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Gene delivery with viral vectors for cerebrovascular diseases

Yu Gan[1,2], Zheng Jing[1,2], R. Anne Stetler[1,2], Guodong Cao[1,2]

[1]Geriatric Research, Education and Clinical Center, Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, PA 15240, U.S.A.; [2]Department of Neurology and Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, Pittsburgh, PA 15260, U.S.A.

TABLE OF CONTENTS

1. ABSTRACT

Recent achievements in the understanding of molecular events involved in the pathogenesis of central nervous system (CNS) injury have made gene transfer a promising approach for various neurological disorders, including cerebrovascular diseases. However, special obstacles, including the post-mitotic nature of neurons and the blood-brain barrier (BBB), constitute key challenges for gene delivery to the CNS. Despite the various limitations in current gene delivery systems, a spectrum of viral vectors has been successfully used to deliver genes to the CNS. Furthermore, recent advancements in vector engineering have improved the safety and delivery of viral vectors. Numerous viral vector-based clinical trials for neurological disorders have been initiated. This review will summarize the current implementation of viral gene delivery in the context of cerebrovascular diseases including ischemic stroke, hemorrhagic stroke and subarachnoid hemorrhage (SAH). In particular, we will discuss the potentially feasible ways in which viral vectors can be manipulated and exploited for use in neural delivery and therapy.

2. INTRODUCTION

Gene transfer provides a powerful approach not only for the study of physiological processes but also for the treatment of various diseases. Gene therapy is a rapidly developing area, and is considered one of the most promising approaches for a variety of inherited and acquired disorders that currently have no alternative remedy. Ongoing gene therapy clinical trials target first and foremost cancer (1); however, neurological disorders, including cerebrovascular disease, are now a valid target. The CNS, in contrast to other organs, faces special obstacles in terms of gene delivery and therapy. For example, mixed cell populations that are highly intermingled in the brain complicate targeted therapeutic strategies. Furthermore, neurons, the major target population in the CNS, are post-mitotic and thus present a twist in the utilization of viruses, which are often dependent on mitotic stages for effective transduction. Finally, and perhaps the most limiting for any brain-targeted therapeutic thus far, the CNS is isolated by the BBB (2). Despite the various limitations in current gene delivery systems, a

spectrum of viral (and non-viral) vectors has been successfully used to deliver genes to the CNS. This review will focus primarily on systems that incorporate viral vectors, and the implementation of viral gene delivery in the context of cerebrovascular diseases including ischemic stroke, hemorrhagic stroke and subarachnoid hemorrhage (SAH). In particular, we will discuss the various ways in which viral vectors can be manipulated and exploited for use in neural delivery and therapy.

3. VIRAL VECTOR-BASED GENE DELIVERY INTO THE CNS

3.1. Lentiviral vectors

Lentiviruses, a subclass of retrovirus, have acquired additional important features rendering them highly attractive as gene therapeutic vectors in brain. Perhaps most importantly, lentiviral vectors are able to infect both mitotic and post-mitotic cells, such as glia and neurons, respectively. Furthermore, lentiviruses possess an ability to translocate across the nuclear membrane and stably integrate into chromosomes, allowing them to mediate long-term gene expression while producing only a minimal immune response (3). These positive features have earned lentivirus-derived vectors a position as one of the principal gene delivery candidates for the CNS, leading to the initiation of a recent clinical trial using a lentiviral vector to treat Parkinson's disease (4).

The first generation of lentiviral vectors was derived from the human immunodeficiency virus 1 (HIV-1) with substantial modifications. The vector was made by co-transfection of a packaging plasmid encoding the viral genes (except for the env and vpu), an envelope plasmid encoding vesicular stomatitis virus glycoprotein protein (VSV-G) in place of the HIV-1 env proteins, and a transfer vector encoding the HIV-1 cis-acting elements, which enable the transgene to be encapsidated into the viral particles (5). This HIV-based viral vector was found capable of mediating stable reporter gene expression in neurons in rat brain (5). In order to further minimize the risk of triggering host immune responses and producing replication-incompetent recombinant viral particles, second and third generations of lentiviral vectors were developed by removing additional viral sequences or separating genes encoding the structural and other components required for packaging the viral genome onto three or four plasmids (6-8). Lentiviral vectors can accommodate up to 16 kb proviral length; however the viral titers are remarkably reduced when the viral genomic size increases (9). To obtain usable titers, the maximal packaging size of lentiviral vectors is estimated to be approximately 11 kb (9). These replication-deficient, self-inactivating vectors lead to long-term expression of transgenes with minimal immune response and inflammation, and provide neuroprotective effects in various models of CNS diseases (10-13). In addition to HIV-1-based lentiviral vectors, vectors derived from alternative lentivirus, such as equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV), have also been developed for gene delivery to the CNS (14-16). Since these viruses in their native forms are non-pathogenic to humans, EIAV-, SIV- and FIV-based vectors theoretically offer the advantage of biosafety for use in human gene therapy.

