TAT fusion proteins against ischemic stroke: Current status and future perspectives

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1. ABSTRACT

The delivery of proteins across the blood-brain barrier is severely limited by the proteins' size and biochemical properties. Thus, a large number of peptides have been characterized in recent years that efficaciously prevent neuronal death *in vitro*, but which may not be applied *in vivo*, since they are unable to cross cell membrane barriers. In the 1990ies, it had been shown that the HIV TAT protein is able to cross cell membranes even when coupled with larger peptides. Subsequent studies with fusion proteins of the 11-amino acid protein transduction domain of HIV-TAT revealed that TAT fusion proteins may successfully be used for therapeutic purposes *in vivo*, even when systemically applied. Indeed, intravenous delivery of TAT proteins linked with anti-apoptotic (Bcl-X_L) and neurotrophic (GDNF) factors resulted in a rapid and highly efficacious transduction of the brain tissue. When administered after focal cerebral ischemia, intravenous TAT-Bcl-X_L and TAT-GDNF significantly reduced brain injury, both when applied after severe and mild ischemic insults. These data provided the first *in vivo* evidence of the efficacy of fusion proteins in the ischemic brain, thus raising new hopes that neuroprotection is feasible after stroke. Yet, molecular biological studies have pointed out more recently that there are also limitations of the TAT protein strategy, which still need to be addressed. The development of clinically-applicable treatments for ischemic stroke based on fusion protein technologies deserves concerted efforts in the future.

2. INTRODUCTION

During the last decade, a huge number of anti-apoptotic and neurotrophic factors have been identified *in vitro*, which protect ischemic neurons against cell demise. Molecular studies allowed us to establish detailed concepts of how cell survival and death are controlled in injured neurons (1-3). Unfortunately, many neuroprotective factors cannot be applied for therapeutic purposes, since they are unable to pass the blood brain barrier, a natural diffusion barrier particularly for larger hydrophilic compounds. The blood brain barrier is formed by the capillary endothelial cells, which are interconnected by tight junctions that prevent the diffusion of molecules with a size of $\geq$600-1000 Da into the brain (4,5). Under physiological conditions, the blood brain barrier protects the brain against the entry of potentially toxic xenobiotic compounds. In neurological disease, however, it clearly impedes pharmacotherapy.

3. BLOOD BRAIN BARRIER

3.1. Blood brain barrier integrity under physiological conditions

In its physiological state, the blood brain barrier allows very limited diffusion of blood-derived molecules into the brain, which may take place either via paracellular tight junction leakage or -possibly also- by vesicle-mediated transendothelial transport (6,7). The physiology of tight junctions has been studied intensively in recent years, involving proteins such as claudin, occludin and

zona occludens-1/-2/-3 that actively control molecular diffusion (7,8). Compared with the tight junctions, the role of trans-endothelial transport systems in blood brain barrier integrity under physiological conditions is less clear and has been a matter of debate in recent years (9,10). However, there appears to be increasing evidence that trans-endothelial transport, which is widely active in other tissues, is suppressed in brain endothelial cells (9). This further explains why pharmacological compounds are often unable to achieve sufficiently high tissue concentrations in the brain parenchyma in order to be therapeutically used.

3.2. Blood brain barrier dysfunction in brain ischemia

Ischemic stress may result in blood brain barrier leakage, both via breakdown of the junctions (8) and also via active transcellular transport (11). *In vitro*, ATP depletion leads to the disintegration of the longitudinal actin networks of the cytoskeleton that then results in altered polarity of membrane proteins (11). In mild hypoxia - ischemia, however, blood brain barrier function remains intact, which may be due to (a) the high resistance of tight junction proteins under conditions in which the actin cytoskeleton is already disturbed (12) and (b) the supportive influence of astroglia which maintains blood brain barrier integrity when tight junctions are injured (13,14). These mechanisms might explain the long time latency noted *in vitro* (13,14) and *in vivo* (15) until blood brain barrier dysfunctions become evident after a stroke. The blood brain barrier leakage appears to late to enable pharmacotherapy.

