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EXPERT REVIEW

Drug Delivery Systems for the Treatment of Ischemic Stroke

Taiyoun Rhim • Dong Yun Lee • Minhyung Lee

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ABSTRACT Stroke is the third leading cause of death in the United States. Reduced cerebral blood flow causes acute damage to the brain due to excitotoxicity, reactive oxygen species (ROS), and ischemia. Currently, the main treatment for stroke is to revive the blood flow by using thrombolytic agents. Reviving blood flow also causes ischemia-reperfusion (I/R) damage. I/R damage results from inflammation and apoptosis and can persist for days to weeks, increasing the infarct size. Drugs can be applied to stroke to intervene in the sub-acute and chronic phases. Chemical, peptide, and genetic therapies have been evaluated to reduce delayed damage to the brain. These drugs have different characteristics, requiring that delivery carriers be developed based on these characteristics. The delivery route is another important factor affecting the efficiency of drug delivery. Various delivery routes have been developed, such as intravenous injection, intranasal administration, and local direct injection to overcome the blood-brain-barrier (BBB). In this review, the delivery carriers and delivery routes for peptide and gene therapies are discussed and examples are provided. Combined with new drugs, drug delivery systems will eventually provide useful treatments for ischemic stroke.

KEY WORDS brain · drug delivery · gene delivery · ischemia · stroke

INTRODUCTION

A stroke, also known as *cerebrovascular accident (CVA)* is defined as the rapid loss of brain function due to a lack of blood supply. A stroke may be ischemic (a blood vessel blocked by thorombosis or arterial embolism) or hemorrhagic (a leaking blood) (1). Ischemic strokes account for approximately 80% of all strokes, and are mainly caused by a blood clot that blocks blood flow to the brain (2). Thorombosis, embolism (3), and systemic hypoperfusion (4) can decrease the blood supply, depriving neural cells of the glucose and oxygen they need to function. The bleeding that causes hemorrhagic stroke suddenly interferes with brain function. This bleeding can occur either within the brain or between the brain and the skull. Hemorrhagic strokes account for about 20% of all strokes, and are categorized depending on the site and cause of bleeding. Since strokes have different causes, the treatments should clearly be different, especially in the first minutes after symptoms begin. For example, lowering the blood pressure during an acute intracerebral hemorrhage may delay hematoma growth (5) and decrease the risk of rebleeding (6,7). Reducing blood pressure, however, may be deleterious in ischemic stroke (8,9). Instead, the blockage should be removed chemically, such as tissue plasminogen activator (t-PA), or mechanically (10).

Various chemical, peptide and genetic therapies have been evaluated to treat ischemic stroke in experimental animal models. The brain is physiologically different from other organs. The blood vessels have a tight endothelial layer, the blood-brain-barrier (BBB), that is difficult for drugs to pass through. The BBB protects the brain from other toxic materials and infections, but is one of the biggest obstacles to drug delivery (11). Therefore, in the brain, the delivery method is the most important factor to determine the efficacy of a drug. To deliver drugs to the brain, they have to pass through or bypass the BBB. Intravenous drug injections are inefficient, thus excess drug should be administered. Specific brain targeting ligands have been used to increase the efficiency of drug delivery to the brain. Directly administering drugs to the brain is more efficient, but the procedures are often invasive and damage the brain. Due to this damage, less invasive procedures, such as epi-cortical application and intranasal administration, have been

T. Rhim • D. Y. Lee • M. Lee (✉)
Department of Bioengineering, College of Engineering
Hanyang University, 17 Haengdang-dong, Seongdong-gu
Seoul 133-791, Republic of Korea
e-mail: minhyung@hanyang.ac.kr

evaluated for drug delivery. All of these procedures, however, still require carriers for efficient delivery. Drug carriers for the brain should fulfill several functions. First, they should protect drugs from degradation until they reach their target site. Peptide and gene drugs for example, are easily degraded by enzymes in the tissues or blood. Therefore, drug carriers should protect the peptides and genes. Second, carriers should increase the delivery efficiency across the BBB. Third, carriers should facilitate drug diffusion in the brain tissue. In this review, therapeutic proteins and genes in experimental ischemic stroke models are briefly introduced and their delivery carriers and routes are discussed.

MECHANISMS CAUSING NEUROLOGICAL DAMAGES AFTER STROKE

Ischemic neuronal cells start to die due to a lack of the nutrients and oxygen provided by blood. First, cellular respiration fails, so neuronal cells ferment glucose to lactate. A major effect of the ATP shortage is a dysfunction of energy-dependent membrane ion pumps, allowing influx of sodium and calcium ion and efflux of potassium ion. The presence of calcium triggers the release of the excitatory neurotransmitter glutamate, and intense glutamate exposure produces immediate neuronal swelling, which can be prevented by the removal of extracellular sodium or chloride (12). Excess calcium can overexcite cells, generating free radicals, reactive oxygen species (ROS), and calcium dependent enzymes, such as calpain, endonucleases, ATPases, and phospholipases, in a process called excitotoxicity (13). This process can cause apoptosis, necrosis of neighbor neurons, or both.

The damage caused by stroke is divided into two or three phases: acute, sub-acute, and chronic (14,15). In the acute phase, damage is mainly caused by ischemia and reperfusion (I/R). I/R results in increases of Zn^{2+} and ROS and secretion of excitotoxic neurotransmitters, causing apoptosis and necrosis of neuronal cells. These processes persist for several hours. In the sub-acute and chronic phases, immune cells infiltrate the infarcted region and activate inflammatory reactions. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1β) are upregulated and induce cell death (15–17). These processes persist for days to weeks, gradually increasing the infarct size.

BARRIERS TO CENTRAL NERVOUS SYSTEM (CNS) DRUG DELIVERY

In general, three structural components separate anatomically brain and blood flow; that is, (i) the network of brain capillaries or BBB, (ii) the choroid plexus (CP) or blood-cerebrospinal-fluid barrier (BCSFB) and (iii) the arachnoid membrane. Both the BBB and the BCSFB can actively regulate molecules transport through the fluids in brain. However, when considering about the surface areas of brain barrier systems, the surface area of the BBB (~20 m^2) is more than 1000-fold greater than that of either the BCSFB or the arachnoid epithelial membrane in the human brain (18–20). Therefore, main issue in drug delivery to brain is penetration through the BBB. So, in order to develop BBB-penetrating drugs with CNS therapeutic effects, it is of great importance to understand the mechanisms involved in uptake into and efflux from the brain.