In order to improve tropism, viral vectors can be pseudotyped by modifications to the envelope proteins. The VSV-G envelope protein is widely used for pseudotyping lentiviral vectors due to its high stability and the potential to generate high-quality and high-titer stocks (17). However, several limitations exist with VSV-G-pseudotyped lentiviral vectors. First, and perhaps foremost, the VSV-G protein has been shown to be cytotoxic (17). Second, although VSV-G-pseudotyped vectors provide a broad tropism toward mitotic and post-mitotic cells, such as neurons and glia (5, 18), they do not undergo retrograde transport, an important mechanism to mediate the entry into the CNS from nerve endings of various viruses such as herpes and rabies (19, 20), and to mediate high-efficiency foreign gene delivery to the CNS via peripheral injection of recombinant viruses. As an alternative to pseudotyping with the VSV-G protein, lentiviral vectors can be pseudotyped using other envelope proteins, such as murine leukemia virus (MuLV), rabies virus and rabies-related virus envelope proteins. These alternatively pseudotyped lentiviral vectors exhibit reduced cytotoxicity and are capable of transducing CNS cells *in vitro* and *in vivo* (21, 22). Furthermore, rabies virus glycoprotein-pseudotyped lentiviral vectors preferentially transduce neurons, undergo retrograde transport and can be expressed in distal neurons *in vivo* after peripheral delivery (22-24). These traits have led to the exploration of gene therapy based on a rabies-pseudotyped vector in a model of motor neuron disease (25).

Lentiviral vectors also have great promise for *ex vivo* gene therapy due to their property of integration of viral genome into host genomes and their ability to mediate stable gene transduction of cell lines and primary cells. In this paradigm, lentiviral vectors are used to efficiently transduce neural stem/progenitor cells *in vitro* (26), and these are then developed as a delivery vehicle of therapeutic genes. NSCs modified with lentiviral vectors carrying various genes have been explored as a treatment in different models of CNS diseases (19, 27, 28).

Overall, lentiviral vectors offer stable and long-term transgene expression in CNS cells, particularly in post-mitotic neurons, with minimal immune response (5). These features allow them to be a highly attractive tool for *in vivo* and *ex vivo* gene therapy for CNS diseases. A lentiviral vector-based clinical trial for Parkinson's disease is currently underway (4). Further studies are focusing on novel non-integrating lentiviral vectors that are deficient in integrase activity. This modification may minimize the risk of integration-induced oncogenic mutations (29, 30). With increased biosafety, the application of lentiviral vectors in the clinical setting may become more prominent in the near future.

3.2. Adenoviral vectors

Adenoviral vectors were one of the first viral vectors employed for gene transfer into the CNS. As

opposed to lentivirus, adenovirus cannot integrate into the host genome and therefore mediates transient, rather than stable, gene expression (31). However, adenoviral vectors are relatively easy to make and can be purified and concentrated up to 10^{12} to 10^{13} viral particles/ml, making them useful for gene therapy application. Adenoviral vectors are the most heavily used viral vectors in clinical trials for human diseases, with the majority of studies focusing on the field of cancer gene therapy (32). However, no adenoviral vector-based clinical trial is currently underway for neurodegenerative diseases.

Adenoviruses contain five early genes (E1A, E1B, E2, E3 and E4) that are involved in viral replication. In adenoviral vectors, the regions of early adenoviral genes are usually replaced with an expression cassette containing the gene(s) of interest. The first generation of adenoviral vectors, which lack E1 and/or E3 and thus lose their ability to replicate, were found to elicit significant immune responses (even in the brain) due to pre-existing anti-viral immune responses (33-35). To avoid adenovirus-trigged immune response, the second generation of adenoviral vectors was generated by making additional deletions of E2 and/or E4 from the viral genome (36, 37). This generation increased cloning capacity compared to the first-generation vectors, but still did not completely avoid *in vivo*-associated immunogenicity due to the remaining viral genes. The third generation, also known as "gutless" adenoviral vectors, are devoid of all coding viral regions and contain only a packaging signal (38). In addition to minimizing the potential immune response (38, 39), these high-capacity adenoviral vectors can accommodate up to 36 kb of foreign DNA (38). Despite not incorporating into the host genome, "gutless" vectors achieve stable gene expression in the brain for up to one year (40, 41). Although these vectors still induce a capsid-mediated immune response in the rat brain (42), they display stability and efficiency in CNS gene delivery, especially for the delivery of larger molecular weight genes.

3.3. Adeno-associated viral (AAV) vector

AAV is a non-pathogenic virus and thus an attractive tool for human gene therapy. AAV vectors can transduce both dividing and non-dividing cells, can offer stable long-term expression, and can be generated at high clinical-grade titers (43). Thus, AAV is currently at the forefront of gene delivery for CNS diseases. Whereas adenoviral vectors are used predominantly in clinical trials for non-neural human diseases, AAV vectors have been applied the most to neurodegenerative diseases.

