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Gene therapy of focal cerebral ischemia using defective recombinant adeno-associated virus vectors

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1. ABSTRACT

This review presents our experience and results concerning cerebral stroke gene therapy with a rat model subjected to rAAV-vector delivered IL-1ra and GDNF. The methodology involving the production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus and the creation of a tri-vessel ligation model of focal ischemic cerebral stroke in rats are described in detail. Furthermore, a literature review of other viral vectors, murine models of focal cerebral ischemia and candidates for therapeutic transgenes used for cerebral stroke gene therapy are presented. Lastly, the potentials and limitations of stroke gene therapy are discussed adding an analysis of possibilities of future experiment designs.

2. INTRODUCTION

Gene therapy is defined as the use of nucleic acid transfer to treat or prevent diseases. Originally envisioned as treatments for hereditary disease, it is now being developed to prevent complex polygenic diseases. Genetic therapy requires the understanding of the disease's pathogenesis, identification of therapeutic genes, the ability to target the tissue of interest (brain, blood vessel), and appropriate animal models to probe the therapy (1). There are a number of degenerative and cerebrovascular occlusive diseases where the resulting CNS pathology may be ameliorated, or even prevented, following the introduction of specific genes into the CNS. Significant advances have been made towards these aims by a number of investigators who have demonstrated successful gene transfer into the adult CNS. Gene therapy has several potential advantages over classical pharmacological therapy. Direct administration of DNA into the brain offers the advantage of producing high concentrations of therapeutic agents in a relatively localized environment. Gene transfer also provides longer effect than traditional drug therapy. Vectors are compounds that encase exogenous complementary DNA (cDNA) and transport it into cells during gene transfer. They are the key determinants of the success of gene therapy (2).

3. VIRAL VECTOR FOR STROKE GENE THERAPY

The three prevailing viral vector systems used for this purpose include the herpes simplex virus type-1 (HSV-1) (3), the adenovirus vector (4) and the adeno-associated

virus (5). The first two viral vectors are limited by their short expression duration. Potential reasons for this include episomal existence in cells (6), immunological rejection of transduced cells (4), or promoter shut-off (7). There also is usually accompanied substantial neurotoxicity (6-9) that to date has not been surmounted (8). Further increased titers of these vectors can lead to neural damage (6).

The third viral vector system utilizes the adeno-associated virus (AAV) which is a common parvovirus that has no known associated human pathology (10). Replication-defective AAV vectors (rAAV) are also nonpathogenic in humans and can efficiently transducer cells in the spinal cord (11), retina (12), lung (13, 14), gut (15), and brain (16-21). 96% of the viral genome are removed from the vector of modern AAV vectors leaving only the two 145-base pairs (bp) inverted terminal repeats (TRs) which are sufficient for packing and integration (22-23). The absence of viral sequences implies that no de novo protein synthesis occurs following transduction, minimizing the amount of foreign protein available to trigger immune response. No detectable cellular (CTL) immune response was observed in these analyses (24-26) and the presence of neutralizing antibodies may not pose a serious problem in brain delivery via rAAV vectors due to the blood-brain barrier. However, the production of neutralizing antibodies following intramuscular and intravenous rAAV deliver has been detected (26). A number of investigators have already shown that rAAV vectors can successfully transfer genes to the CNS of mice and rats. Such information favors AAV as a promising vector for CNS gene transfer.