The BBB separate circulating blood stream from the brain extracellular fluid in the CNS. Endothelial cells form along all capillaries and consists of tight junctions around the capillaries, while it do not exist in normal circulation. It restricts the diffusion of large or hydrophilic molecules, while allowing the diffusion of small hydrophobic molecules. The endothelial cells of BBB have active transporter proteins for transporting metabolites. This barrier also has a thick basement membrane and astrocytic end-feet. On the other hand, brain microvascular endothelial cells (BMvECs) constitute the BBB. They form a dynamic interface between cerebral blood flow and the CNS and also generate the coordinated regulation of intercellular adherens and tight junctions, thereby restricting paracellular diffusion of molecules from the circulation into the brain (21–23). Both junctions mutually cooperate to stabilize and regulate the BBB in response to tissue condition. Interestingly, much clinical evidence demonstrates that disruption of microvascular endothelial barrier is a hallmark of various diseases such as stroke, multiple, sclerosis, and diabetic retinopathy (21,24,25). On the other hand, the epithelial ultrastructure of choroid plexus (CP) is very similar to the renal proximal tubules, and transports isotonic fluid across its epithelium like the kidney (26). The most important CP functions are CSF secretion and an effective detoxification system within the brain (27,28). In fact, the CP is one of the main sites of xenobiotic metabolism in the brain (29). Many compounds are transported across the CP epithelium because eleven transporter families with almost 30 individual transport proteins are found in the CP (30). The transporters are members of the solute carrier family (SLC) and ATP-binding cassette (ABC) transporter family.

THERAPEUTIC PROTEINS AND GENES FOR EXPERIMENTAL ISCHEMIC STROKE

Various proteins and genes have been investigated as therapeutic agents in experimental stroke animal models. The proteins and genes are summarized in Tables I and II. There are good reviews for therapeutic proteins and genes

Table I Neurotrophic Factors as a Therapy for Ischemic Stroke

Family	Protein	Delivery route	References
Neurotrophin	Nerve growth factor, NGF	Local injection	(42)
	Brain-derived neurotrophic factor, BDNF	Local injection	(49,50)
		Systemic delivery	(163,173)
	Neurotrophin 3, NT-3	Local injection	(56,57)
	Neurotrophin 4/5, NT 4/5	IV infusion	(58)
GDNF family	Glial cell line-derived neurotrophic factor, GDNF	Local injection	(60)
	neurturin, NTN	Local injection	(174)
	artemin, ART	Local injection	(174,175)
	persephin, PSP	Local injection	(176)

for ischemic stroke (31–33). Some of the therapeutic proteins and genes are discussed here.

Nerve Growth Factor and Related Factors

Neurotrophins, also known as neurotrophic factors, are a family of proteins that regulate the neuronal survival, differentiation, proliferation, and death in the peripheral nerve system (PNS) and central nerve system (CNS) (34). During development, neutrophins play an important role in the ability of a target to prevent neuronal cell death (35). In the adult CNS, neurotrophins are thought to be involved in maintenance and survival of neuronal cells by activating cell survival genes and suppressing suicide genes (36). Nerve growth factor (NGF), the first neurotrophin discovered (37), stimulates nerve growth and maintains sympathetic and sensory nervous systems. NGF is as an extracellular ligand for the high affinity NGF receptor (NTRK1) and low affinity NGF receptor (NGFR), and it activates cellular signaling cascades to regulate neuronal proliferation, differentiation, and survival.

Proteins homologous to NGF were subsequently identified. NGF, Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT 4/5)

Table II Therapeutic Genes for Gene Therapy of Ischemic Brain

Genes	Vectors	Delivery route	References
BDNF	AAV	Local injection	(51–54)
	MSC-Adenovirus	IV infusion	
GDNF	AAV	Local injection	(61)
	Adenovirus		(62)
	HSV		(64)
	Lentivirus		(65)
	Sendai virus		(66)
VEGF	Liposomes	Systemic injection	(154)
	AAV	Local injection	(78)
FGF2	Liposomes	IV injection	(86)
	AAV	Local injection	(87)
HO-1	Polymer	Local injection	(148,153,157)
Kallikrein	Adenovirus	Local injection	(177,178)
		IV injection	(136)
HSP27	HSV	Local injection	(138)
PIGF	Genetically modified MSC-adenovirus	Systemic injection	(146)
Netrin-1	AAV	Local injection	(179)
MANF	AAV	Local injection	(180)
SOD1	AAV	Local injection	(181)
Del-1	AAV	Local injection	(182)
Adrenomedullin	Adenovirus	IV injection	(183)
TIMP1, TIMP2	Adenovirus	IV injection	(184)
Glutamate trasporter-1	AAV	Local injection	(185)
SDF-1α	Adenovirus	Local injection	(186)

share 50% sequence homology and are found in a wide range of vertebrates (38). All NGF-related proteins first exist as precursor protein. They are cleaved by various intracellular proteases, such as plasmin or matrix metalloproteinases, to create mature proteins of 118-120 amino acids that form active homodimers (39). ProNGF has a higher affinity for the common neurotrophin receptor p75NGFR than mature NGF (kd≈10^{-9}M), and induces apoptosis in cultured neurons. While mature NGF binds with high affinity (kd≈10^{-11}M) to tropomyosin-related kinase A (TrkA) (40). The 3 Trk (tropomyosin receptor kinase) receptors (TrkA, TrkB, and TrkC) are receptor tyrosine kinases expressed in mammals (40). Unlike p75NGFR, each neurotrophin molecule has a preferred receptor. NGF prefers TrkA, while BDNF and NT4/5 prefer TrkB (41).

NGF and trkA are reportedly involved in ischemic neuronal injury after focal cerebral ischemia (42). NGF expression decreased in the peri-infarct penumbra area where heat shock protein 70 (HSP70), a marker of injury in the ischemic cortex, is induced. Eliciting NGF expression may help rescue these neurons (42).

BDNF is more common therapeutic agent for neurodegenerative disease. Increasing innate BDNF levels by stimulating BDNF expression has been shown to improve diseases in several Huntington's disease (HD) mouse models (43,44). In Parkinson's disease, BDNF applied directly to dopamine cell-rich grafts increased the survival rate of grafts (45). Many reports have shown that BDNF prevents hippocampal neuronal death in a rat model of transient forebrain ischemia (46–48). Administering intraventricular BDNF before focal cerebral ischemia and intraparenchymal BDNF after significantly reduced the infarct volume, primarily in the cortex (49,50). The BDNF gene has also been evaluated as a gene therapy for stroke (51–54).

