Gene Transfer for Cerebrovascular Disease

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Gene transfer is a powerful, evolving technique that uses a biologic vehicle (*eg*, an engineered adenovirus) to introduce a specific gene of interest (*ie*, a recombinant gene) into a target tissue. This approach, which has considerable therapeutic potential, underlies the concept of gene therapy. Several studies have characterized the morphologic, biochemical, and functional effects of recombinant gene expression in animal and human cerebral arteries, and support the possibility of gene therapy for cerebrovascular disease. However, for successful integration into future clinical practice, key issues concerning vector safety, delivery methods, and transduction specificity need to be addressed. Alongside completion of the Human Genome Project, transfer of novel genes into the central nervous system is likely to impact greatly on our ability to favorably modify diseased human tissue. Knowledge of the fundamental concepts of cerebrovascular gene transfer is therefore useful to understanding both its molecular basis and potential clinical utility.

Introduction

Since the first human gene transfer study reported by Rosenberg *et al*. [1] in 1990, it has become apparent that, despite recent clinical setbacks [2], this novel and rapidly evolving technology is likely to play an important role in the future treatment of conditions including cancer, immune, hematologic, and metabolic disorders, and cardiovascular disease [3,4••,5–8]. Today, there are several major gene therapy journals, dozens of gene therapy companies, hundreds of gene therapy investigational protocols, and thousands of patients involved in clinical gene therapy trials. Under the umbrella of the Human Genome Project, billions of dollars have been invested in research, development, and utilization of this technology [8,9•]. In aiming to achieve the optimal gene therapy paradigm from a biologic perspective (Fig. 1), due consideration must be given to the complex and highly dynamic nature of this field, including its ethical aspects and the momentous task of standardizing, monitoring, and coordinating efforts between individuals, research and clinical departments, institutions, government regulatory bodies, and the biotechnology industry at large [10•].

Critical Issues in Cerebrovascular Gene Transfer

The goals of gene transfer for cerebrovascular disease are to safely, specifically, and efficiently introduce genetic material into the wall of a cerebral blood vessel in order to produce a substance that favorably modulates vascular growth or function, or both [5,11]. However, for successful gene transfer in the cerebral circulation, particular technical difficulties must first be addressed (Table 1). Following this, it is envisaged that gene therapy may become a useful alternative for the treatment of conditions such as atherosclerosis-thrombosis, vasospasm, diabetes mellitus, and hypertension, all of which can affect the cerebral vasculature.

Choice of vector

The choice of biologic vehicle to deliver a recombinant gene into an artery can be broadly divided between viral versus nonviral versus hybrid vectors [5,9•,11,12]. Viral vectors may be RNA viruses (*retroviridae*) such as mouse Moloney leukemia virus (MoMLV) or lentiviruses including human (HIV), bovine (BIV), and simian (SIV) immunodeficiency viruses. DNA viral vectors, on the other hand, include strains linked to the common cold pathogen (adenovirus) or parvoviruses (such as adeno-associated virus [AAV]). Nonviral vectors include naked DNA (plasmids) and DNA-containing cationic lipid particles (liposomes). Hybrids such as plasmid-liposome [13] and Sendai virus-liposome [14] conjugates have also been developed and used in vascular gene transfer. Each of these classes of vectors has a characteristic profile related to DNA integration, efficiency of transduction, cell avidity, and induced inflammatory response. In general, viral vectors demonstrate appreciably greater gene transduction efficiency than nonviral vectors [5,9,11]. Incorporation into the host cell genome (referred to as DNA integration) following entry into the nucleus is a feature of RNA viruses such as MoMLV and HIV, and the DNA-containing AAV, but not adenovirus (which remains epichromosomal). Although the benefit of DNA integration is