4. METHOLOGY OF STROKE GENE TERAPY

4.1. Production of high titer recombinant adeno-associated virus

The rAAV-LacZ viral vectors were produced by co-transfection methods according to published protocols with modifications (27). Briefly, 1-2 hr before transfection, twenty 15-cm-diameter dishes of 293-cells (human embryonic kidney cells) at 80% confluence were fed with 25 ml of fresh Iscove modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal calf serum (FCS - HyClone, South Logan, UT) without antibiotics. A total of 49μg of plasmid DNA (16μg rAAV-LacZ plasmid, 8μg of pXX2 which encode Rep and Cap proteins, and 25μg of pXX6 which encode adenovirus gene products) were dissolved in 2 ml of 0.25 M $CaCl_2$ and then quickly mixed with 2 ml of HEPES-buffered saline (50mM HEPES, 280 mM NaCl, 1.5 mM Na2HPO_4 of pH 7.2) and added to the transfect 293-cells in each 15-cm-diameter dish. At 8 to 12h after transfection, the medium was placed with fresh Dulbecco modified Eagle medium (DMEM) (Gibco, BRL, Gaithersburg, MD, USA) containing 10% FCS and antibiotics. Cells from 80 dishes were harvested 48 hr post-transfection and resuspended in 40 ml of OptiMEM medium (Gibco, BRL, Gaithersburg, MD, USA), lyzed with three repeats of freeze and thaw. Cell lysates were digested with 4000 units of DNase (Sigma chemical Co., St. Louis, MO, USA) and 1 mg of RNase (Sigma chemical Co., St. Louis, MO, USA) at 37 ^{0}C for 30 min and deoxycholate (Sigma chemical Co., St. Louis, MO, USA) was added to a final concentration of 1%. The mixture was then homogenated and CsCl was added to a final density of 1.37 g/ml. CsCl density gradient purification was then carried out as previously published (26). Titers of rAAV-LacZ were determined by slot blot analysis to calculate concentration of viral particles. 20μl of rAAV-LacZ from the CsCl gradient fractions was digested with DNase I and Proteinase K (Sigma chemical Co., St. Louis, MO, USA). Then single strand DNA from rAAV-LacZ was extracted by phenol/chloroform extraction and precipitated by ethyl alcohol. The pellet was dissolved in 0.2 ml 0.4N NaOH/10mM EDTA and then immobilized on Hybond positively charged nylon membrane (Amersham Biosciences Co, Piscataway, NJ, USA) by using a vacuum slot blot apparatus (Schleicher & Schuell Co, Riviera Beach, Florida, USA). Recombinant AAV vector plasmid (pXX/LacZ) was serially diluted in 10-fold with 0.4N NaOH/10mM EDTA and immobilized on the same filter to create a standard curve of DNA concentration. The membrane was hybridized with non-radioactive LacZ probes Dig (Boeringer Mannhem Co, Indianapolis, IN, USA) for 1 hour at 60^{0}C, and washed to remove non-specifically bound probe, reacted with anti-Dig serum and substrate according to the manufacturer's instruction and exposed to Kodak XAR film. Preparation of rAAV-GDNF or rAAV-IL1-ra was the same as described above except for pXX/LacZ, which was replaced by pXX/GDNF or pXX/IL-1-ra plasmid (28).

While conducting our study, we found that the most critical step of the experiment was to establish a reproducible cerebral ischemic damage and to synthesize high titer quality rAAV. The latter was found to be relay crucially on DNA transfection efficiency since three plasmids had to be simultaneously delivered into the same cells for the synthesis of viral particles. DNA was purified with endotoxin free purification system (Qiagen Inc, Valencia, CA, USA). 293-cells were maintained in log phase growing condition. The transfection condition was refined by comparing different batches of HeBS solution. Transfection efficiency was maintained above 90%. A successful transfection indicator was a uniform cytopathic morphologic changes of the transfected 293-cells caused by XX6 plasmid encoding adenovirus genes at 48 hr after transfection.

4.2. Tri-vessels occlusion (3VO) model of focal cerebral ischemia

Adult male Sprague-Dawley rats weighing between 450 to 600 gm were used. The rats were allowed free access to water and rat chow until surgery. Surgery leading to focal cerebral ischemia was conducted under anesthesia with 10 ml/kg i.p. 4% chloral hydrate. Rectal temperature was monitored with thermocouples and was maintained at 37.0 ± 0.5? with a heating pad. Physiological parameters including the plasma glucose concentration, arterial blood pressure, and pulse rate were monitored and maintained within normal ranges as described previously (28,29). Cerebral ischemia was induced y clamping the carotid arteries. The bilateral common carotid arteries were identified and isolated by passing a suture around each

artery through an anterior ventral midline cervical incision. This procedure was completed prior to MCA ligation. The right medial cerebral artery (MCA) was exposed using microsurgical techniques.

Animals were placed on a stereotactic frame (Stoelting, Wooddale, IL USA) and operated under a surgical microscope. Following a 2-cm vertical scalp incision midway between the right eye and ear, the temporalis muscle was split and retracted in order to expose the zygoma and squamosal bone. A craniotomy of around 2 X 2 mm^2 was drilled at the junction of zygomatic arch and the squamosal bone. Care was taken as described above to avoid thermal or physical injury to the cortex during preparation of the craniotomy. The right MCA trunk was then isolated and ligated immediately above the rhinal fissure with Nylon 10-0. Then, the bilaterally isolated common carotids were occluded temporary with non-traumatic aneurysm arterial clips for 90 minutes. During this time, four burr holes were drilled in the skull (from bregma: AP=0.4, ML=0.9, DV=3.5; AP=0, ML=4, DV=2; AP=-3, ML=4.2, DV=2; AP=-6, ML=4.4, DV=2).

