNKI-1 delivered onto the round window membrane (RWM) separating the inner ear from the middle ear space, using an osmotic minipump [129]. They demonstrated that RWM-delivered D-JNKI-1 prevents hair-cell death and the development of a permanent shift in the hearing threshold caused by sound trauma in a dose-dependent manner, and that the therapeutic window for protection of the cochlea from sound trauma extended to 12 h after sound exposure.

5.3.4. Smaller JNK inhibitor; cerebral ventricular injection for brain ischemia

Guan et al. designed a smaller cell-permeable peptide inhibitor of JNK, named Tat-JBD, where TAT peptide was linked to a 11-mer peptide corresponding to residues 153-163 of murine JIP-1 (JNK binding domain) [124]. Pre-injection of Tat-JBD into the cerebral ventricle inhibited the activation of JNK induced by transient forebrain ischemia in the vulnerable hippocampal CA1 sub-region, resulting in decreasing phosphorylation of c-Jun and expression of the Fas ligand. At the same time, the inhibition of JNK activation resulted in inhibiting the phosphorylation of Bcl-2 (a cytosolic target of JNK) and the release of Bax from Bcl-2/Bax dimers, which in turn suppressed Bax translocation to mitochondria, the release of cyt. *c*, the activation of caspase-3 and hydrolyzation of poly(ADP-ribose)polymerase. They showed that Tat-JBD infusion before or after ischemia exhibited

neuroprotective effects on ischemic brain damage *in vivo*. It remains uncertain to what extent systemic administration of Tat-JBD peptide exhibits neuroprotective activity.

5.4. Glutamate receptor sub-unit 6 (GluR6) C-terminus derived peptide

5.4.1. GluR6 as a therapeutic target for seizures

As mentioned above, kainate has been widely used to induce seizures in rodents as a model of human temporal lobe epilepsy. Kainate receptors, a type of ionotropic glutamate receptors, are composed of five sub-units, one of which is GluR6. One reason for targeting GluR6 is that GluR6-deficient mice showed phenotypes similar to those of *Jnk3* gene knockout mice, including resistance to kainate-induced seizures and neuronal toxicity [122,130]. The 9-mer RLPGKETMA motif of the C-terminus of GluR6 can bind to the postsynaptic density protein PSD95/SAP90 [131], whereas PSD95 can bind to MLK3 (mixed lineage kinase 3), which is one of the MAPKKKs and an upstream kinase of JNK [132]. It is suggested that PSD95 plays a critical role in GluR6-mediated JNK activation and excitotoxicity by anchoring MLK to the receptor complex [133].

5.4.2. Cerebral ventricular injection for ischemia- and kainate-induced brain injuries

Zhang's group designed Tat-GluR6-9c, in which the RLPGKETMA motif of the C-terminus of Glu6R was conjugated with TAT peptide [134,135]. It was expected that Tat-GluR6-9c entered cells to perturb the association between Glu6R and PSD95 required for JNK activation. Although Tat-GluR6-9c was able to perturb the association of Glu6R with PSD95, Tat-GluR6-9c did not affect kainate receptor-mediated currents [134]. The authors demonstrated that transient forebrain ischemia followed by reperfusion enhanced the assembly of the triple complex of GluR6, PSD95 and MLK3 to activate JNK, resulting in Bax translocation to induce the mitochondrial apoptotic pathway. Pre-infusion of Tat-GluR6-9c into the cerebral ventricle suppressed the assembly of the triple complex, and inhibited neuronal degeneration in the hippocampal CA1 region [134]. Another study demonstrated that kainate also enhanced the assembly of the triple complex of GluR6, PSD95 and MLK3 to activate JNK in rat hippocampal CA1 and CA3/dentate gyrus sub-fields [135]. It was shown that kainate-induced neuronal loss in hippocampal CA1 and CA3 sub-regions was prevented by intra-cerebroventricular pre-injection of Tat-GluR6-9c via inhibiting the assembly of the triple complex. In these studies, Tat-GluR6-9c was used to examine the molecular mechanism(s) by which kainate receptors participate in ischemic brain injury and neuronal cell death induced by seizure, and may provide a new approach for seizure therapy.