AAV is a single-stranded DNA virus. Its genome encodes two proteins, rep and cap. Rep is responsible for the replication, integration and rescue of virus, whereas cap encodes capsid structural proteins required for housing the viral genome. The rep-cap genome is flanked by inverted terminal repeats (ITRs), which are also required for the replication, rescue, integration and packaging of AAV (44). However, efficient replication of AAV genome and production of infective particles requires co-infection with a helper virus, such as adenovirus or herpes virus. After entering the host cell by receptor-mediated endocytosis, AAV engages in a lytic life cycle only in the presence of a helper virus. In the absence of helper functions, AAV genomes integrate into the host chromosome in a site-specific manner (45-47) to establish the latent infection, which does not lead to obvious consequences for the host cell (47). Thus, this requirement for the lytic cycle can be exploited for vector propagation and/or control of subsequent gene delivery.

Using adenovirus as a helper virus, Kaplitt *et al.* (48) were the first to develop an AAV vector for CNS gene delivery. They generated an AAV vector by replacing the rep and cap genes with lacZ and tyrosine hydroxylase (TH) gene, and demonstrated that administration of AAV vectors resulted in foreign gene expression up to four months in neurons as well as in glia *in vivo*. In contrast, AAV vectors may also be generated via a "helper-free" system which avoids the helper virus infection. All elements required for AAV production can be provided by transient transfection of helper genes containing plasmids in host cells (49, 50), or by established stable cell lines (51). Recently, self-complementary AAV (scAAV) vectors, sometimes called double-strand AAV, have been developed. The dimeric genomes packaged in scAAV are formed by deleting the terminal resolution site sequence from ITR (52, 53). This results in earlier onset of gene expression and higher transduction efficiency compared with regular single-strand AAV (ssAAV) genomes (54). For gene delivery to the CNS, scAAV vectors have been shown to achieve a better foreign gene transduction than ssAAV (55, 56). However, the insert capacity of scAAV vectors is only half that of ssAAV vectors.

Various cap genes, which determine the AAV serotype, are associated with differential tropism. Several serotypes of AAV have been used for gene delivery to the CNS. Among them, AAV serotype 2 (AAV2) is the predominantly studied and the best characterized serotype in AAV biology. AAV2 exhibits a strong and specific neuronal tropism (57). In addition, AAV2 has a low potential for insertional mutagenesis and appears to be nontoxic when directly injected (58). Most ongoing clinical trials for CNS diseases use AAV2 vectors, and preliminary data suggests that these vectors are well tolerated (59, 60). However, AAV2 has a relatively limited spread through the brain parenchyma (61). Apart from AAV2, several other serotypes have also been tested for gene delivery to the CNS and appear to possess unique traits that can be exploited in different disease contexts. AAV5 transduces both neurons and astrocytes not only at the site of injection but also at more distant sites (62). AAV4-mediated gene delivery is preferential to ependymal cells and astrocytes in SVZ (63). In addition, AAV1, 6, 8 and 9 enable transgene delivery to the neonatal and adult mouse brain with greater efficiency and distribution than AAV2 (64-66). Recently, efficient gene transfer mediated by AAV8 was also observed in monkey brains with a preferential tropism for neurons but not for glia (67). AAV8 and AAV9 have both been studied for gene therapy in various models of CNS disease (68, 69).

Overall, their biological features allow AAV-derived vectors to be promising gene transfer vectors for use in gene therapy. They currently represent the

Table 1 Key features of different viral vector systems

	Lentivirus	**Adenovirus**	**AAV**	**HSV**
Vector genome	ssRNA	dsDNA	ssDNA	dsDNA
Insert capacity	~11kb	8~36kb	~4.7kb	40~150kb
Host chromosomal integration	Yes	No	Low frequency	No
Immunogenicity	Low	Moderate	Low	Recombinant virus: high Amplicons: low
CNS tropism	Glia and neurons	Glia > Neurons	Neurons > Glia	Neurons
Duration of expression	Months ~ years	Weeks ~ months	Months~years	Weeks~year
Clinical trial for CNS diseases	Yes	No	Yes	Yes

predominant vector for gene therapy in clinical trials for neurodegenerative diseases. Despite a recent study demonstrating that the AAV vector capacity can be expanded from approximately 4.7 to 6 kb by removing the capsid protein subunit Vp2 (70), a significant limitation of AAV vectors is their relatively small cloning capacity compared to other viral vector systems. Thus, the development of alternative larger capacity viral vectors remains a necessity.

3.4. Herpes simplex virus

Herpes simplex virus (HSV) contains a double-stranded DNA genome enclosed in a protein capsid surrounded by a lipid membrane envelope. The 152-kb genome of HSV is the largest and most complex of all the viruses being developed for gene therapy. However, vectors derived from HSV, which is a naturally neurotropic human virus, can maintain their presence in sensory neurons for long periods after infection. Thus, they are very attractive and suitable for CNS gene therapy (71-73).