4. OVERCOMING THE BLOOD BRAIN BARRIER WITH GENE THERAPY

Intense efforts have been made in the past in order to overcome the blood brain barrier for therapeutic purposes. As such, viral gene therapy techniques have been used by others and us as treatment trying to resolve this problem (16-19). The major advantage of gene therapy as a treatment strategy is that therapeutic proteins are continuously supplied to the brain tissue, once the tissue has been successfully transduced (17-19). Yet, the delivery of genes into brain tissues and expression of therapeutic proteins are time-consuming processes, which may pose a problem in a rapidly developing pathology like stroke (17). Moreover, viral gene therapies may also have immunological side effects, particularly when gene vectors are systemically applied (20,21). Immunological effects were particularly pronounced with first generation adenoviral vectors. This lead to the generation of more advanced vectors, such as 'gutless' viruses (22), adeno-associated viruses (23) or lentiviruses (24), and to the development of non-viral gene vectors, such as liposomes and nanoparticles (25), as strategies for brain tissue transduction.

4.1. Limitations of gene therapy in ischemic stroke

Unfortunately, gene therapy strategies encounter additional conceptional problems particularly in brain ischemia. Ischemia is a powerful suppressor of cerebral protein synthesis both *in vitro* and *in vivo* (26). After focal cerebral ischemia, protein synthesis remains suppressed over several hours in the ischemic tissue, even in brain areas resistant to ischemia (27). In vulnerable brain regions, protein synthesis exhibits a particularly long-lasting inhibition, which usually does not recover until cell death evolves (27,28). Inhibition of protein synthesis by ischemia is induced by disruption of ribosomal mRNA translation, via phosphorylation of the eukaryotic initiation factor (eIF)-2α (29). As the disruption of mRNA translation impedes not only the expression of endogenous, but also of viral genes, the efficacy of gene therapies is invariably compromised. This may explain why gene therapy approaches are efficacious when applied before or immediately after an ischemic event, but not when given at more delayed time points (30).

5. *IN VIVO* TISSUE TRANSDUCTION USING HIV-TAT FUSION PROTEINS

In view of these aspects, the transduction of the brain tissue with proteins may represent a much more promising strategy, compared with gene transfer tools. It has already been shown in 1988 by two groups that the HIV-TAT protein can cross cell membranes (31,32). Many years later, it was then demonstrated that heterologous proteins chemically cross-linked to the so-called protein transduction domain (PTD) of the TAT protein are able to transduce into cells (33) and that intraperitoneal injection of the 120 kDa ß-galactosidase protein, fused to the PTD of the TAT protein, results in delivery of biologically active fusion protein to various tissues in mice, including the brain (34). The same authors determined the distribution of fusion peptides in brain sections and were able to show a progressive translocation of TAT proteins into the brain parenchyma (34). They also showed that the blood brain barrier remained intact during the transduction process.

When analyzing the biophysical nature of the PTD, the authors showed that it represents an amphipathic alpha helix carrying basically charged arginine residues on its surface (35). With these arginine residues, the TAT protein is thought to attach to cell membranes. The mechanism of membrane passage, however, is still a matter of debate. It has been suggested that TAT proteins pass the cell membrane via endocytotic-like mechanisms (36-38). Besides, direct membrane penetration in a denatured, energy-rich state, followed by refolding of the protein in the cell interior (39,40), or membrane passage via an inverted micelle formed by the ruptured membrane lipid bilayer (41,42) have been discussed. It is conceivable that more than one mechanism is involved and that the mechanism of cell entry depends on the fusion construct's size, its primary and secondary structure as well as its electrical surface charges.

5.1. TAT-BCL-X_L and TAT-GDNF for ischemic stroke treatment

Based on *in vivo* data by Schwarze *et al.* (34), we became interested whether a systemic delivery of a TAT fusion protein may therapeutically be used for stroke treatment. Thus, we made use of a fusion protein of the TAT-PTD with the Bcl-X_L protein, which we applied
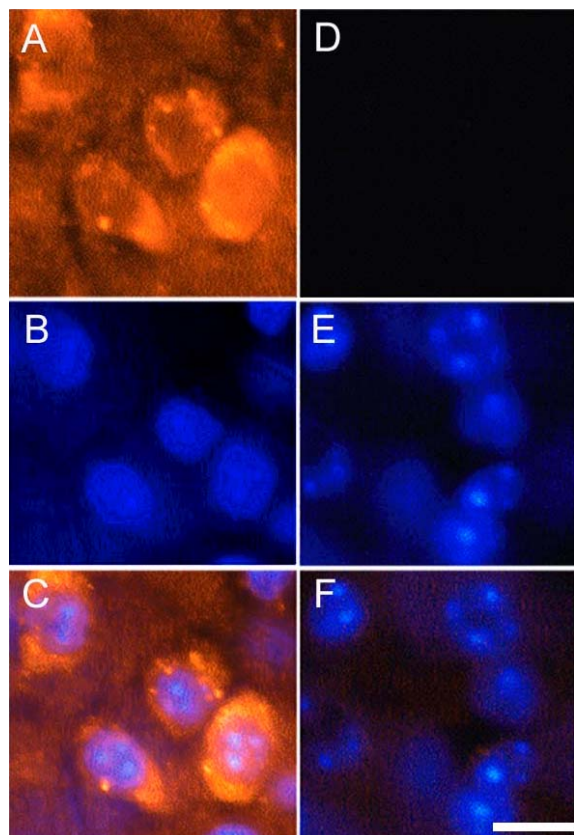
Figure 1. Bcl-X immunopositive cells in the lateral part of the striatum of mice at the bregma level 4 hours after *i.v.* infusion of TAT-Bcl-X$_L$ (0.6 nmol) (A), contrasted with the corresponding area in an animal treated with TAT-GFP (0.6 nmol) showing no labeled cells (D). In (B) and (E), the histomorphology of the brain regions in (A) and (D) is depicted by DAPI staining. In (C) and (F), photomicrographs (A) and (B) as well as (D) and (E) are superimposed. Bar, 25 μm.