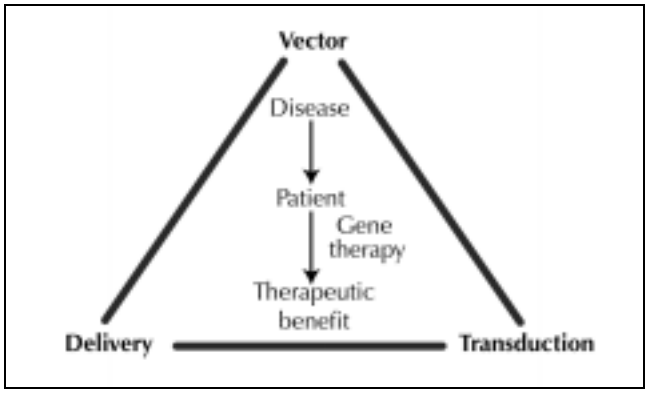

Figure 1. Key biologic considerations for an optimal gene therapy paradigm include a disease of known molecular pathogenesis, with supportive animal and human tissue experimental models; an informed, consenting patient whose condition and treatment meets the rigorous criteria defined by government and institutional regulatory bodies; a clinically safe vector comprised of a suitable biologic agent (such as a minimal-genome adenovirus), a therapeutic gene of interest, and a regulatory element to control gene expression; a delivery device (*eg*, an effective catheter system) and appropriate mode of delivery (*ie*, ex vivo versus in vivo; intraoperative versus nonoperative; intraluminal versus periadventitial approach); efficient and tissue-specific transduction; and a therapeutic benefit that must be objectively and clinically measurable.

relatively long-term recombinant gene expression, there is an appreciably higher risk of insertional mutagenesis [5]. With regard to the type of cell infected by viruses, MoMLV exclusively targets dividing cells (thereby limiting tissue selection and in vivo applications), whereas lentiviruses can transduce some, but not all, types of dividing and nondividing cells. On the other hand, adenoviruses and AAV have the broadest cell avidity known. The major disadvantages of adenoviruses are their propensity to induce a brisk inflammatory response in vivo, their immunogenicity upon re-exposure, and relatively short-term transgene expression [15–19]. The advent of newer generation "gutted" adenoviruses (with minimal native viral genome) is a move toward less immunogenic and cytotoxic vectors [2,9•,12]. Hybrid vectors such as virus-liposome conjugates may yield certain advantages in terms of improved efficiency of gene transfer compared to the use of liposomes alone, and reduced inflammatory response compared with viruses alone [5,9•,14].

To date, the adenovirus (particularly serotype 5) remains the predominant vector used in cerebrovascular gene transfer studies (Table 2), most likely due to its broader cell avidity, greater efficiency of transduction, and ability to be generated in relatively high titers (*ie*, between 10^{11} and 10^{12} infectious or plaque-forming units (PFU)/mL) [4••,5,20]. As elaborated below, a considerable amount of information has been acquired regarding this vector and its applications. Briefly, for use in gene transfer, the adenoviral genome is combined with a gene of interest whose expression (*ie*, transcription followed by translation into a particular protein) is driven by a promoter, frequently a cell-nonspecific one derived from cytomegalovirus (CMV). The adenovirus is rendered replication-incompetent through the deletion of certain replication-associated genetic sequences (*eg*, "early" regions E1 and E3) [11,20,21]. Entry of the modified virus into target cells typically involves attachment of the viral fiber knob to the host-cell plasmalemma facilitated by the coxsackie virus-adenovirus receptor (CAR) [22], and is followed by α_v-integrin-mediated internalization [23]. Once it has entered the cell, the adenovirus retains an epichromosomal (nonintegrated) position in the nucleus, and uses the biosynthetic machinery of the host to generate the (recombinant) protein of interest. In experimental models, expression of such proteins is detectable morphologically, biochemically, and functionally (see below). It is important to note that most cerebrovascular gene transfer studies involving adenoviruses (Table 2) have utilized early generation vectors that contain considerably more viral genome than the more recent adeno-associated and gutted vectors, thereby accounting for increased immunogenicity and cytotoxicity (particularly from the biosynthesis of peptides derived from nondeleted late-region adenoviral genome sequences) [3,5,11,12,15–19].

Choice of gene

The pivotal role played by nitric oxide (NO) in cerebral vasomotor function, and its implication in the pathogenesis of a wide variety of cardiovascular diseases including atherosclerosis-thrombosis, diabetes mellitus, vasospasm, and hypertension, make this molecule a prime candidate for potentially therapeutic gene transfer [4,11,24,25•,26–30]. This is certainly substantiated in the cerebrovascular gene transfer literature, in which over half of the studies to date have involved a vector encoding the endothelial isoform of nitric oxide synthase (eNOS; Table 2). It should be noted that the choice of eNOS over the inducible NOS isoform (iNOS) is principally based on an association between iNOS activity and cytotoxic free radical generation, paradoxically related to overzealous production of NO by the latter isoform (for further information pertaining to the choice of NOS isoforms in cardiovascular gene transfer, see Chen *et al.* [4••]).