Two types of HSV vector systems have been developed: recombinant virus and amplicons. Recombinant virus can be divided into replication-competent attenuated vectors and replication-defective vectors. Replication-competent attenuated vectors contain essential genes for *in vivo* replication and retain the ability to multiply in actively dividing cells. Replication-defective vectors, on the other hand, are created by deleting immediate early (IE) genes that are required for replication, including the viral thymidine kinase gene. Nonessential viral genes in recombinant HSV vectors can be replaced by transgenes of interest at different sites in the viral genome, providing a large capacity for use in gene therapy. These vectors can usually accommodate up to 40 kb of transgenic DNA. Replication-competent attenuated vectors have been used in Phase I and II human trials for malignant brain tumors (74). However, these vectors exhibit cytopathic effects due to the presence of IE gene products (75). Replication-defective HSV vectors exhibit diminished toxicity, but result in severely reduced transgene expression (76, 77). These vectors are currently being used in a Phase I clinical trial for the treatment of pain (78).

Also known as defective helper-dependent vectors, amplicon vectors contain plasmid DNAs with only minimal HSV replication (ori) and packaging (pac) DNA sequences in the viral vector genome. Amplicons require HSV helper functions, which usually are provided by recombinant viral vectors. To improve safety, a series of helper-free amplicon vectors has been developed. Today, bacterial artificial chromosome and hybrid amplicons (containing the ori and pac sequences from other viral vectors) can be used for amplicon packaging, and have been produced in high titers (79). Compared to recombinant HSV vectors, amplicons have reduced cellular toxicity and lower immune response (80). In addition, the capacity to carry large fragments of foreign DNA allows amplicons to be used not only for the insertion of entire genomic loci but also for the addition of various elements, including promoters, inducible systems for regulated gene expression or several separate expression cassettes (81).

HSV vectors have been used in CNS clinical trials for brain tumor therapy (82), but HSV amplicon vectors have not yet been tested in human clinical trials. However, the potential of HSV amplicon vectors for CNS gene therapy is promising based on animal studies (83-87). Overall, the HSV vector system, with its large transgene capacity, offers a useful tool for gene delivery to the CNS that allows for cell-specific and regulated gene expression of multiple genes. Table 1 summarizes key features of different viral vector systems.

3.5. Promoter effects on viral vector-mediated transgene expression

The promoter is clearly one of the major elements that influence transgene expression levels. Most viral vector-mediated gene transfer is under the control of strong constitutive promoters. The human cytomegalovirus (CMV) immediate early promoter has been widely used in almost all viral vector systems and is one of the strongest promoters available for gene therapy in most tissues and cells. However, this promoter can be silenced following *in vivo* gene therapy (88), especially in lentivirus-mediated gene delivery. Although it has been reported to drive neuronal gene expression *in vitro* (89), data from our lab (unpublished data) and others (90, 91) demonstrated only weak CMV promoter activity in neurons but strong activity in astrocytes in lentivirus-mediated gene transfer. Other pan-cellular promoters that have been applied to gene transduction in the CNS include phosphoglycerate kinase 1 (PGK1), elongation factor 1-α (EF1-α), the viral promoter Rous Sarcoma Virus (RSV), and a hybrid CMV/β-actin (CAG) promoter. All these promoters can successfully drive gene expression in the brain (92-94) but with potential individual differences in efficacy and/or regional preference. For example, the CAG promoter appears to lead to higher transduction efficacy in either striatum or white matter compared to CMV and EF1-α (94). The RSV promoter was shown to exert stronger activity compared to the CMV promoter (90, 92) and produces a region-specific expression in the brain when used with an adenoviral system (92). However, these promoters have not been

systematically analyzed in the same vector background. To better understand the efficacy of lentiviral vectors driven by different promoters and the effect of the phenotype of transduced cells in the CNS, we constructed a series of lentiviral vectors driven by CMV, RSV, CAG, EF1-α, ubiquitin C, PGK1, synapsin I and neuron-specific enolase (NSE) promoters. Our results indicate that the synapsin I promoter has the highest transduction efficacy, followed by the constitutive promoter PGK1, in neurons both in primary culture and in the brain. Interestingly, the CMV promoter had the lowest transduction efficacy (unpublished data). As opposed to the differences observed in transduction of neurons, the various viral promoters drove equally high-efficiency gene transduction in glial cells.

The use of cell-specific promoter elements is one approach to achieve targeted transduction in the CNS. NSE, synapsin I, tubulin and platelet-derived growth factor β-chain promoters were commonly chosen for neuron-specific expression. All of these promoters drive persistent and selective neuronal expression *in vitro* and *in vivo* (90, 94-97). However, several lines of evidence (95, 96) and our unpublished data indicate that the synapsin I promoter has the highest specificity for neuronal expression. Our laboratory has tested a wide array of promoters in the context of lentiviral vector gene delivery. We found that synapsin I promoter is the best and strongest promoter in lentivirus-mediated gene delivery for neurons, in the setting of both primary cultures and *in vivo*. As an example of neuronal subtype-specific targeting, tyrosine hydroxylase (TH) upstream promoter can be used as a dopamine neuron-specific promoter. Oh *et al.* (98) demonstrated that TH promoter could specifically drive transgene expression in dopaminergic neurons of the substantia nigra *in vivo*, and may be useful for tracing dopamine neuronal circuits in the brain. In addition, the glial fibrillary acidic protein (GFAP) promoter was demonstrated to selectively drive transgene expression in astrocyte *in vitro* and *in vivo* (94, 99, 100). It was reported that astrocyte-specific expression of GDNF by GFAP promoter could prevent DA neuron degeneration in a Parkinson's disease model (101). Our study also found that viral promoters such as CMV and RSV prefer to transduce astrocytes in lentivirus-mediated gene transfer (unpublished data). Interestingly, the combination of a CMV enhancer with neuronal-specific promoters yielded a higher transgene expression compared to the neuron-specific promoter alone (96, 102). However, for most neuron-specific promoters, the fusion of CMV enhancer significantly decreased their neuronal specificity (96).