5.5. Peptide inhibitor of NF-κB: IκB kinase inhibitor NEMO-binding domain peptide

5.5.1. NF-κB as a therapeutic target

Proinflammatory cytokines, such as IL-1, which are released by resident macrophages in islets in response to a variety of stimuli, have been shown to stimulate NF-κB activity in human and rodent islets *in vitro*, resulting in β-cell impairment [136]. NF-κB binds to a family of naturally occurring repressors termed IκB, resulting in retention of the transcription factor complex as the inactive form in the cytoplasm. The NF-κB essential modifier (NEMO), also known as I-B kinase (IKK)-γ, is a scaffold protein that binds IKK-α and IKK-β and is essential for forming a functional IKK complex. Increases in the activity of the IKK complex result in the phosphorylation and subsequent degradation of IκB and allow translocation of NF-κB to the nucleus where it participates in transcriptional regulation. The NEMO-binding domain (NBD) of IKK-β was shown to be within the α_2-region, consisting of 11 amino acid residues, of the COOH terminus [137].

5.5.2. Islets for transplantation

Rehman et al. constructed the cell-permeable peptide PTD-5-NBD, in which NBD was conjugated with the cationic PTD-5 peptide [138], to protect islets from IL-1β-mediated dysfunction by inhibiting the functional interaction of IKK-β and NEMO, and subsequent activation of NF-κB [139]. They showed that transduction of PTD-5-NBD almost completely inhibited NF-κB activation in islets treated with IL-1β. Pre-infusion of PTD-5-NBD into the common bile duct was shown to protect islets from loss of viability due to osmotic, mechanical and ischemic stresses during the isolation procedure, resulting in improvement in glucose-stimulated static insulin release. The percentage of viable cells compared with non-viable cells in the PTD-5-NBD treatment group was around 97%; meanwhile, those in the control groups (no infusion and infusion of PTD-5 alone) were 80 to 90%. They emphasized the feasibility of using peptide transduction domains to modify tissues *in situ* to improve their viability prior to transplantation.

6. Conclusions

In vivo delivery of anti-cell death proteins/peptides exhibits therapeutic effects to mitigate various diseases in animal models. When full-sized proteins are used, the therapeutic proteins would interact only with the targeted molecule(s) for which they were designed, so they could be administered in substantially lower doses, resulting in fewer side effects. In this regard, genetically engineered therapeutic proteins with enhanced activity are more advantageous. The therapeutic full-length proteins described in this review were prepared from bacterial cells, which calls attention to endotoxin contamination in the preparations. In addition, this is not adequate for proteins that require secondary post-translational modifications, for example, glycosylation, to exhibit full activity. Nonetheless, to our knowledge, no report on the manufacture of PTD-fused proteins for human use is available. To be safe for human use, one needs to thoroughly investigate biodistribution and pharmacokinetics of PTD-fused proteins, the immune response to the proteins, an effective mode of delivery to target cells and intra-cellular targets, such as mitochondria, in addition to solving difficulties with large-scale and low-cost purification. Moreover, urea denaturation required for TAT-mediated transduction probably removes metal(s) from metal-containing enzymes, which may result in loss

of its therapeutic activity. In this regard, PEP-1- or other PTDs-mediated transduction, like PEP-1-SOD, may provide an alternative to TAT-mediated transduction.

PTD-fused peptides are small enough to be chemically synthesized without endotoxin contamination. In addition, like D-isomers of TAT and (Arg)-oligomer, D-isomer-containing PTDs can be synthesized without affecting internalization efficiency, and are resistant to proteolytic degradation, leading to substantially lower doses; however, as shown in the section on TAT-BH4, it is possible that peptide inhibitors does not necessarily exhibit the same effect, in terms of dose and/or function, as their full-length proteins.