Meanwhile, 1.0 x 10^{10} viral particles of rAAV-GDNF, rAAV-IL1ra and rAAV-LacZ in 5µl of phosphate buffered solution (PBS) or PBS (mock) was injected into the right cerebral hemisphere through a microliter syringe (Hamilton Company, Reno, NV, USA) fitted with a 27-gauge needle at 0.5µl/min perfusion rate. After the injection, the needle was left in place for 4 minutes before being slowly withdrawn from the brain. After the predetermined duration of ischemia (90 minutes) was reached, the MCA ligation was untied and the clips at the common carotids were removed to restore internal carotid artery and MCA blood flow. Restoration of blood flow in all three arteries was observed directly under the microscope. The craniotomy was covered with a small piece of gel foam. The temporalis muscle and the overlying skin were allowed to fall back and were sutured individually. Free access to food and water was allowed after recovery from anesthesia (28).

5. MURINE MODELS OF FOCAL CEREBRAL ISCHEMIA

Laboratory reproduction necessitates standardizing the degree and variance in the animal model of focal cerebral ischemia. This is also essential for evaluation of the absolute risk reduction and its estimation in clinical studies.

5.1. Conventional 3VO model

Similarly to the technique employed in our study, a reproducible induction of temporary cerebral ischemia was achieved by performing a temporary suture ligation for about 90 minutes. The ligated vessel and its reperfusion after suture removal were both confirmed by direct visualization under the microscope. The temporary 3VO-model with complete three-vessel-release was a more complicated procedure, especially when ligating and releasing the MCA with Nylon 10-0. It's more difficult to perform in the mice model than in the rat one, and more microsurgical skills and practice were needed.

5.2. Modified 3VO model

The modified 3VO temporary cerebral ischemia model in rats and in mice was developed by H. Yanamoto *et al* (30). The technique consists of a permanent occlusion of the proximal MCA with reversible occlusion of bilateral common carotid arteries (CCAs) for 2 hours. The induced temporary focal cerebral ischemia was reproducible with successful achievement of neocortical infarction in normotensive rats and mice. The skill needed for permanent cauterization of MCA with bipolar forceps was easier than the ones for transient ligation of MCA with Nylon 10-0, especially in the mice animal model.

5.3. Intraluminal thread-induced MCA occlusion model

Koizumi et al. (31) and Longa et al. (32) introduced the intraluminal thread occlusion model without the need for craniotomy. This technique aimed to obstruct the orifice of the MCA along with the proximal internal carotid artery (ICA) in normotensive rats, producing a consistent infarction lesion in the basal ganglia.

Analysis of the infarction size showed that it expanded into the neocortex. The reported data demonstrated that the absolute infarction size in the neocortex varied with no correlation to ischemic duration. In order to exclude cases of thread-derived traumatic subarachnoid hemorrhage (in 44% of cases), which resulted in a large variability of the infarction size, bilateral monitoring of cerebral perfusion in each rat was deemed essential. To obtain a less variable infarction value, the authors concluded that appropriated exclusion of a subgroup (34%) that underwent ischemic challenge in experimental groups was necessary in the thread occlusion model (33).

The conventional 3VO and/or modified 3VO stroke models targeted the neocortex, which basically did not require exclusion in experimental animals or monitoring of the cerebral perfusion during ischemia as did the intraluminal thread-occlusion model. While the modified 3VO stroke model required less complicated microsurgical procedures than the conventional one, nevertheless it still could consistently produce temporary neocortical ischemia.

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6. THERAPEUTIC TRANSGENES IN STROKE

Ischemia-induced brain injury is a major cause of mortality worldwide (34-36) for which there is no effective therapy to date. The pathogenic processes involved in neuronal cell death are increasingly better understood resulting in experimental gene transfer applications to prevent ischemia-induced neuronal cell death. Currently, gene therapy for cerebral ischemia is primarily concerned with the fundamental mechanisms of neuronal loss: depolarization-induced calcium entry via NMDA receptors (37-40), intracellular free radical generation (41-42), damage to mitochondrial respiratory enzymes (43-45), energy failure (46,47), nitric oxide synthesis (48-52),

inflammatory cytokine network (53-55), and induction of programmed cell death (56-62).