The therapeutic effects of the neurotrophin 3 (NTF3) on neurological diseases have been evaluated. An adenoviral vector expressing NT-3 (55) or a protein injection (56,57) into the spinal cord, regenerated injured dorsal root axons, sensory neurons, or both. NTF 4/5 has also been tested as a candidate therapeutic molecules, but it is less potent than other neurotrophins in promoting cell survival after stroke (58).

Glial Cell Line-Derived Neurotrophic Factor and Related Factors

A second family of neurotrophic factors is called the glial cell line-derived neurotrophic factor (GDNF) and includes the related factors neurturin (NTN), artemin (ART), and persephin (PSP). The GDNF family proteins are basic, dimeric, secreted proteins with a cysteine knot structure (59). These proteins promote survival of many types of neurons including peripheral autonomic and sensory neurons, as well as midbrain dopaminergic neurons (59). Since GDNF and related proteins protect neural cells, their roles in ischemic stroke have been studied. An intrastriatal infusion of GDNF after stroke promoted striatal neurogenesis. GDNF delivery enhances cell proliferation in the subventricular zone (SVZ) and neuroblast recruitment in the striatum first, then it increases the number of new mature neurons (60). The GDNF gene has also been extensively studied for stroke gene therapy (61–66).

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor and has been extensively studied to treat various ischemic diseases (67–70). The pro-angiogenic effect of VEGF facilitates blood flow recovery into damaged areas of the brain (71). Furthermore, VEGF is also neuroprotective and facilitates the growth of neuronal progenitor cells (72). The VEGF gene has been evaluated as a gene therapy for spinal cord injury and stroke (73,74).

However, it should be noted that VEGF increases the permeability of the BBB and induces brain edema. Previously, it was reported that gene transfer of anti-VEGF inhibitor, soluble Flt-1 (sFlt-1) reduced infarct volume and brain edema (75). In addition, non-specific VEGF expression might induce endothelial cell outgrowth and promote the formation of endothelial cell derived tumors (76,77). Therefore, tightly regulating VEGF expression may reduce the side effects. The hypoxia inducible VEGF genes have been evaluated as therapies for stroke (78). Specific promoters, untranslated regions (UTR) of the RNA, and oxygen-dependent degradation (ODD) domains of the proteins have been evaluated to facilitate hypoxia inducible gene expression (74,78–83). Hypoxia inducible VEGF genes have had some success in ischemic heart and spinal cord injury gene therapy (74,79,80,82,83), suggesting that hypoxia inducible VEGF genes are also useful for stroke gene therapy.

Fibroblast Growth Factor 2

Fibroblast growth factor 2 (FGF2) has a cytoprotective effect and has been widely investigated as a therapeutic agent for ischemic heart disease and limb ischemia (84,85). The FGF2 gene was also used as a therapeutic gene for ischemic brain therapy (86,87). FGF2 is a mitogen for neural stem cells and is involved in neurogenesis (88). The FGF2 gene has been delivered with AAV and liposomes (86).

Heme Oxygenase-1

Heme oxygenase-1 (HO-1) is an anti-oxidant enzyme involved in degrading the heme group (89). The products of

heme degradation are CO and biliverdin. HO-1 attenuates adhesion molecule and leukocyte recruitment in ischemic tissues (90,91). Furthermore, HO-1 reduces pro-inflammatory cytokines and chemokines (92,93). HO-1 expression is induced in ischemic tissue, suggesting that it is cyto-protective (94).

BCL Family

Bcl-2 and Bcl-w are members of Bcl-2 protein family. Bcl-2 and Bcl-w are anti-apoptotic and promote neuronal cell survival under hypoxic conditions (95–99). Bcl-2 has been investigated for various ischemic disease gene therapies (100,101). Bcl-2 is also over-expressed in various tumors, suggesting that tight regulation of the Bcl-2 gene therapy is required to avoid side effects such as tumor formation (102,103).

Small Interfering RNAs (siRNAs) and microRNAs (miRNAs)

Recently, siRNAs have been also suggested as therapeutic agents for ischemic brain. The caspase-3 siRNA was evaluated as a therapeutic gene to reduce apoptosis in ischemic brain (104). High mobility group box-1 (HMGB-1) protein was reported as a pro-inflammatory cytokine in the acute and sub-acute phases of stroke (15). Silencing the HMGB-1 gene with an siRNA reduced the infarct volume (15,105).

Antagomirs to specific miRNAs were also suggested as therapeutic agents. Insulin-like growth factor-1 (IGF-1) is a neuroprotectant and involved in ischemic heart disease and stroke. Since it has a binding site in the IGF-1 3′-untranslated region (3′-UTR), it was proposed as a potential negative regulator for IGF-1, (106). Indeed, delivery of an antagomir to miRNA Let7f reduced the infarct volume in stroke animal model (106).

DELIVERY CARRIERS

Carriers for Proteins

Modification of Therapeutic Proteins

Proteins have been modified with polyethylene glycol (PEG) and functional peptides to increase the delivery efficiency. Examples include TAT-linked proteins. The TAT peptide is a cell-penetrating peptide (CPP) that facilitates drug transport across the plasma membrane (107). The HIV TAT peptide has been linked to therapeutic proteins to allow efficient translocation across the BBB for ischemic stroke therapy. Hsp70 was linked with TAT by genetic engineering (108). TAT-Hsp70 was administered intravenously in a transient MCAO animal model at the time of reperfusion and again 14 days later. The TAT-Hsp70 decreased the infarct size dramatically, compared to controls. TAT-hemaglutinin, however, did not have this effect confirming that TAT-Hsp70 was transported across the BBB and had therapeutic effect in the ischemic brain.

PEGylation to proteins is another strategy to increase their therapeutic effects. PEGylated-hemoglobin (PEG-hemoglobin) was evaluated in an animal model of stroke (109). PEG-hemoglobin, named SB1, was intravenously infused through the femoral artery after transient MCAO. SB1 reduced the infarct size, compared with the MCAO control. Furthermore, SB1 improved motor functions in the stroke model. PEGylation has some advantages for drug delivery. PEGylation increases the stability and circulation time of drugs. Furthermore, PEGylation of proteins increases their diffusion. In epi-cortical applications, the inefficient protein diffusion to target sites is a barrier to therapeutic effects (110). PEGylation of EGF increased the diffusion distance of EGF (110). As a result, PEG-EGF reached the subventricular zone (SVZ) efficiently and facilitated the proliferation and migration of neuronal stem progenitor cells (NSPC). PEGylation, however, also has disadvantages such as reduction of the transport efficiency across the BBB (111).