Repeated administration of PTD-fusion protein would definitively raise its cognate antibodies; therefore, in the first studies on the delivery of anti-cell death proteins/peptides, acute diseases, such as ischemia/reperfusion injury, were used as the experimental model, where a single dose was given to evaluate its therapeutic effects. However, the results of the TAT-PNP study suggest that PTD-protein delivery might be useful to constantly supply therapeutic enzymes as an alternative to gene therapy. Finally, we would like to emphasize that, besides as a device for therapeutic delivery, PTD-mediated transduction can be applied to maintain and/or cryopreserve biological materials for transplantation.

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Therapeutic Benefits of Intrathecal Protein Therapy in a Mouse Model of Amyotrophic Lateral Sclerosis

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When fused with the protein transduction domain (PTD) derived from the human immunodeficiency virus TAT protein, proteins can cross the blood–brain barrier and cell membrane and transfer into several tissues, including the brain, making protein therapy feasible for various neurological disorders. We have constructed a powerful antiapoptotic modified Bcl-X_L protein (originally constructed from Bcl-X_L) fused with PTD derived from TAT (TAT-modified Bcl-X_L), and, to examine its clinical effectiveness in a mouse model of familial amyotrophic lateral sclerosis (ALS), transgenic mice expressing human Cu/Zn superoxide dismutase (SOD1) bearing a G93A mutation were treated by intrathecal infusion of TAT-modified Bcl-X_L. We demonstrate that intrathecally infused TAT-fused protein was effectively transferred into spinal cord neurons, including motor neurons, and that intrathecal infusion of TAT-modified Bcl-X_L delayed disease onset, prolonged survival, and improved motor performance. Histological studies show an attenuation of motor neuron loss and a decrease in the number of cleaved caspase 9-, cleaved caspase 3-, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells in the lumbar cords of TAT-modified Bcl-X_L-treated G93A mice. Our results indicate that intrathecal protein therapy using a TAT-fused protein is an effective clinical tool for the treatment of ALS. © 2008 Wiley-Liss, Inc.

Key words: TAT; Bcl-2; SOD1; spinal cord; ALS

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disease that is characterized by selective loss of central and peripheral motor neurons. There are many hypotheses to explain the cause of this disease; for example, glutamate toxicity (Bruijn et al., 2004; Ganel et al., 2006), axonal transport deficiency (Farah et al., 2003; Rao and Nixon, 2003; Kieran et al., 2005), protein misfolding and aggregation (Bruijn et al., 1998; Julien, 2001), and mitochondrial dysfunction (Menzies et al., 2002; Kirkinezos et al., 2005; Manfredi and Xu, 2005). In approximately 15–20% of familial ALS cases, a variety of dominant missense mutations or small deletions in the Cu/Zn superoxide dismutase (SOD1) gene have been identified (Deng et al., 1993; Rosen et al., 1993; Brown and Robberecht, 2001). Several lines of transgenic (Tg) mice expressing a mutant human SOD1 gene have been established, and these act as valuable models of human ALS (Gurney et al., 1994; Wong et al., 1995). Although wild-type SOD1 exerts antiapoptotic effects (Rabizadeh et al., 1995), mutant SOD1 promotes apoptosis in neural cells (Rabizadeh et al., 1995; Ghadge et al., 1997). Furthermore, caspase 1 and caspase 3 are activated in mutant SOD1 Tg mice (Vukosavic et al., 2000); thus, apoptosis is considered to underlie the motor neuron death in ALS. Therefore, antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-X_L, which are localized mainly in mitochondria, where they regulate the release of cytochrome c (Reed, 1997; Halestrap et al., 2000), can be considered as therapeutic proteins for the treatment of ALS. Moreover, Bcl-2 delays activation of caspases (Vukosavic et al., 2000), and transgenic overexpression of Bcl-2 or intra-

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spinal infusion of adeno-associated virus encoding Bcl-2 shows neuroprotective effects in ALS model mice (Kostic et al., 1997; Azzouz et al., 2000). However, gene therapy approaches cannot exclude the possibility of hazardous insertions of the transgene.