6.1. Reduction of brain edema

Cranial fluid imbalances, including edema and elevated intracranial pressure, are major influential factors in the pathophysiology of acute head trauma, stroke and numerous other neurological diseases (63, 64). Recent studies have indicated that recently identified molecular water channels called aquaporin, recently identified in animals (65, 66), may play an important role in brain edema. This presents new therapeutic possibilites. Aquaporins are found in several cell types, including the kidney, lung and other fluid-transporting tissues where water flow is driven by osmotic gradients and hydrostatic pressure differences. These small integral membrane proteins function primarily as bidirectional water-selective transporters in many cell types in the. Aquaporin-4 (AQP4) is abundantly expressed throughout the brain, particularly at the blood-brain and brain-cerebrospinal fluid (CSF) interface. The specific localization of AQP4 to these anatomical and cellular regions of the central nervous system suggests a role for AQP4 in cerebral water balance. Well-characterized experimental models of water intoxication (67) and ischemia stroke (68, 69) were used to produce cerebral edema in wild-type ($AQP4^{+/+}$) and AQP4 null ($AQP^{-/-}$) mice. New promising therapies for controlling brain edema and other cranial water transport imbalances in a wide variety of neurological disorders (70) may be possible through the inhibition of AQP4.

6.2. Amelioration of reactive oxygen species effects

There is much new research providing evidence of improvements in reactive oxygen species effects in cerebral ischemia. The heat shock protein, which is one of the immediately early genes expressed after stroke (71, 72), have putative therapeutic potential for limiting cerebral damage. Also, the exogenous administration of melatonin in experimental stroke models demonstrated reduction of infarct volume, lower frequency of apoptosis, increasing number of surviving neurons, reduction of reactive gliosis, lower oxidation of neural lipids and oxidative damaged DNA, induction of bcl-2 gene expression (the activity of which improves cell survival), upregulation of excision repair cross-complementing factor 6 (an essential gene for preferential DNA excision repair), restrained poly (ADP ribose) synthetase (which depletes cellular NAD resulting in the loss of ATP) activity, and improvements in neurophysiologic outcomes. Under no circumstances did melatonin exacerbate the damage associated with ischemia/reperfusion injury (73).

SAG (sensitive to apoptosis gene) is a recently cloned antioxidant protein which experiments demonstrate its ability to reduce stroke extent in a MCA occlusion mouse model (42). This gene was delivered by direct injection via an adenoviral vector into ischemia putamen. Its overexpression protected ischemic neurons against apoptosis by reducing the level of reactive oxygen species. Moreover, such antioxidants as superoxide dimutases, catalase, or glutathione peroxidase, may all reduce the damage produced by oxygen radicals (74).

6.3. Improving energy delivery

Neuroprotection against ischemia and excitotoxic insults may be achieved through the overexpression of the glucose transporter GLUT-1 via herpes simplex virus or adenoviral vectors (75, 76).

6.4. Reduction of intracellular calcium effect

Yenari et al. demonstrated that herpes vector-mediated delivery of calbindin protein to the rat striatum, which acts as an intracellular buffer, could improve the survival of striatal neurons. (77)

6.5. Anti-inflammation peptides

Interleukin-1 (IL-1) has been identified as an important mediator for neurodegeneration induced by experimental cerebral ischemia (stroke), excitatory or traumatic brain injury in rodents (78-80). The interleukin-1 receptor antagonist (IL-1ra) is an endogenous molecule that inhibits the activity of interleukin-1 (81). Local synthesis of IL-1ra achieved by adenovirus-mediated gene delivery could attenuate ischemic inflammatory response and reduce both infarction volume and cerebral edema induced by ischemic brain injury. These findings not only confirmed the involvement of IL-1 in the pathogenesis of ischemia-induced damage, but also established the feasibility of gene therapy for ischemia-induced injury using IL-1ra (82-84). In our study, we injected recombinant adeno-associated virus (rAAV) vectors expressing the interleukin-1 receptor antagonist (rAAV-IL-1ra) into the cortex of rats experiencing transient cerebral ischemia.

An accumulation of IL-1ra in the cortical tissues of rAAV-IL-1ra-injected animals was confirmed by ELISA. Triphenyltetrazolium chloride (TTC) staining of viable brain tissue revealed that the rAAV-delivered IL-1ra gene could rescue the brain tissues from ischemia-induced injury. Cortical tissues that received rAAV-IL-1ra injections exhibited significantly smaller total volumes of infarction and smaller areas of infarction on each brain slice when compared with the control models (1).