intravenously in mice subjected to focal cerebral ischemia. The TAT-Bcl-X$_L$ protein appeared particularly suitable for our studies, as Bcl-X$_L$ exhibits very pleiotropic anti-apoptotic and anti-necrotic actions after ischemia. As such, Bcl-X$_L$ dimerizes with pro-apoptotic Bcl-2 family members (i.e., Bax, Bcl-X$_S$ and Bid), keeping them from exerting their death-promoting influence in injured neurons (43,44). Whenever Bcl-X$_L$ levels decrease and the balance of Bcl-2 proteins is shifted towards pro-apoptotic members, cytochrome c, Smac/DIABLO and apoptosis-inducing factor (AIF) are released from the mitochondria into the cytosol (44,45), where they induce DNA fragmentation via caspase-dependent and -independent pathways. Upon downregulation of Bcl-X$_L$, mitochondrial permeability transition pores are also formed by pro-apoptotic Bcl-2 members, which, unless antagonized, lead to a breakdown of the mitochondrial energy state (46).

Using a TAT-Bcl-X$_L$ fusion protein, we were able to confirm the data by Schwarze *et al.* (34), showing that TAT proteins indeed accumulate in the brain tissue in a highly efficacious manner (5). Thus, we observed a

sequential transduction of the cerebral vasculature and brain parenchyma with TAT-Bcl-X$_L$ (see Figure 1). In animals subjected to focal cerebral ischemia, TAT-Bcl-X$_L$ significantly reduced the ischemic infarct size and also ameliorated neurological deficits (see Figure 2). Notably, TAT-Bcl-X$_L$ was similarly effective when applied before and after stroke, both when severe and mild ischemias were imposed (5). Our data validated the key role of mitochondrial signaling in the ischemic injury process, at the same time providing first *in vivo* evidence that fusion proteins of the TAT-PTD with anti-apoptotic peptides may therapeutically be used for stroke treatment.

We subsequently performed experimental studies using a TAT-GDNF protein, which we again applied intravenously to mice subjected to focal cerebral ischemia. In these studies, TAT-GDNF also protected against ischemic injury (47). Particularly strong rescue effects were seen in animals treated with TAT-GDNF prior to ischemia (~90% injury reduction), while TAT-GDNF's neuroprotection was less pronounced when delivered after the stroke (47). Taken together, our results demonstrate the efficacy and potential therapeutic usefulness of TAT fusion proteins in brain ischemia. In the meantime, our TAT-Bcl-X$_L$ and TAT-GDNF results were confirmed by a number of other groups delivering fusion proteins of the TAT-PTD with (a) Bcl-X$_L$ (48), (b) its gain-of-function mutant FNK (49), (c) the Jun kinase (JNK) inhibitor, JNK interacting protein (JIP)-1 (50) and (d) copper/zinc-super oxide dismutase (SOD) (51). Using models either of focal or global cerebral ischemia, the authors showed that neuronal injury after brain ischemia may indeed be targeted *in vivo* by these fusion constructs.

5.2. Methodological problems and limitations of TAT fusion protein technologies

After great enthusiasm in initial studies, anticipating that it will soon be possible to develop fusion proteins in almost unlimited numbers, it turned out more recently that the cloning, expression and purification of fusion proteins has also some methodological limitations which considerably slow down progress in the field. As such, it has been shown that not all proteins can be expressed successfully in *E. coli* (52). When using prokaryotes as expression system, eukaryotic peptides may lack post-translational modifications required for their proper function. In such cases, fusion proteins may not successfully be produced, at least not at sufficiently high concentration levels.