Overall, the choice of promoter is crucial for efficiency, specificity and longevity of transgene expression in viral vector-mediated CNS gene delivery. Neuron or glia selective gene delivery can be achieved by using cell-specific promoters, which may be more useful than constitutive promoters in the context of future gene therapy applications for CNS diseases.

4. CEREBROVASCULAR DISEASES

4.1. Ischemic stroke

Stroke is the nation's third leading cause of death and the most common cause of permanent disability in adults. About 80% of strokes are ischemic and 15% are hemorrhagic. Thus far, the only drug that has been used successfully to treat acute ischemic stroke in the clinic is the clot-dissolving drug tissue plasminogen activator (tPA). However, tPA must be administered within 3 hours of the onset of an ischemic stroke, making it a viable treatment for less than 5% of patients (103, 104). Thus, strategies providing a wider inclusion of patient populations as well as combinatorial approaches targeting neuroprotective signaling are needed for the treatment of stroke. Delivery of exogenous genes to the brain is believed to be able to alter the post-ischemic pathophysiologic process, and thus has become an attractive treatment for ischemic stroke. Several viral vectors have been used for gene therapy in experimental stroke models. Here we review the application of viral vectors in ischemic stroke based on the type of vector.

4.1.1. HSV vectors

HSV vectors containing neuroprotective genes were first tested. Lawrence *et al.* showed that HSV-mediated overexpression of glucose transporter following transient focal cerebral ischemia improved the survival of striatal neurons (105). Since it is well established that apoptotic cell death occurs during cerebral ischemic injury (106), anti-apoptotic genes are another class of candidates for gene therapy against stroke. Bcl-2 is one of the most studied anti-apoptotic genes. Overexpression of this gene by HSV either before (107) or immediately after focal cerebral ischemia (108) was able to prevent stroke-induced loss of neurons in the striatum. In addition to anti-apoptotic proteins, heat shock proteins (HSPs) can also attenuate cerebral ischemic injury. Injection of HSV vectors expressing the inducible form of HSP70 after ischemia significantly improved striatal neuron survival (109). However, HSP70 or Bcl-2 overexpression by HSV vectors were no longer protective when delivered 5 hours after ischemia (108, 109). Thus, other genes and vector systems are still under investigation in order to further expand the post-ischemic therapy window.

4.1.2. AAV vectors

AAV vectors have become the most commonly used vehicles for ischemic stroke gene therapy. AAV vector-mediated Bcl-2 overexpression prevented ischemia-induced DNA fragment in the CA1 region of gerbil hippocampus (110). Similarly, AAV-mediated Bcl-w overexpression in cerebral cortex and striatum significantly reduced infarct volume and improved neurological function (111). In addition, our laboratory (112) identified a novel anti-apoptosis protein designated as Apaf-1-interacting protein (AIP), which functions as an inhibitor of the Apaf-1-caspase-9 pathway. AAV-mediated overexpression of AIP in the hippocampus significantly promoted neuronal survival after transient global cerebral ischemia.

Neurotrophins are another class of candidate genes for stroke gene therapy. These genes include brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor

(NGF), all of which have substantial evidence supporting a role in neuroprotection and attenuation of ischemic neuronal injury. AAV-mediated delivery of BDNF or NGF to the striatum 4-5 weeks prior to ischemia moderately mitigated neuronal death following stroke and led to detectable functional sparing in a rat transient MCAO model (113). Similarly, AAV-based GDNF gene delivery immediately after ischemia was reported to rescue cortical neuronal cells by preventing apoptosis (114). GDNF delivery by adenovirus or HSV was also shown to decrease apoptotic cells and protect against ischemic injury in various ischemic animal models (84, 115, 116). However, long-term AAV-mediated high levels of GDNF were also shown to provide no protection to striatal neurons after MCAO; in fact, they exacerbated ischemia-induced neuronal loss and, moreover, failed to promote functional recovery (117). In addition, AAV-mediated BDNF delivery to hippocampus was shown to suppress ischemia-induced neurogenesis in a rat stroke model (118). However, conflicting results were observed by Gustafsson *et al.*, who found that high levels of BDNF release via AAV retrograde axonal transport increased the vulnerability of striatal interneurons to stroke-induced damage (119). The above results indicate that viral vector-based neurotrophin gene therapy for stroke may be more complex than previously assumed. More research is needed to further assess its effects on stroke.