The leukocyte adhesion receptors, P-selectine, ICAM-1 and E-selectin, are expressed in sequence by microvascular endothelium with in the ischemic territory (85-88). Studies using selective inhibitors to these receptors have shown the importance of these adhesion molecules in the stroke model. Smaller lesions were observed in rats treated with antibodies against MAC01 the leukocyte counterpart to ICAM-1) or ICAM-1 after transient MCA occlusion (89, 90). Further, the combination of tissue plasminogen activator and anti-CD18 provides significantly improved outcomes, and may increase the therapeutic time window in stroke (91).

6.6. Apoptosis mediators

The neuroprotective effects of overexpressing neuronal apoptosis-inhibiting proteins, including Bcl-2, which inhibits cytochrome-c release, have been demonstrated in several reports (92, 93). Y-Sun et. al. injected the vector-delivered BCL-w, an anti-apoptotic protein in the Bcl-2 family, into the cerebral cortex and the striatum of experimental animals. Three weeks later,

temporary focal ischemia was induced by occluding the ipsilateral MCA for 90 min, followed by reperfusion for 24h. Recipients of the rAAV-BCL-w vector showed a 30% reduction in infarct size and 33~40% improvement in neurological function, compared to the control groups. The result provided evidence that BCL-w can influence the overall histological and functional outcomes after focal cerebral ischemia (94).

6.7. Neurotrophic factors

Glial cell line-derived neurotrophic factor (GDNF) is a member of the TGF-ß superfamily (95). It is involved in the differentiation of neurons during normal development as well as the survival and recovery of significant number mature neurons. In vitro and in vivo research has demonstrated the protective effects of GDNG to the central and peripheral nervous system after being subjected to various injuries. Intra-cerebroventricular and intra-parenchymal administration of GDNF significantly protects the cerebral cortex from ischemia-induce injury (28, 96-98). We injected rAAV vectors expressing GDNF (rAAV-GDNF) into the cortex of rats experiencing transient bilateral CCA and right MCA ligation for 90 minutes. GDNF levels in the cortical tissues of rAAV-GDNF-injected animals were significantly higher than in the control ones injected with rAAV-expressing lacZ (rAAV-lacZ). This result indicates that rAAV can successfully deliver and express the GDNF gene in cerebral cortical tissues. Triphenyltetrazolium chloride tissue stain analysis revealed that the rAAV-delivered GDNF gene could rescue the brain tissues from ischemia-induced injury. Cortical tissues which received rAAV-GDNF injections had both significantly smaller total volumes and areas of infarction on each brain slice than those ones which were injected with rAAV-lacZ. An in situ labeling analysis demonstrated significantly less apoptotic cells in cortical tissues rescued by rAAV-GDNF, indicating prevention of apoptosis as the mechanism of cortical cell protection (28).

Neurotrophic growth factors, including basic fibroblast growth factor, cilliary neurotrophic factor, vascular endothelial growth factor (99-102), and Neuregulin-1 (NRG-1), are all potential gene transfer agents that await further study.

Neuregulin-1 (NRG-1), for example, is expressed throughout the immature and adult central nervous system. Its influence on the migration of a variety of cell types in the developing brain has been demonstrated. Elevated levels of NRG-1 and erbB4 in the peri-infarct regions of the ipsilateral cortex were found in the adult brain after injury 24 h post MCA occlusion. This suggests that NRG-1 is involved in the physiological response to neuronal injury. Increased motor performance and reduced cerebral infarction were observed in rats which were pre-treated with the NRG-1 protein 30 min prior to undergoing experimental cerebral ischemia. Ischemia in the cortex of NRG-1 treated rats induced a decrease in caspase-3 immunoreactivity and a reduction in the density of cells positive for terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end-labeling. Improvement in behavioral assays was also found in animals treated with NRG-1(103). The reduced ischemia or reperfusion injury by NRG-1, indicates it is a possible endogenous neuroprotective agent against stroke and therefore presents a promising research target in the development of new treatments for stroke.