Recently, it was shown that proteins can cross the blood–brain barrier and cell membrane and transfer into several tissues, including the brain, when fused with the protein transduction domain (PTD) derived from the human immunodeficiency virus TAT protein (Schwarze et al., 1999; Cao et al., 2002). In mouse or gerbil models of focal ischemia and traumatic injury, full-length therapeutic proteins fused with TAT, including TAT-Bcl-X_L, show strong therapeutic effects when delivered intraperitoneally, intravenously, or intraocularly (Cao et al., 2002; Dietz et al., 2002; Kilic et al., 2002, 2003, 2004; Kim et al., 2005). We have constructed a modified Bcl-X_L protein (originally constructed from Bcl-X_L), which has a powerful antiapoptotic effect rather than original Bcl-X_L (Asoh et al., 2000, 2002), fused with TAT (TAT-modified Bcl-X_L; Asoh et al., 2000, 2002), which also shows strong prevention of neuronal death in a gerbil model of focal ischemia when delivered intraperitoneally (Asoh et al., 2002). Protein therapy using TAT-modified Bcl-X_L can be widely applicable for various neurological disorders, including ALS. However, therapeutic proteins fused with TAT need to be efficiently delivered into the spinal cord to be used as therapies in ALS model mice, and a TAT-fused protein has not been shown to be able to transfer into neurons of the spinal cord efficiently when delivered intraperitoneally or intravenously. We have reported that the technique of intrathecal infusion improves drug delivery into spinal cord and strengthens the efficacy of therapeutic proteins in the spinal cord (Nagano et al., 2005). Therefore, intrathecal infusion of TAT-modified Bcl-X_L can be expected to deliver this therapeutic protein efficiently into spinal cord neurons. Here, we describe the intrathecal infusion of TAT-modified Bcl-X_L in G93A SOD1 Tg mice and show that this form of therapy provides therapeutic benefits in these mice.

MATERIALS AND METHODS

Preparation of TAT-GFP and TAT-Modified Bcl-X_L Proteins

pTAT-HA-GFP was generously provided by Dr. S. Dowdy. Plasmid was transformed into a BL21 (DE3) pLysS (Novagen, Madison, WI) bacterial strain. A 50-ml overnight culture of highly expressing BL21 (DE3) pLysS bacteria was inoculated into 1 liter of LB containing 1 mM isopropylthiogalactoside (IPTG; Wako, Osaka, Japan) and shaken for 2 hr at 37°C. The culture was pelleted, resuspended in a lysis buffer (100 mM phosphoric acid, 300 mM NaCl, pH 7.8), and sonicated. The sonicate was pelleted, resuspended in another lysis buffer (100 mM phosphoric acid, 500 mM NaCl, 8 M urea, pH 7.8), and applied to an Ni-NTA resin (Novagen). Fusion proteins were eluted using 50 mM imidazole. The eluant was dialyzed against urea and concentrated using Amicon filters (Millipore, Bedford, MA). Protein concentration was determined by Coomassie brilliant blue staining after SDS-PAGE, followed by comparison with bovine serum albumin (BSA) standards. TAT-modified Bcl-X_L was prepared as described previously (the construct of TAT-modified Bcl-X_L; see supplemental data; Asoh et al., 2002; Ozaki et al., 2004).

Cell Culture and Staurosporine (STS)-Induced Apoptosis

Neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagles' medium (DMEM; Invitrogen, San Diego, CA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained in a humidified incubator at 37°C under 5% CO_2. TAT-modified Bcl-X_L was incubated at 37°C for 7, 14, or 28 days. SH-SY5Y cells were pretreated with 3 nM PTD-FNK for 1 hr, incubated with 50 nM STS for 1 day, and then 10 μM propidium iodide and 10 μM Hoechst 33342 (Vybrant Apoptosis Assay Kit 5; Molecular Probes, Eugene, OR) were added to each well. The number of stained cells per well was counted. Cells with blue round nuclei, red round nuclei, and blue or red fragmented nuclei were judged as living cells, necrotic cells, and apoptotic cells, respectively (Shimizu et al., 1996). Cell viability was calculated as follows: 100 × living cells/total cells.