Another modification approach is to fuse proteins with Trojan horse molecules to allow efficient translocation across the BBB. For stroke therapy, the human insulin receptor monoclonal antibody (HIRMab) was used to modify therapeutic proteins (112–114). Trojan horse molecules, such as HIRMab, bind to receptors and facilitate transport across the BBB. For example, the extracellular domain of the tumor necrosis factor receptor (TNFR ECD) was used as a decoy to reduce the effect of TNF-α in inflammation reactions in the ischemic brain. However, TNFR ECD is large and translocated across the BBB inefficiently. Thus, HIRMab was linked to the C-terminus of TNFR ECD (114). Similarly, GDNF was linked to HIRMab (113). In an MCAO model, intracerebral injection of HIRMab-GDNF reduced the infarct volume, suggesting that HIRMab increased the drug delivery efficiency.

Nanoparticles and Microparticles

Nanoparticles (NPs) are an attractive option for the drug delivery into the brain. NPs may protect therapeutic agents from denaturation and increase the translocation efficiency across the BBB. NPs produced with poly-DL-lactide-coglycolide (PLGA) have been evaluated as drug delivery carriers for stroke therapy (115,116). PLGA-NPs were produced by the double-emulsion method. The erythropoietin (Epo) and superoxide dismutase (SOD) proteins were loaded into the PLGA-NPs (115,116). PLGA-Epo-NPs were administered to animal models intraperitoneally every 24 h. The PLGA-

Epo-NPs had a higher therapeutic effect, reducing the infarct size, than Epo without a carrier. At the same dose, PLGA-Epo-NP was approximately 16 times more effective than Epo without a carrier. PLGA-SOD-NPs were administered to animals intravenously (116). In this case, three injection routes were evaluated: the internal carotid artery, the tail vein, and the jugular vein. Administering NPs through the internal carotid artery resulted in more efficiency delivery to the brain than the tail or intrajugular veins. PLGA-SOD-NPs administered though the internal carotid artery, reduced the infarct volume and improved motor activity.

Microparticles have also been used as drug carriers to the ischemic brain. The high mobility group box-1 A box domain (HMGB-1A) was loaded into gelatin microspheres (117). HMGB-1A is an HMGB-1 antagonist (118). HMGB-1 is a key player in inducing inflammatory reactions in the post-ischemic brain. Therefore, inhibiting HMGB-1 is a strategy to reduce the I/R infarct volume. In the experimental animals with transient MCAO, gelatin microspheres containing HMGB-1A were injected locally into the striatum using stereotaxic equipment (117). HMGB-1A without carriers were also anti-inflammatory and cytoprotective in post-ischemic brains. Their therapeutic time range, however, was very short; mainly because HMGB-1A denatured quickly (117). Therefore, protecting the HMGB-1A protein with gelatin microparticles improved the therapeutic effects. In addition, slow HMGB-1A release from the gelatin microspheres increased the therapeutic time range (117). As another example, PLGA microparticles have been loaded with fenofibrate (119). Fenofibrate is a peroxisome proliferator activated receptor (PPARa) activator. PPAR activators have been suggested to be neuroprotective in post-ischemic brains by reducing oxidative stress and inflammation (120,121). Local delivery of fenfibrate-loaded PLGA microparticles protected and increased the therapeutic effects of the drug.

Hydrogels

Hydrogels were used to create a depot of drug in the brain. Injectable alginate hydrogel was developed to delivery drugs to the brain locally (122). *In vitro* tests showed that VEGF was released from the gel over 2 weeks. Locally administering VEGF-loaded hydrogel to the striatum reduced the infarct size and improved motor activity in an animal model. The animals receiving hydrogel only did not have this effect. Although hydrogel is an attractive carrier for the brain, local injection may cause brain damage. Therefore, epi-cortical hydrogel delivery was investigated as a less invasive procedure (110,123,124). In this procedure, the drug-loaded hydrogel was applied to the cortical surface in a simple surgical procedure. In this procedure, the damage to brain tissue was minimal, compared with intraparenchymal injection into the brain. The main issue for this procedure was whether the drug could diffuse to the target site efficiently. The diffusion distance of drugs in the brain tissue is not efficient enough to penetrate from the cortical surface to target sites. The diffusion depends on drug characteristics, such as size and surface charge (125). In particular, protein and DNA drugs can degrade or denature during diffusion, which may reduce their therapeutic effects. PEGylating drugs protects them during diffusion. Furthermore, PEGylation reduces the surface charge of drugs and may increase the diffusion distance. PEG-EGF was loaded into a hyaluronan and methylcellulose (HAMC) hydrogel (110). The hydrogel was applied to the cortical surface. PEG-EGF reached the SVZ and facilitated NSPC proliferation more efficiently than EGF. Epo was also delivered by epicortical application using HAMC hydrogel, and reached the SVZ efficiently (124).

Carriers for Gene Therapies

Viral Vectors

The carriers used to deliver genes to ischemic brains can be divided into 2 groups, viral vectors and non-viral vectors. Various viral vectors have been used for gene delivery in treating stroke. AAV, adenoviral vector, HSV, and lentiviral vector have been evaluated for gene delivery to the ischemic brain. Since therapeutic genes are anti-apoptotic or growth factors, long-term expressions of them may induce deleterious effects such as tumor growth. Therefore, it is important to regulate the genes for short-term expression.

AAV is a single-stranded DNA virus and has been used to deliver various genes, including the GDNF, BDNF, VEGF, and FGF-1, to the brain (Table II). Although antibodies against AAV have been found after systemic injection, AAV does not cause human diseases (126–128). This suggests that AAV is a relatively safe gene carrier. AAV can integrate into the host genome at hot spots (129), However, the integration rate is not high and most transduced AAV genomes exist as episomes or concatamers in the cells (127,130). Furthermore, no oncogenic recombination effects of AAV integration have been reported to date (131). One disadvantage of AAV is the size limit of DNA (5 kb) it can accommodate, which is not big enough for some genes. AAV delivery can be achieved by directly injecting the vectors into the brain (Table II). Glia and neurons were transduced by AAV and expressed the genes at therapeutic levels.