Not only the expression of the fusion proteins, but also the detection of successful tissue transduction may pose technical difficulties. When assessed by FACS analysis, the presence of TAT fusion proteins in transfected cultures does not necessarily imply that a protein has entered the cell interieur, since TAT proteins may remain attached to the cell membrane surface without passing into the cytosol (52). This may lead to the false positive impression that TAT proteins transduced the cells successfully, which may not be the case. Subsequent *in vivo* experiments with frustrating results may then follow. Extensive treatment with proteases might help in that
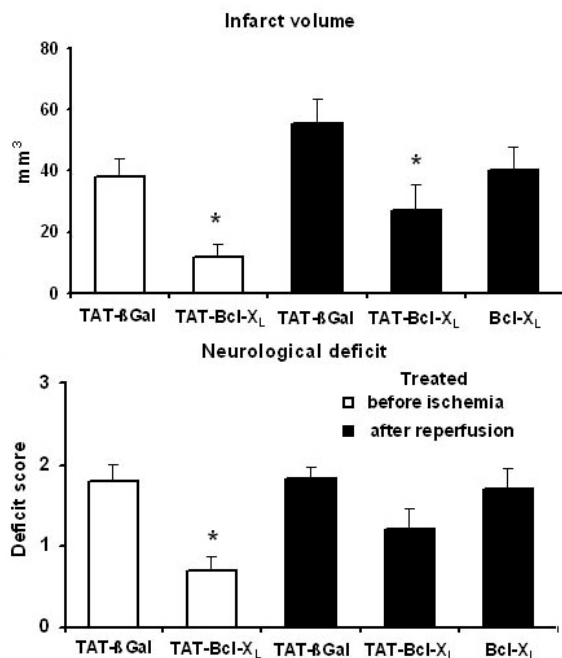
Figure 2. Infarct volumes (A) and neurological deficit scores (B) of mice subjected to 90 minutes of MCA occlusion followed by 24 hours of reperfusion. TAT-ß-galactosidase or TAT-Bcl-X_L (0.6 nmol each) was administered *i.v.* either one hour before ischemia or immediately after reperfusion. Note that TAT-Bcl-X_L significantly reduces brain injury and ameliorates neurological performance. In contrast with transducible Bcl-X_L protein, nontranstucible Bcl-X_L protein had no effect on neuronal injury when delivered after reperfusion. Values are given as means ± S.E.M. * P< 0.05 compared with TAT-ß-galactosidase treated mice.

context, allowing us to remove the extracellular protein from the cell culture.

Unsuccessful cell transduction has been described for a number of fusion constructs in the meantime, linking the TAT-PTD with diphtheria toxin A (53), with *Leishmania* gp63 (54) or with aromatic carboxylic acid (55). The lack of membrane passage observed for these molecules may be related to their primary or secondary structure, or to surface charges of the constructs that prevent membrane adhesion and passage. Another problem may be that a fusion protein may pass inside a cell through a vesicle-mediated mechanism, but that it is unable to be released from its transport vesicles. This failure of intracellular release has recently been reported for a TAT-calpastatin construct (56), which failed to exhibit calpastatin activity inside the transduced cells.

6. FUTURE PERSPECTIVES

Despite these technical difficulties, there is a good reason that fusion protein technologies might gain therapeutic potential in neurodegenerative diseases and, more specifically, in stroke. Not only the TAT-PTD, but also synthetic PTDs may be used for such purposes. Synthetic PTDs have recently been identified based on 3D models calculating secondary structures of oligopeptides and their interactions with cell membranes (35). Clearly, a number of issues need to be addressed before fusion protein delivery in humans may be considered. First of all, it is well known that the TAT protein induces immunological reactions in its host organism. Thus, detailed studies are needed in order to rule out potential inflammatory or allergic risks. Secondly, as TAT proteins enter various cell types in a non-selective manner, carcinogenic effects must carefully be tested, particularly as fusion proteins with cell death-inhibitory activity are used. Yet, in stroke patients, TAT fusion proteins will be administered only over a few hours to days, as stroke is a highly acute event. This might reduce the risk of allergic and carcinogenic side effects markedly. Nonetheless, experimental studies in primates will clearly be needed in order to address these issues. Only with such information further decisions can be made on whether TAT proteins should enter their way into clinical studies.

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