In addition to rescuing ischemia-induced neuronal loss, viral vector-based gene therapy for stroke also can aim to promote post-ischemic angiogenesis and neurogenesis. It is established that vascular endothelial growth factor (VEGF) plays
multiple roles in post-ischemic pathophysiologic progress. In a gerbil global cerebral ischemic model, injection of AAV-VEGF into the lateral ventricle was reported to significantly attenuate CA1 delayed neuronal death and post-ischemic learning deficits (120). However, systemic delivery of VEGF increased BBB damage and induced brain edema in the early phase after ischemia (121). To reduce VEGF-induced BBB disruption in cerebral ischemia, an AAV vector containing VEGF under the control of hypoxia-responsive element was constructed (122). Injection of this VEGF vector reduced the infarct volume and neuronal loss induced by transient MCAO. Inclusion of the hypoxia-responsive element also restricts VEGF expression to areas of ischemia and minimizes its side-effects. Another approach to reduce VEGF-induced BBB damage is the combined use of AAV encoding VEGF and Angiopoietin 1 (Ang1), which can reduce permeability of endothelial cells induced by VEGF (123). Co-transduction of AAV-Ang1 enhanced VEGF-mediated formation of mature neovessels and protection against ischemic injury, with reduced side-effects on BBB permeability. Apart from VEGF, insulin-like growth factor-1 (IGF-1) was recently used for stroke gene therapy. Overexpression of IGF-1 prior to ischemic injury through an AAV transduction system promoted long-term functional recovery due to enhanced neovascularization and neurogenesis (124). A similar effect was also achieved by injection of IGF-1 expressing AAV vector at 24 hours after stroke (125), suggesting AAV-based IGF-1 gene delivery may provide a long therapeutic time window.

4.1.3. Lentiviral vectors

Lentiviral vectors also provide potential therapeutic tools for use in cerebral ischemic stroke. Human anti-apoptotic Bcl-2 or GDNF genes overexpressed by lentiviral vectors protected against excitotoxic damage during ischemia in both cultured hippocampal neurons and rat hippocampus (9). Lentiviral vector-delivered matrix metalloproteinase (MMP-9) shRNA also ameliorated ischemic brain injury in rats by increasing BBB integrity (126). Besides direct injection, lentiviral vectors can also be delivered to ischemic brains via neural stem cell transplantation. Transduced neural stem cells survive and secrete neurotrophic factors, such as NT-3, and enhance functional recovery after stroke in rats (19). In addition, Daadi and colleagues combined bioluminescence and magnetic resonance imaging (MRI) to show that neural stem cells survived for 2 months after transplantation and differentiated into neurons, oligodendrocytes and astrocytes in stroke-damaged rat brains (127). Real-time imaging provides an important non-invasive tool useful for tracking and monitoring neural stem cell fate and function in ischemic brain environments, and this is very important for successful delivery and safety in the clinical setting.

Accumulating evidence indicates that viral vector-mediated gene therapy may be a therapeutically useful strategy for stroke therapy. Exogenous gene expression alters post-ischemic pathophysiologic progress and improves recovery from ischemic injury. However, the ability to use viral vector-mediated gene transfer for treating stroke will be dependent upon the optimization of the selection of targeted gene(s) as well as the development of non-neurosurgical approaches to administer viral vectors. Further studies focusing on the delivery method of viral vector will be helpful to determine the viability of virus-based gene therapy for human stroke.

4.1.4. **RNA interference**

RNA interference (RNAi) has been developed as a promising tool to specifically downregulate candidate genes. RNAi-mediated gene silencing can be initiated by synthetic double-stranded small interfering RNA (siRNA) or by short hairpin RNA (shRNA), which can be processed to siRNA inside cells. Viral vectors are ideal vehicles for shRNA delivery. By using proper viral vectors, it is possible to achieve persistent shRNA expression and targeted gene knockdown *in vivo*. For the CNS, lentiviral and AAV vectors have been used in this capacity and are able to produce long-term genetic knockdown (128-131). The most commonly used promoters for shRNA focus on ubiquitously expressed polymerase III promoters, such as U6 and H1. Both of these promoters have been successfully utilized to drive viral vector-mediated shRNA expression in the brain (126, 132). Recently, lentiviral vectors expressing shRNA under the control of a polymerase II promoter from CMV knocked down gene expression in the brain (129). In particular, this study also showed neuron-specific gene knockdown using the NSE promoter, offering the possibility of specifically targeting RNAi in the brain

(129). In the context of cerebral ischemia, recent studies demonstrated the therapeutic potential of viral vector-based RNAi. Since the calpain family of cysteine proteases plays a causal role in neuronal cell death following acute brain injury (133, 134), Bevers *et al.* (135) constructed AAV vectors expressing shRNAs targeting micro- and m-calpain. They subsequently demonstrated that knockdown of micro-calpain could decrease neuronal death and preserve hippocampal function in a global cerebral ischemic model (135).