Adenovirus is a double stranded DNA virus that can incorporate much larger genes than AAV (up to 36 kb) (132). Adenovirus enters cells by endocytosis mediated by the coxasckie and adenovirus receptor (CAR) (130). CAR is

expressed in various cell types, suggesting that adenoviral vectors can transduce various cell types, including neuron and glia. Adenoviral vectors induce a relatively high immune response, which limits its gene therapy applications (128). Coating the vector with inert polymers, however, may reduce immune response (133,134) and can change its tropism (135). Various genes, including BDNF and GDNF, have been delivered to ischemic brains using adenoviral vector (Table II). Most gene deliveries using adenoviral vectors were locally injected into the brain, but some studies used IV injection. For example, adenoviral vectors containing the kallikrein gene were injected into tail veins (136), reducing the infarct volume in the ischemic brain. I/R opens the BBB transiently (137), which might provide adenoviral vectors a chance to enter the brain.

HSV is a double stranded DNA virus. HSV has natural neurotropism, which is useful for stroke gene therapy (132). After transduction, HSV exists as an episome or concatamer in cells (130). HSV accommodate relatively large genes, up to 150 kb. The GDNF and HSP27 genes were delivered using HSV into the brain and the gene expression protected the ischemic brain (64,138). Like the adenoviral vector, HSV has a relatively high immune response, which is not desirable and can cause inflammation in the ischemic brain (128,130,139). Therefore, modifying the vector surface may be useful to reduce inflammation.

Lentivirus is a single-stranded RNA virus. The lentiviral vector efficiently transduces non-dividing and dividing cells (140,141). Therefore, it can transduce various cell types, including neuron and glia. Lentivirus is a retrovirus and randomly integrates its genome into a host cell chromosome (132). Stable integration of lentiviral genome into the host genome enables long-term expression, but it may also induce oncogenic recombination. The lentiviral vector can accommodate genes up to 9 kb. The lentiviral vector was used to deliver GDNF for stroke gene therapy (65). The lentiviral vector has also been evaluated as an shRNA gene carrier, and efficiently expresses shRNA in the brain (142). Over-expressing shRNAs may interfere with normal miRNA production by occupying the endogenous miRNA machinery (143,144). Therefore, tissue specific shRNA delivery is desirable to avoid side effects. A lentiviral vector expressing MMP-9 shRNA was injected directly into the brain. The local injection was enough to reduce the infarct volume and minimize liver toxicity due to non-specific shRNA expression (142).

Genetically Modified Stem Cells

Genetically modified stem cells have been used to deliver genes. Mesenchymal stem cells (MSCs) were transduced with an adenoviral vector (53,145,146). Adenoviral vectors have the highest transduction efficiency, and the *ex vivo* approach minimizes the immune response by adenoviral vectors. Furthermore, MSC infused into the blood stream target ischemic areas. Therefore, MSC are useful to deliver therapeutic genes to ischemic brains. Gene expression from an adenoviral vector is transient and persists for several weeks (130). Long-term or permanent expression of therapeutic genes is not always beneficial for stroke gene therapy. Gene therapy for ischemic diseases, such as stroke and ischemic myocardium, may require transient expression. After recovery, continually inhibiting apoptosis and angiogenesis may promote the endothelial cell-derived tumor growth. Thus, transient expression of therapeutic genes in ischemic tissue may be safer.

Genetically modified MSCs were developed to deliver BDNF and PIGF (53,146). MSCs carrying the BDNF gene (BDNF-MSCs) were injected intravenously at 6 h after transient MCAO (53). Unmodified MSCs increased the BDNF level in the ischemic brain, showing that MSCs enter the ischemic tissues and elevate endogenous BDNF expression. BDNF-MSCs, however, had higher BDNF expression and further reduced the infarct volume. Similarly, PIGF-MSCs were infused intravenously induced higher PIGF expression and angiogenesis in the ischemic brain (146).

Non-viral Vectors

Non-viral vectors such as liposomes and polymers have been developed for stroke gene therapy. Non-viral vectors have some advantages over other vectors. Non-viral vectors have low immunogenicity and toxicity. In addition, non-viral vectors can be chemically modified easily to target brain cells (147). Conjugating targeting ligands to vectors can increase the efficiency of gene delivery to brain cells. In addition, conjugating ligands can increase the gene delivery efficiency across the BBB. Some non-viral carriers can be used as drug carriers by simple chemical modification, allowing combined drug and gene delivery.

We previously delivered dexamethasone and the HO-1 gene to ischemic brains (148). Dexamethasone decreased the inflammatory reactions and reduced the infarct volume in the ischemic brain (149,150). To combine this effect with gene therapy, dexamethasone was directly conjugated to a polymeric gene carrier, polyethylenimine (PEI) (Fig. 1a) (151). Dexamethasone-conjugated PEI (PEI-Dexa) functioned as both a gene carrier and a drug carrier. Gene and drug combined delivery using PEI-Dexa had a synergistic effect, with each component enhancing the delivery efficiency of the other (Fig. 1b). The enhanced gene delivery efficiency may have two possible causes. First, dexamethasone conjugated to PEI facilitated nuclear translocation of PEI/DNA complex through the dexamethasone receptor (151). Dexamethasone binds to the glucocorticoid receptor (GR) in the cytoplasm. Upon binding, the dexmathasone/