7. SUMMARY AND PERSPECTIVES

A benefit of adeno-associated virus vectors is that they produce minimal immune response post intra-cerebral injection (24). While the gene sequence that can be inserted into this vector is severely limited by its relatively small size, the majority of genes encoding therapeutic factors are within the size parameter. Further, simultaneous gene transfer using two separate AAVs carrying different gene inserts (104-106) overcomes this problem. Also, the difficulty in producing rAAVs on a large scale fortunately has be reduced by several recent technological advances in vector production, purification, and titration which enable significant increases (>10-fold) in production capacity. Several current methods for the production of high titer rAAV (10^4 particles/cell level) should enable widespread use of this technology for clinical applications (107,108). Since CNS gene therapy requires relatively smaller amounts of viruses in comparison to that needed in other organs, rAAV-mediated gene therapy in the CNS system is preferred.

Achieving selective expression of the transferred genes in target tissues without widespread effects on other organs is one challenge that researchers are addressing. Targeting tissue-specific cell-surface makers (receptor targeting) for selectively binding viral vectors and using promoters that are active only in specific cell types linked to transgenes (transcriptional targeting) are some of the newest methods currently being developed for tissue-specific expression of transgenes. Both methods have proven successful offering increased promise for eventual use in the clinic (109-112).

Investigations into other potential gene therapy applications for cerebral ischemia are underway. For example the use of gene therapy to promote of angiogenesis in regions of the brain exposed to chronic ischemia thereby preventing stroke or cognitive impairment is being explored. Recent clinical trials for ischemic heart disease using vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in animal models also show promise for cerebral therapeutic angiogenesis (113). Maria et al. showed that treatment with rAAV-VEGF, 6 days or 12 days before the ischemic event, significantly improved survival rates, brain edema, CA1 delayed neuronal death, and post-ischemic learning. Animals treated with rAAV-VEGF showed a significant immunostaining for VEGF in the thalamus and the cortex, similar to those ones subjected only to brain ischemia (114).

Gene therapy may help in the prevention of post angioplasty re-stenosis due to intimal hyperplasia (115,116). Neo-intimal hyperplasia may be inhibited by gene transfer of endothelial nitric oxide synthases, cell

cycle regulators such as P53, antisense oligonucleotides, or transcription factors. VEGF can induce the production of endothelial nitric oxide and prostacyclins, leading to inhibition of vascular smooth muscle cell proliferation. Localized gene expression was successfully achieved with adeno-associated viral vector delivered into common carotid artery of rats (117). Jiang et al. (118) subjected rats to MCA occlusion, followed by continuous peritoneal injections of bromodeoxyuridine (BrdUrd). Their research showed that newly dividing progenitors incorporated BrdUrd, which were then found to be localized in the ischemic penumbra, suggesting an endogenous repair activity. Evidence from in vitro and in vivo systems indicates that bFGF, epidermal growth factor (EGF) and transforming growth factor-a (TGF-a) increase the proliferation of neural stem cells. Both EGF and bFGF expand the SVZ progenitor population after infusion into the lateral ventricle of the adult rat brain. Lastly, gene therapy may be an important tool to manipulate the endogenous precursors of growth factors, which is complementary to standard pharmacological therapies such as drug or protein infusion.

There is still much work to be done in the understanding and development of gene therapy for human cerebrovascular disease. New techniques must be developed in order to optimize targeting and vector delivery, enhance expression and viability of transgenic products, and minimize immune response. Further the development of safer and more effective vectors, improvement of vector delivery to cerebral penumbra or cerebral blood vessels, extended transfection period in chronic vascular disease and very early transfection in acute ischemia are all areas which need significant improvement. Finally, there must be a better understanding and recognition of the optimal genes selected for delivery in stroke gene therapy.

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Abbreviations: 3VO: three vessels occlusion, BrdUrd: bromodeoxyuridine, CCA: common carotid artey, cDNA: complementary DNA,CNS: central nervous system, CTL: cytotoxic T-lymphocyte, GDNF: **G**lial cell line-derived neurotrophic factor, HSV-1: herpes simplex virus type-1, ICAM-1: Intercellular adhesion molecule-1, IL-1: Interleukin-1, IL-1Ra : interleukin-1 receptor antagonist, ICA: internal carotid artery, MCAO: middle cerebral artery occlusion, SAG: sensitive to apoptosis gene, TGF-ß: tumor growth facto-ß, NRG-1: Neuregulin-1, rAAV: recombinant adeno-associated virus, VEGF: vascular endothelial growth factor.

Key Words: Brain, Nervous system, Neruron, Adenovirus, Adeno-associated virus, Cytokine, GDNF, IL-1Ra, Stroke, rat, Apoptosis, Review

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