4.1.5. Global cerebral ischemia

Global cerebral ischemia occurs when blood flow to most or all of the brain is halted or drastically reduced, commonly caused by cardiac arrest. Global brain ischemia leads to delayed neuronal death and cognitive impairment. The symptoms may be transient or permanent dependent on whether sufficient circulation is restored within a short period of time. Compared to studies using focal ischemic models, significantly fewer viral therapy studies have been investigated in the context of global cerebral ischemia. With the exception of one study utilizing HSV-amplicon to express human Bcl-2 in the CA1 region of the gerbil hippocampus to protect CA1 pyramidal cells from delayed neuronal death (136), and another study using a Sendai viral vector (developed by Hasegawa's group) to mediate neurotrophic factor expression in yielding neuroprotection in a transient global cerebral ischemia model (137, 138), most viral therapies in global cerebral ischemia have been conducted using adenoviral and AAV vectors. Adenovirus-mediated expression of GDNF and tissue inhibitors of matrix metalloproteinases have been shown to significantly prevent the loss of hippocampal CA1 pyramidal neurons following global cerebral ischemia (116, 139). Moreover, Matsuoka and colleagues demonstrated that administration of adenovirus expressing fibroblast growth factor 2 (FGF-2) promoted progenitor cell proliferation more efficiently compared to continuous intraventricular infusion of FGF-2 recombinant protein following transient global ischemia (140). AAV vectors are also widely used for gene expression or suppression in global cerebral ischemia animal models by injection into the hippocampal CA1 region in most cases. With the use of such AAV-mediated gene regulation, roles for the transient receptor potential melastatin 7 (141), Apaf-1 interacting protein (112), and calpain (134, 135) have been explored in delayed neuronal death following global cerebral ischemia. Interestingly, use of viral vectors can alter the effects of a target protein when compared to recombinant protein delivery. For example, Larsson and colleagues showed that long-term expression of BDNF via viral vector in hippocampus inhibited the formation of new dentate granule cells triggered by global cerebral ischemia in rats (118), contrasting with many previous reports demonstrating that recombinant BDNF administration in the lateral ventricle or ventricular zone stimulates neurogenesis. These apparently conflicting reports warn us that long-term expression of neuroprotective molecules by viral vector may exert differential effects compared to protein delivery, and thus must be tested carefully before clinical application.

4.2. Hemorrhagic stroke

While ischemic stroke is caused by interruption of the blood supply, hemorrhagic stroke results from the rupture of a blood vessel or an abnormal vascular structure. Although few studies focus on gene therapy for hemorrhagic stroke, emerging findings have revealed alterations in gene expression and protein after hemorrhagic stroke such as intracerebral hemorrhage (ICH) (142), and indicate that gene-targeted strategies may be an attractive treatment to promote recovery from hemorrhagic stroke.

ICH, caused by bleeding within the brain tissue, is one of the main types of hemorrhagic stroke. A hematoma in the brain may produce edema through chemical toxicity and/or mass effect. Furthermore, ICH can result in the disruption of the BBB surrounding the hematoma, and lead to cerebral edema. These events are coincident with inflammation, characterized by production of pro-inflammatory cytokines, activation of resident brain microglia and migration of peripheral immune cells into the brain. Upregulation of inflammatory genes, such as IL-1β, is associated with BBB damage. Adenovirus can be used as a vector to transfer the IL-1 receptor antagonist (IL-1ra), a cytokine competitively inhibiting the binding of IL-1 to its receptor, to the brain subject to experimental ICH. Adenovirus-mediated overexpression of IL-1ra attenuated brain edema formation and thrombin-induced intracerebral inflammation (polymorphonuclear leukocyte infiltration) following ICH (143). In addition, studies have provided several attractive candidates for ICH gene therapy. Peroxisome proliferator-activated receptor gamma (PPAR-γ) may be one of these candidate genes because it acts as an important gene in promoting phagocytosis of microglia and hematoma absorption, resulting in protection of surrounding brain cells from ICH-induced damage (144). PPAR-γ activator also increases PPAR-regulated gene expression (*e.g.*, catalase and CD36), thus reducing pro-inflammatory gene expression and extracellular H_2O_2 level (144). Heat-shock proteins (HSPs) may also be good candidates for use against ICH damage, due to their ability to reduce inflammation and apoptosis in a variety of brain insults. Supporting this idea, pharmacological induction of HSP70 was indicated to reduce brain edema and exert neuroprotective effects in experimental ICH (145).

Viral vector-mediated gene transfer has been applied to *ex vivo* gene therapy for ICH. By using a retroviral vector encoding v-myc, Lee *et al.* generated stable, immortalized cell lines of human neural stem cells from primary human fetal telencephalon cultures. Transplantation of these NSCs promoted functional recovery in a mouse ICH model (146). Furthermore, retroviral vectors were used to transfer various genes, including VEGF (147), GDNF (148), BDNF (149), and Akt (150), to this NSC line.These genetically modified NSCs increased survival and differentiation of grafted NSCs, and improved behavioral recovery in a mouse ICH model as compared to parental NSCs.