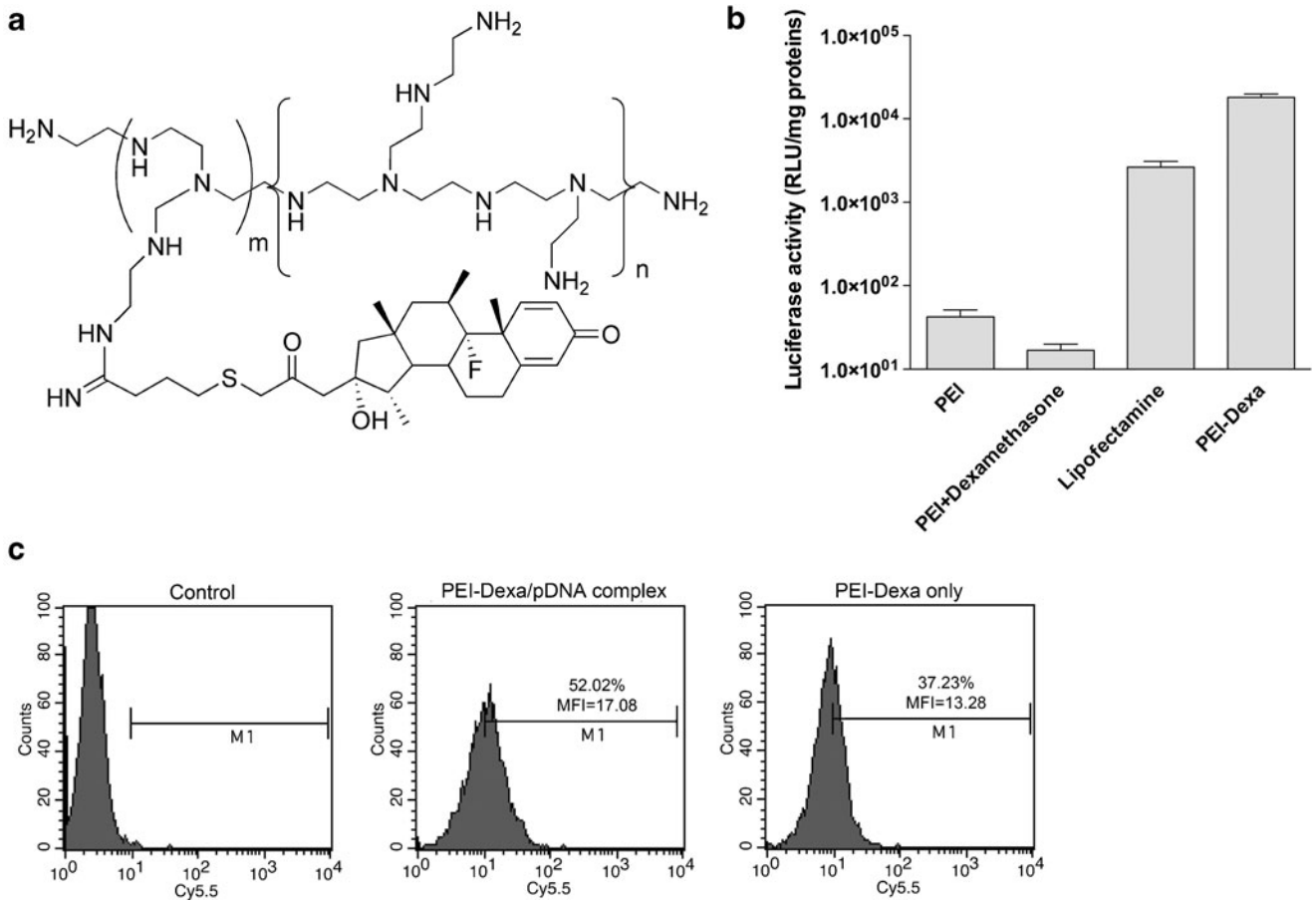

Fig. 1 Drug and gene delivery efficiency of PEI-Dexa. **(a)** Structure of PEI-Dexa. **(b)** Gene delivery efficiency of PEI-Dexa. PEI-Dexa/pβ-Luc complexes were used transfect Neuro2A neuroblastoma cells. After 24 h, the gene delivery efficiency of PEI-Dexa was measured by luciferase assay. PEI and lipofectamine were used as control carriers. **(c)** Cellular uptake of PEI-Dexa/pβ-Luc complex. PEI-Dexa was labeled with Cy5.5. PEI-Dexa/pβ-Luc and PEI-Dexa only were added to Neuro2A cells. The cellular uptake of PEI-Dexa/pβ-Luc complex was measured by flow cytometry (Reprinted from Ref. 148 with permission from Elsevier).

GR complex is transported into the nucleus, because GR is a nuclear receptor and acts as a transcription factor when bound to its ligand. In this process, the PEI and DNA were co-transported to the nucleus. Plasmid DNA larger than 2 kb is not mobile in the cytoplasm (152). Therefore, active transport to the nucleus can increase gene expression level dramatically. Second, GR dilates the nuclear pores during translocation, which facilitates the nuclear translocation of DNA.

Nanoparticles of PEI-Dexa with DNA also increased the dexamethasone delivery efficiency. Dexamethasone alone can enter cells by simple diffusion. However, nanoparticles of PEI-Dexa and DNA enter cells more efficiently by endocytosis (148). Endocytosis is an active process and more efficient than simple diffusion. Therefore, PEI-Dexa nanoparticles with DNA increased the cellular entry rate of PEI-Dexa. Indeed, the PEI-Dexa nanoparticles with DNA had higher cellular entry efficiency than PEI-Dexa alone (Fig. 1c) (148). Due to synergistic effect, the infarct volume was reduced by the combination of dexamethasone and the HO-1 gene (Fig. 2a and b). Furthermore, PEI-Dexa efficiently delivered the HO-1 gene and dexamethasone into the ischemic brain and reduced inflammation reaction (Fig. 2c and d) (148).

Similarly, the amphiphilic peptides comprising 3 arginines and 6 valines (R3V6) were used as a carrier for the combined delivery of dexamethasone and therapeutic DNA (153). The R3V6 peptides formed micelles in aqueous solution. Dexamethasone can be loaded into the hydrophobic core of the R3V6 micelles. In particular, dexamethasone provides a hydrophobic core for micelle formation and facilitated the formation of the R3V6 peptides micelles. Therefore, the dexamethasone loaded R3V6 (R3V6-Dexa) formed a more stable complex with DNA and delivered genes more efficiency than R3V6 micelles without dexamethasone. R3V6-Dexa had a higher HO-1 gene delivery efficiency than PEI in the ischemic brain.