Animal Model

All experimental procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine, Dentistry and Pharmaceutical Sciences of Okayama University. Tg mice with the G93A human SOD1 mutation (G1H/+) were obtained from Jackson Laboratories (Bar Harbor, ME; Gurney et al., 1994) and maintained as hemizygotes by mating Tg males with C57BL/6J females.

Intrathecal TAT-GFP and TAT-Modified Bcl-X_L Infusion

Artificial cerebrospinal fluid (NaCl 122 mM, KCl 3.1 mM, $NaHCO_3$ 5 mM, KH_2PO_4 0.4 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.0 mM, D-glucose 10 mM, pH 7.4) as vehicle (aCSF), TAT-GFP (3.5×10^{-2} nmol or 3.5×10^{-1} nmol, administered over 7 days) or TAT-modified Bcl-X_L (3.5×10^{-1} nmol, administered over 7 days) was intrathecally infused into a lumbar site of nontrangenic (Tg) wild-type B6SJL male littermates (wild type; WT) at 91 days of age, for 7 days, using a 200-μl-volume osmotic minipump (Alzet minipump model 2001; ALZA Corporation, Palo Alto, CA) as described previously (Nagano et al., 2005; Ohta et al., 2006). The minipump was designed for a nominal infusion rate of 1 μl/hr for 7 days, was attached to sterile polyethylene tubing (Becton Dickinson, Sparks, MD), and was placed subcutaneousuly at a lumbar site. A small pit was made at L5, and then a rostrally directed cannula was inserted and its tip was placed in the spinal subarachnoid space at the level of L6–S1. Cannula and osmotic minipump were sewn to the muscle to close the insertion site. At 7 days after minipump placement, mice were sacrificed.

After dose-determination experiments, aCSF (vehicle), TAT-GFP (1.4 nmol, administered over 28 days), or TAT-modified Bcl-X_L (1.4 nmol, administered over 28 days) was intrathecally infused into a lumbar site of G93A SOD1 Tg male mice at 91 days of age, for 28 days, using a 200-μl-volume osmotic minipump (Alzet minipump model 2004; ALZA), which was designed for a nominal infusion rate of 0.25 μl/hr for 28 days. Forty-six G93A mice were randomly assigned to aCSF (n = 13), TAT-GFP (n = 12), or TAT-modified Bcl-X_L (n = 21) treatment and used for the assessment of clinical scores, disease onset, and survival. Among them, six G93A mice treated with TAT-modified Bcl-X_L were to be sacrificed at 133 days for histological analysis.

Clinical Scores

The clinical scores of the mice were evaluated twice per week starting at 90 days of age. Body weight was measured, and disease onset was judged by an investigator blinded to the treatment conditions, who examined mice for shaking of the hindlimbs by hanging them from the tail. The time point at which mice showed a fine tremor in at least one hindlimb over a 20-sec period was recorded as the time of disease onset (Kostic et al., 1997). For behavioral studies, rotarod tests were performed and wheel-running activity was measured, based on our previous method (Abe et al., 1997; Manabe et al., 2002; Nagano et al., 2005). Rotarod testing was begun with the mice trying to stay on a rod that was rotated at 1 rpm; the speed was then increased by 1 rpm every 10 sec. The length of time for which mice stayed on the rod (to a maximum of 5 min) was recorded as an indicator of its grasping power. Three trials were performed, and the best result was recorded. Wheel-running activity was measured as voluntary movement of the mice, over a 30-min period, in a unilateral direction (forward or back) in a narrow circular cage. The time-point when mice were unable to roll over within 20 sec of being pushed on their side was recorded as the time of death.