4.3. Subarachnoid hemorrhage

SAH is another main type of hemorrhagic stroke, resulting from blood extravasations into the subarachnoid space. Vasospasm is an important complication of SAH. Adenovirus has been shown to be an attractive vehicle for

vasospasm-targeted SAH gene therapy. Intracisternal injection of adenoviral vectors containing β-gal under the control of the CMV promoter successfully expressed the reporter gene in adventitia of blood vessels after SAH (151), suggesting recombinant viral vectors can be used for gene transfer to cerebral blood vessels even in the presence of cisternal blood, and may be useful for preventing cerebral vasospasm after SAH. One of the possible candidate genes for vasospasm-targeted gene therapy is nitric oxide synthase (NOS) because the attenuation of vasospastic response could result from enhanced production of nitric oxide via activation of NOS. Likewise, expression of heme oxygenase-1 or constitutive heme oxygenase-2 can lead to the synthesis of carbon monoxide and similarly attenuate the vasospastic response in rats subjected to experimental SAH (152). However, iNOS knockout mice were shown to have significantly less brain edema than their littermates after ICH when hematoma size was similar, suggesting the induction of iNOS may promote edema formation (153). Therefore, careful investigation concerning the pathological contexts must be performed prior to applying these genes in hemorrhage therapy.

5. NON-VIRAL GENE DELIVERY COMPARED TO VIRAL VECTOR

Although viral delivery has dominated the gene therapy field, non-viral gene delivery has made significant strides recently and, if efficiency improves, may yield significant advantages over traditional viral vectors, such as convenient delivery and reduced risk of pathogenic and immunological complications (154). Non-viral vectors include naked DNA itself or chemical carrier-mediated transgenic DNA ferried to the cellular target (155). Owing to rapid clearance by serum nucleases and the mononuclear phagocyte system, the application of naked DNA delivery is limited. Thus, several kinds of chemical carriers have been designed to improve gene expression and delivery efficiency. Lipids and polymers are the primary carriers for non-viral gene delivery. Recent progress in non-viral vector gene delivery *in vivo* has been made in the use of nanoparticles. Several nanoparticles have been demonstrated to transfer report gene to the brain efficiently. For efficient brain-targeting gene delivery, biodegradable polyamidoamine (PAMAM) esters are being explored and developed (156). Kim *et al.* (157) showed that the transfection of HMGB1 siRNA with PAMAM ester markedly reduced infarct volume in the postischemic brain. Modification of the PAMAM to transferrin (Tf)-conjugated, pegylated PAMAME gives the advantage of accessing the brain from blood via transferrin-receptor-mediated transcytosis and delivering exogenous genes into the brain without damaging the BBB or requiring intracerebroventricular injections. Widespread expression of an exogenous gene was observed in mouse brain after i.v. administration of Tf-conjugated pegylated nanoparticle. However, non-viral vectors still have significant disadvantages when compared to viral vector gene therapy. Typically, viral vectors lead to a higher level of transfection and subsequent expression of inserted genes. Viral-mediated gene transfer may overcome the obstacles to CNS gene delivery due to a natural ability to cross cellular membranes efficiently and to infect post-mitotic cells. The ability of some viral vectors to integrate into the host genome and lead to stable long-term expression render them quite suitable for the treatment of CNS diseases, especially chronic neurological disorders. Furthermore, higher transduction efficacy and long-term stable expression of some classes of viral vectors, especially lentivirus, AAV, and HSV, make them attractive carrier systems for introducing genes into the brain, both for purposes of neurobiological research and for gene therapy of neurological diseases (158).

In addition to direct application of genes to the brain, genes can be transferred to target tissue using genetically modified cells which have been transduced with viral vectors encoding therapeutic genes. Although non-neural cell types were first used for *ex vivo* CNS gene delivery (159), the strong tropism of neural stem cells (NSCs) for the nervous system (160) make them an ideal vehicle for this approach. NSC-mediated gene delivery as a therapeutic tool has been tested in various neurological disease models. As discussed above, transplantation of retroviral vector immortalized human NSCs promoted functional recovery in a mouse ICH model (146). Administration of gene-modified NSCs was able to not only prevent neuronal death but also improve neurological recovery following focal cerebral ischemia (146-150, 161-163). Although NSC-mediated gene delivery combining gene and cell therapy has great potential for the treatment of CNS disease, there are still many hurdles that need to be overcome for its use in therapy.

5. SUMMARY

In recent decades, gene therapy has become a highly feasible clinical option for cerebrovascular diseases. This field will continue benefiting from improvements in elucidating mechanisms of molecular neuropathology and optimizing sophisticated technologies, including RNA interference and optogenetics. Although the CNS faces specific obstacles in terms of gene delivery, various viral vectors have been successfully utilized to deliver genes to the CNS, indicating viral vectors as ideal vehicles for CNS gene therapy. However, the delivery and diffusion of viruses throughout large brain structures and the sustained expression of gene candidates via viral vectors still need to be improved significantly. Further studies on the safety and toxicity of viral vectors, and potential inflammatory and immune responses to them, are required. Carefully addressing these hurdles may move gene therapeutic approaches for cerebrovascular diseases from pre-clinical neuroscience to a clinical reality.

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Send correspondence to: Guodong Cao, Department of Neurology, S505 BST, University of Pittsburgh, School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15261, Tel: 412-648-8953, Fax: 412-648-1239, E-mail:caog@upmc.edu