Two other considerations about determining the most appropriate choice of gene to be inserted into a vector should be noted. First, NO may be only one of a number of important mediators underlying the pathogenesis of cerebrovascular disease; other candidates include endothelins, and altered enzymatic activities or byproducts of cyclooxygenase, superoxide dismutase, and heme oxygenase isoforms [11,26,31–33]. Therefore, gene transfer using cDNAs encoding one or more of these proteins may need to be considered in addition NOS gene transfer alone. Second, relative insufficiency of enzymatic substrates or cofactors following, *eg*, NOS gene transfer may potentially limit the efficacy of this technique if uncorrected by exogenous means [4••]. Further in vivo and ex vivo investigation along these lines will aid in addressing these important issues, as will information gleaned from the Human Genome Project and

Table 1. Critical issues in adenovirus-mediated cerebrovascular gene transfer

	Problem	Solution
Disease pathogenesis	Precise molecular basis unknown.	Ongoing ex vivo and in vivo laboratory investigations.
Gene of interest	Choice of gene depends on pathophysiology; *eg,* endothelins, NOS, heme oxygenase, cyclooxygenase, superoxide dismutase.	Completed Human Genome Project and use of differential gene displays may aid in elucidating precise genetic basis of disease.
Regulatory element	At present, cannot control recombinant gene expression in vivo.	Promising developments in oral drug-responsive regulatory elements. Transient gene expression may be helpful in a disease such as cerebral vasospasm whose time course is temporally limited.
Vector	Remains immunogenic and cytotoxic (limiting gene expression).	Newer "gutted" vectors being developed with minimal adenoviral genome.
Target tissue	Nonspecificity of tissue transduction remains a problem.	Use of promoters such as SM22α (specific for smooth muscle) or direct mechanical techniques may aid in more specific targeting.
Route of delivery	Limited efficacy of intravascular (catheter-based) gene delivery systems; some require interruption of cerebral blood flow; adluminal transduction may be attenuated by blood-barrier and elastic lamina.	Improvement in catheter design; optimization of periadventitial delivery methods (cerebrospinal fluid infusion; endoscopic delivery).
Therapeutic benefit	Currently no in vivo human model for cerebrovascular gene therapy.	Recent ex vivo human tissue studies helpful; ongoing development of safe and effective clinical grade vectors will facilitate future human trials.

NOS—nitric oxide synthase.

the use of gene microarrays to precisely identify genes differentially expressed in normal versus diseased cerebral arteries.

Route of delivery

For cerebrovascular gene transfer in vivo, the route of delivery of a vector may be either intravascular or periadventitial [4••,11,34•,35–37]. Intravascular transduction involves use of a catheter- or stent-based delivery device. Appreciable limitations include the risk of vascular injury during navigation or deployment of the device, the need for temporary interruption of cerebral blood flow (leading to cerebral ischemia) when using certain (nonperfusion) catheters, and diffusion of vector distal to the target vessel segment [11]. Periadventitial delivery, on the other hand, may be a sound alternative for several reasons: first, for a rapidly diffusible gas such as NO, this route of delivery may be as effective as, if not more effective than, intraluminal delivery in terms of access of NO to smooth muscle cells of the tunica media, somewhat akin to physiological NO release from perivascular nitrergic nerve endings [11,28]. Second, from the periadventitial side, transduction is unhindered by the blood-brain barrier and subendothelial elastic lamina. Third, choices of periadventitial delivery include injection or infusion of vector into the cerebrospinal fluid (CSF; *ie*, intrathecal delivery), the efficacy of which has been repeatedly demonstrated in animal experiments [11,24,38•,39–42], or direct application of vector on cerebral arteries ex vivo or during open surgery. Last, although adventitial fibroblasts do not express endogenous eNOS, they do express receptors for NO-mediated agonists such as bradykinin [31,43,44]. This is fortunate given that these cells are primary targets for adenovirus entry and recombinant eNOS synthesis, as demonstrated using immunoelectron microscopy in animal cerebrovascular gene transfer studies [4••,43] and, more recently, in a study involving intact human cerebral arteries [34•].