Histological Evaluation

WT mice treated with aCSF, TAT-GFP, or TAT-modified Bcl-X_L for the analysis of TAT-fused protein delivery, sacrificed at 98 days of age (7 days after minipump placement), and six G93A mice treated with aCSF or TAT-GFP that died before 133 days of age, as well as six G93A mice treated with TAT-modified Bcl-X_L and six WT control mice that were sacrificed at 133 days of age were perfused transcardially with heparinized saline. The lumbar cord spanning L4–L5 was removed, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and frozen after cryoprotection with a series of phosphate-buffered sucrose solutions of increasing concentration (10%, 15%, and 20%). Transverse sections of 10 μm thickness were cut through the middle of the L4 segment using a cryostat.

The sections for the analysis of TAT-GFP or TAT-modified Bcl-X_L delivery into the lumbar cord were incubated with a rabbit polyclonal anti-GFP antibody (1:1,000; Molecular Probes) or a mouse monoclonal anti-Bcl-X_L antibody (1:1,000; No. 35-32; Asoh et al., 2002), respectively, which was detected by using the avidin-biotin complex method as described previously (Warita et al., 2001). Immunoreactivity was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Additionally, sections were incubated with a mixture of the same anti-GFP antibody and SMI32, a mouse monoclonal antibody (1:5,000; Sternberger, Lutherville, MD), overnight at 4°C. After rinsing in PBS, the sections were incubated with fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (1:100; Chemicon, Temecula, CA) and Texas red-labeled horse anti-mouse IgG (1:100; Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. The specificity of immunostaining was confirmed by omission of the primary antibody. The sections for double immunofluorescence studies were scanned using a confocal microscope equipped with argon and HeNe1 lasers (LSM-510; Zeiss, Jena, Germany).

The sections for analysis of TAT-modified Bcl-X_L treatment were stained with cresyl violet (Nissl stain). One hundred consecutive sections were cut through the middle of the L4 segment, and Nissl-stained motor neurons in L4 were counted in every fifth transverse sections (20 sections in total) from each lumbar cord (Nagano et al., 2005). All cells in both ventral horns below a lateral line across the spinal cord from the central canal were microscopically video-captured, and only cells with a diameter greater than 20 μm that showed clear nucleoli were counted by the investigator, who was blinded to the treatment conditions (Warita et al., 1999; Manabe et al., 2003). A separate set of every fifth section (20 sections in total) from each lumbar cord was immunostained with a rabbit polyclonal anticleaved caspase-9 antibody (1:1,000; Cell Signaling Technology, Danvers, MA) and a rabbit polyclonal anticleaved caspase-3 antibody (1:4,000; Cell Signaling Technology), which were also detected using the avidin-biotin complex method. Additionally, sections were incubated with a mixture of the same anticleaved caspase-9 or cleaved caspase-3 antibodies and a mouse monoclonal antineuronal nuclear antigen (NeuN; 1:400; Chemicon), or mouse monoclonal antiglial fibrillary acidic protein (GFAP) antibodies (1:200; Chemicon), overnight at 4°C. After rinsing in PBS, the sections were incubated with fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (1:100; Chemicon) and Texas red-labeled horse anti-mouse IgG (1:100; Vector Laboratories) for 1 hr at room temperature. Additionally, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed in a separate set of every fifth section (20 sections in total) from each lumbar cord, using the TACS 2TdT DAB in situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD).

Statistical Analysis

Data are expressed as means ± SD. Statistical comparisons of data from the STS-induced apoptosis test in vitro and histological data were performed by using a one-factor ANOVA, and clinical data were compared by repeated-measures ANOVA, followed by a Tukey-Kramer post hoc comparison. A Kaplan-Meier survival analysis and the log-rank test were used for onset and survival comparison. Statistical significance was accepted at $P < 0.05$.