Liposomes were evaluated for brain targeting delivery after systemic administration (86). The genes were complexed with lipofectin, DOTAP, or Fugen6 and injected

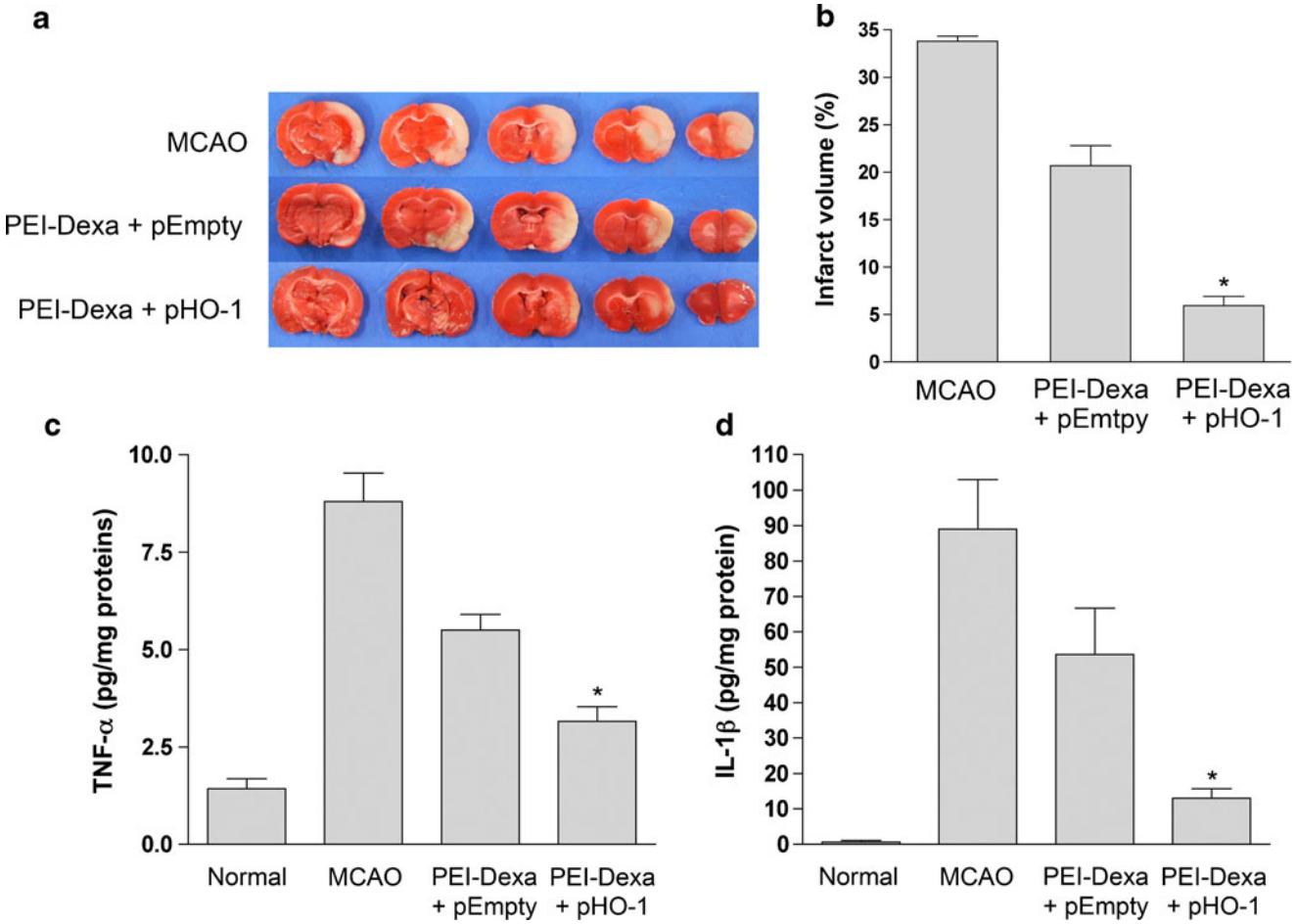

Fig. 2 Neuroprotective effect of PEI-Dexa/pHO-1 complex. **(a)** TTC staining. PEI-Dexa/pEmpty and PEI-Dexa/pHO-1 complexes were injected into the brain 1 h before MCAO/reperfusion. The infarct size was evaluated by TTC staining. The typical staining pattern of each group is presented. **(b)** Infarct volume. **(c)** TNF-α suppression. The brains from each group were harvested and proteins were extracted 24 h after injection of PEI2k-Dexa/pHO-1 complex. TNF-α levels were measured by ELISA. **(d)** IL-1β suppression. IL-1β levels were measured by ELISA. *$P < 0.01$ as compared with MCAO and PEI-Dexa + pEmpty groups (Reprinted from Ref. 148 with permission from Elsevier).

intravenously into animal models. The liposomes delivered the VEGF and FGF2 genes to macrophages in the blood stream. The transfected macrophages then infiltrated the ischemic brain. As a result, macrophages expressing VEGF and FGF2 were detected only in the ischemic region, not in normal brain. Another strategy to increase gene delivery to the brain after systemic administration is to conjugate specific ligands to liposomes and polymers for higher BBB permeability (154). For example, the transferrin receptor is expressed on the BBB and has been used to target drug delivery to the brain. Transferrin was conjugated to the surface of liposome/DNA complexes. Systemically administering the liposome/DNA complexes resulted in delivery of the VEGF gene across the BBB.

Cationic polymers and liposomes may be cytotoxic due to excessive positive charges of the carrier/DNA complexes. The positively charged the complexes can interact with and bind to negatively charged proteins in the cells. This nonspecific binding may interfere with normal protein function. In addition, carrier/DNA complexes with excessive positive charge may interact with and aggregate on the surface of negatively charged cellular membranes. The aggregates may rupture the membrane, inducing cytotoxicity (155). Therefore, carriers should have low charge density to reduce toxicity. Carriers with low charge density, however, cannot form stable complexes with DNA, because the charge interactions between the carrier and DNA are weak. One solution to this problem is biodegradable carriers. When biodegradable carriers form complexes with DNA, the complexes are stable with a high charge density. Upon gene delivery, the biodegradable carriers are easily degraded to reduce charge density and toxicity. Polyamidoamine ester (ePAM-R) is an example of a degradable polymeric carrier for gene delivery to the ischemic brain (156). ePAM-R is a degradable version of the arginine grafted polyamidoamine dendrimer (PAMAM-Arg). PAMAM-Arg

is synthesized by conjugating arginines to primary amines on the PAMAM surface (15). ePAM-R has ester bonds in its backbone and is easily degraded in an aqueous environment. This carrier is less degradable when complexed with DNA. When the carrier releases DNA, it is easily degraded, since the carbonyl groups of the ester bonds become exposed to nucleophilic attack by bases. ePAM-R has been evaluated as an HMGB-1 siRNA carrier (105,156). Another degradable polymer is reducible poly(oligo-D-arginine) (rPOA). rPOA was synthesized with C-9R-C by oxidation between thiols of cysteines (157). The disulfide bonds of rPOA are stable outside of cells. After gene delivery, rPOA is rapidly degraded, since the cytoplasm is a reducing environment with a high glutathione concentration. rPOA was used to deliver the HO-1 gene into the ischemic brain (157). After local administration, rPOA expressed the HO-1 gene more efficiency than PEI, reducing the interleukin-1β level and infarct volume more efficiently.