Specificity and efficiency of transduction

Targeting vectors to specific cells or tissues remains a major obstacle that needs to be overcome before clinical implementation of cerebrovascular gene transfer techniques is achieved. Heistad *et al.* [11, 40] first reported the use of a mechanical method, namely controlled animal head-tilt, to assist in localizing vectors injected into the CSF via the cisterna magna to arteries in the circle of Willis. Although this technique is indeed helpful in this regard, it remains relatively nonspecific and operator-dependent. A molecular targeting technique using a cell-specific promoter such as SM22α (selective for smooth muscle cells; cf. cell-nonspecific CMV-derived promoters) has been demonstrated to be effective in vitro [45], and may be useful in vivo via selectively targeting vascular versus neuronal or glial tissue. However, at present, there is no way to reliably distinguish between smooth muscle cells in different cerebral arteries, and therefore the question of being able to target specific vascular territories remains unanswered using this approach.

With regard to gene transfer efficiency, it is apparent from calculations based on ex vivo peripheral and cerebrovascular gene transfer studies that the ratio of infectious particles to target-tissue cells is in the order of 1000:1; *ie*, there is a considerable overabundance of infectious

Table 2. Gene transfer studies in the cerebral vasculature

Study	Species	Cerebral arteries	Vector	Genes studied	Delivery mode	Type of study
Ooboshi *et al.* [40]	Rat	Basilar, middle cerebral	Adenovirus	*LacZ, CFTR*	in vivo	Morphology
Chen *et al.* [20]	Canine	Basilar	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, biochemistry
Chen *et al.* [38]	Canine	Basilar, middle cerebral	Adenovirus	*LacZ, eNOS*	in vivo	Morphology, biochemistry, function
Christenson *et al.* [39]	Mouse	Basilar, middle cerebral	Adenovirus	*LacZ*	in vivo	Morphology, biochemistry
Ono *et al.* [13]*	Rabbit	Basilar	Plasmid-Liposome	*NF-κB*	in vivo	Angiography, morphology, biochemistry
Onoue et al. [6]*	Canine	Basilar	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, function
Tsutsui *et al.* [43]†	Canine	Basilar	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, biochemistry, function
Onoue *et al.* [31]	Canine	Basilar	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, biochemistry, function
Suzuki *et al.* [32]*	Rat	Basilar	Anti-sense oligodeoxy-nucleotide	*HO-1*	in vivo	Angiography, biochemistry, function
Khurana *et al.* [34]	Human	Pial (intact arteries)	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, function
Stoodley *et al.* [41]*	Canine	Basilar	Adenovirus	*LacZ, eNOS*	in vivo	Angiography, morphology, biochemistry, function
Toyoda *et al.* [33]	Rabbit	Basilar	Adenovirus	*LacZ, CGRP*	in vivo	Morphology, biochemistry, function
Toyoda *et al.* [42]	Rabbit	Basilar	Adenovirus	*LacZ*	in vivo	Morphology, biochemistry
Tsutsui *et al.* [44]	Canine	Basilar	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, function
Tsutsui *et al.* [37]	Canine	Small brainstem arteries	Adenovirus	*LacZ, eNOS*	ex vivo	Morphology, biochemistry, function

CFTR—cystic fibrosis transmembrane conductance regulator; CGRP—calcitonin gene-related peptide; eNOS—endothelial nitric oxide synthase; HO-1—heme oxygenase-1; LacZ—β-galactosidase; NF-κB—nuclear factor κB.
*Studies carried out in animals following experimental subarachnoid hemorrhage.
†Study also involved endothelium-denuded arterial segments.

particles. Despite this, experiments involving recombinant β-galactosidase- or luciferase-based quantification of adenovirus-mediated gene transfer efficiency demonstrate somewhat poor transduction of arteries ex vivo, likely to be even poorer in vivo [11]. To some extent, this phenomenon may be attributable to a relative paucity of CAR in cerebral arteries [11]. However, regardless of the underlying reasons, development of techniques to greatly reduce the number of infectious units delivered to blood vessels ex vivo, and ultimately in vivo, is required in order to reduce the likelihood and severity of an adverse response to the vector due to the shear number of particles delivered to the host. In this light, Toyoda *et al.* [42] have recently reported that precipitation of adenovirus with calcium phosphate crystals greatly increases the efficiency of vascular transduction both ex vivo and in vivo in rabbits. Whether this important finding holds true for human arteries remains to be determined.

Gene Transfer Studies in the Cerebral Vasculature

Following the pioneering study by Davidson *et al.* [46] describing in vivo delivery of adenovirus into murine CNS, Ooboshi *et al.* [40], using Sprague-Dawley rats, reported the first in vivo adenovirus-mediated gene transfer to cerebral arteries. Subsequently, numerous cerebrovascular gene transfer studies have been carried out (Table 2). Notably, 1) approximately one half of these studies have been published in the last 18 months; 2) transduced, intact arteries from numerous species have been studied including, more recently, humans; 3) both large-diameter (basilar; middle cerebral) and small-diameter (pial; brainstem secondary) arteries have been studied following gene transfer; 4) nearly all studies involved adenoviral vectors; 5) LacZ or eNOS cDNA (or both) were most frequently the genes of interest; 6) numerous ex vivo and in vivo studies have been carried out in animals, although to date the only cerebrovascular gene transfer involving intact human arteries was carried out ex vivo; and 7) it is apparent that the morphologic, biochemical, and functional features of cerebrovascular gene transfer have been extensively characterized.

Although it is beyond the scope of this review to detail each and every cerebrovascular gene transfer study carried out to date, we have selected four particular studies [20,34•,38•,40] as being representative of the general nature of the investigations carried out in this field. As indicated above, Ooboshi *et al.* [40] carried out the first gene transfer to cerebral arteries in vivo. In their purely morphologic study, the investigators delivered a replication-incompetent adenoviral vector (expressing recombinant β-galactosidase) into the CSF of Sprague-Dawley rats held in various anatomical positions. One to 7 days following injection, the transduced brains were examined histochemically after appropriate staining. The authors reported 1) distribution of recombinant protein staining consistent with the anatomic position in which the rat was held (an example of mechanical targeting); 2) good transduction of the adventitial layer of large and small cerebral arteries (consistent with perivascular gene delivery); and 3) undetectable β-galactosidas expression by day 7 following injection (*ie*, indicative of short-term recombinant gene expression). In the first functional study of transduced intracranial arteries, Chen *et al.* [20] reported the morphologic, biochemical, and vasomotor effects of ex vivo transduction of canine basilar artery with an adenoviral vector expressing recombinant eNOS (AdeNOS). Their principal findings were 1) recombinant protein was expressed mainly in the adventitia and, to a lesser extent, in the endothelium of transduced arteries (consistent with ex vivo transduction); 2) expression of recombinant eNOS in the arterial wall was associated with beneficial vasomotor effects including significantly enhanced relaxations to calcium ionophore A23187, a compound whose receptor-independent relaxing actions are NO-mediated, and reduced contractions to uridine triphosphate; and 3) basal production of cyclic 3'5'-guanosine monophosphate (cGMP; the second messenger for NO-mediated signaling) was significantly increased in AdeNOS-transduced arteries. Immediately following this study, similar findings were reported by Chen *et al.* [38] in vivo in dogs. Together, these studies indicated that cerebral arterial tone could be favorably modulated by recombinant eNOS expression in the vessl wall. That these findings are reproducible in nonpostmortem human cerebral arteries has been recently demonstrated by Khurana *et al.* [34•]. In our ex vivo study, intact pial arteries of outer diameter 500 to 1000 μm were freshly isolated from 30 patients undergoing temporal lobectomy for intractable seizures. These vessels were studied morphologically (by histology, histochemistry, and electron microscopy) and functionally (by isometric force recording) before and 24 hours after adenovirus-mediated recombinant LacZ and eNOS gene transfer. The principal findings of our study follow. In transduced human pial arteries, expression of recombinant protein occurred mainly in the adventitial layer; the main cellular target for virus entry and recombinant protein synthesis following periadventitial transduction was the adventitial fibroblast; and expression of recombinant eNOS was associated with significantly augmented relaxations to the endogenous peptide, bradykinin (consistent with an increased absolute amount of eNOS facilitating the action of this NO-dependent agonist), and augmented contractions to the NOS inhibitor, L-N^g-nitroarginine methylester (L-NAME; consistent with an increased absolute amount of eNOS being inhibited by this compound).

Conclusions

Although still in its infancy, gene transfer technology is likely to become a feasible therapeutic approach to the treatment of human diseases, including those affecting the cerebral vasculature. Its three principal biologic objectives

remain the construction of safer vectors, development of more effective gene delivery techniques, and optimization of conditions for specific and efficient cellular transduction. As a measure of growing interest in cerebrovascular gene transfer, of the studies carried out in the past 5 to 6 years, approximately one half have in fact been published in the last 18 months including, most recently, the first gene transfer study in the human cerebral circulation. Together, these investigations suggest that gene transfer may be beneficial in future clinical applications of gene therapy for the treatment or prevention of cerebrovascular disease.

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