One common characteristic of polymeric carriers for gene delivery to the ischemic brain is a high arginine content. PAMAM-Arg had a remarkably higher transfection efficiency to primary neuronal and glial cultures than any other liposome or polymer (158). Arginines may increase transfection of brain cells specifically and polymers with high arginine contents may behave like PTDs. Considering that poly-L-arginine had lower transfection efficiency to neurons, however, the carrier shape and arginine distribution in the polymers may also affect the transfection efficiency (158).

DELIVERY ROUTES

Drugs can be delivered to the brain through local or systemic delivery. Local injection to the brain is simple and efficient, since drugs are directly delivered into the brain. However direct injection is invasive, causing damages to the brain. Therefore, the improved methods such as epi-cortical and intranasal applications have been developed. Systemic delivery is relatively non-invasive, compared with local delivery. However, delivery efficiency to the brain is much lower than local delivery. For systemic delivery, targeting carriers with specific ligands and ultrasound application have been developed for high efficiency.

Local Delivery to the Brain

Local delivery is a simple and direct method to deliver therapeutic agents to the brain, bypassing the BBB. The intraventricular and parenchymal injections are widely used for local delivery to the brain (125,159). The intraventricular injection infuses therapeutic agents into cerebro-spinal fluid (CSF). Diffusion of therapeutic agents to their target, however, is inefficient, especially for large particles such as viruses and polymer/DNA complexes. Therefore, acute-parenchymal injections are commonly used for local delivery. Brain injections are performed directly into the core or penumbra of the infarct region. The injection is performed using stereotaxic equipment and a micro-injector. Samples are usually injected slowly, because the dense brain tissue leads to backflow upon rapid injection. For example, our group injects animal at a rate of 1 μl/min. Injection into the core or penumbra of brain ischemia localizes therapeutic agents to the target region where they can have immediate effects.

The local injections may damage brain tissue. A less invasive local delivery method, epi-cortical application, has also been studied. A hydrogel containing therapeutic agents was applied directly to the cortical surface. The drugs then penetrated into the brain. For this method, drugs need to have high diffusibility to reach their targets without losing activity.

Drugs do not diffuse efficiently in the brain, due to its dense tissue. Localizing drugs in a small area of the brain may reduce their efficiency. Therefore, wide distribution of therapeutic agents in the brain is an important factor in determining therapeutic effects. The distribution of the therapeutic agents after local delivery depends on diffusion (125). The convection-enhanced delivery (CED) can also drive wide distribution of therapeutic reagents. CED depends on a pressure gradient created by local injection (125). Depending on the pressure, the bulk flow can carry the reagents through extracellular matrix. Surface characteristics and size of drugs and carriers are the main determinants of CED and diffusion (125). Particles with high positive charged have low mobility in the brain because they bind the extracellular matrix tightly. Particles smaller than 100 nm are relatively free in the brain, but particles larger than 200 nm lose diffusibility.

Intranasal delivery has also been studied to deliver drugs to the brain. Intranasal delivery is non-invasive, compared with other local delivery methods. After intranasal application, drugs can reach the brain by direct transport from olfactory region to the brain. Chemical drugs, proteins, DNA, and cells have been successfully delivered to the brain by intranasal delivery (160). Drugs can be delivered to the brain by olfactory and trigeminal pathways. Olfactory pathway was initially suggested as a drug delivery pathway to the brain. However, trigeminal pathway was also reported as an important pathway to the brain (161). Therapeutic proteins such as IGF, FGF, Epo, and VEGF were delivered to ischemic brains by intranasal delivery. Adenoviral vectors containing the β-gal gene were successfully delivered by intranasal administration (162). Therapeutic gene delivery has also been studied with HMGB-1 siRNA in the ischemic brain (105). HMGB-1 siRNA was delivered by intranasal administration using ePAM-R. HMGB-1 expression was

decreased in various tissues including the amygdala, hypothalamus, cortex, and striatum. This HMGB-1 knockdown reduced the infarct volume in ischemic brains. This suggests that intranasal gene delivery is clearly a promising route for stroke therapy.

Systemic Delivery

Systemic delivery by intravenous injection is less invasive and more acceptable clinically than local injection. However, drug delivery to the brain following systemic injection is inefficient due to the BBB. Therefore, large amounts of drug should be administered, which may cause systemic side-effects. To increase drug delivery to the brain after systemic administration, specific ligands (such as transferrin, a glycoprotein from rabies virus and Tat peptide) are conjugated to the drug or carrier, which induces transcytosis across the BBB (163–168).

Delivery efficiency across the BBB can also be increased with ultrasound (169–172). Focused ultrasound bursts microbubbles of biocompatible materials, producing micro-jets into the endothelial layer that transiently open the BBB. The microbubbles can carry drugs, which are then released at the ultrasound site. Repeated, focused ultrasound with low energy may be useful to target drug delivery to the ischemic brain.

CONCLUSIONS

Current progress in the field of drug delivery to the brain may create new efficient and safe stroke therapies. Some issues remain to be addressed before applying these methods clinically. First, the carriers should be optimized, depending on the delivery methods. The carriers have different conditions between local and systemic administrations. This suggests that the characteristics of the carriers such as charges, sizes, and components should be optimized depending on these conditions. Second, carrier toxicities should be minimized. Some gene carriers, such as adenoviral vectors or HSVs can induce inflammation. Because severe inflammation may cause cell death in the ischemic brain, an immune response to the carrier is undesirable. Similarly, the excessive charge densities of polymers and liposomes may also induce cell death. Third, the delivery efficiency of carriers should be improved. Parenchymal injection into the brain is the most efficient method, but it is an invasive procedure that damages brain tissue. Systemic administration is less invasive than local delivery, but has a low delivery efficiency and is not applicable to therapeutic settings. Various research has focused on increasing targeted brain delivery after systemic administration. For example, ultrasound can increase the delivery efficiency to the brain. Targeted carriers and physical methods, such as ultrasound, should achieve more efficient and less invasive drug delivery to the brain. Finally, therapeutic genes should be carefully selected. A better understanding of the pathophysiology of stroke may help choose appropriate therapeutic genes. In addition, gene expression should be regulated to reduce the side effects.

Although many issues should be addressed for drug delivery to the brain for the treatment of stroke, significant progress has already been made in all of these areas. This suggests that a safe and efficient drug delivery system will likely be available eventually for the treatment of stroke.

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