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GENE THERAPY FOR ERECTILE DYSFUNCTION

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TABLE OF CONTENTS

1. ABSTRACT

The past decade has seen an explosion of new information on the physiology of penile erection, pathophysiology of erectile dysfunction (ED), and development of new oral agents (e.g., three PDE5 inhibitors) to manage ED. Although all three selective PDE5 inhibitors are effective in the majority of ED cases, these oral medications have failed in certain disease states, such as diabetic ED, postprostatectomy ED, and severe veno-occlusive dysfunction. Only about 50% to 60% of these cases benefit from PDE5 inhibitor therapy, prompting the development of new approaches, including gene-based therapies for the treatment of ED. The penis is a convenient tissue target for gene therapy because of its external location and accessibility, the ubiquity of endothelial lined spaces, and low level of blood flow, especially in the flaccid state. Initially, gene therapy has been reserved for the treatment of life-threatening disorders including cancer, hereditary and acquired diseases. However, gene therapy is an attractive therapeutic possibility for the treatment of ED. Evolution of nitric oxide (NO), a small gaseous, lipophilic signaling molecule that is produced by nitric oxide synthase (NOS) activates guanylate cyclase (GC), resulting in increased cyclic guanosine monophosphate (cGMP) production, plays a significant role in our understanding of cavernosal smooth muscle physiology. Many gene therapy strategies have focused on the NO/GS/cGMP pathway. All three NOS isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS), and iNOS have been used for gene therapy in order to modulate erectile response. Various viral and nonviral vectors have been used to date for the transfer of genetic material to the target cell or tissues with various degrees of success. Recently, second generation or "gutless" (helper-dependent) adenovirus vectors have been developed in order to reduce cellular toxicity and immune response, while increasing efficient gene therapy. Varieties of other gene therapy trials have also been undertaken for the treatment of ED and are the focus of this review.

2. INTRODUCTION

Penile erection is a neurovascular event that depends on neural integrity, a functional vascular system, and healthy cavernosal tissues (1). Physiologic erectile function involves three synergistic and simultaneous processes: 1) a neurogenically mediated increase in penile arterial inflow, 2) relaxation of cavernosal smooth muscle, and 3) restriction of venous outflow from the corpora cavernosa. The corpus cavernosum of the penis is composed of a meshwork of interconnected smooth muscle cells lined by vascular endothelium. In addition, endothelial cells and underlying smooth muscle line the small resistance helicine arteries that supply blood to the corpora cavernosa during penile tumescence. Pathological alterations in the anatomy of penile vasculature or impairment of any combination of neurovascular processes can result in erectile dysfunction (ED). ED has traditionally been classified as psychogenic, organic, or a combination of these two entities. More recent data shows >80% of ED as having an organic basis, with vascular disease being the most common etiology (2). Although ED is a natural consequence of aging, its severity is directly related to the number and degree of vascular risk factors, such as hypertension, cigarette smoking, atherosclerosis, hypercholesterolemia, and diabetes mellitus (3). Hence, endothelial dysfunction in the penile vascular tree can lead to ED (4).

ED is defined as the consistent inability to obtain or maintain an erection for satisfactory sexual intercourse (5). The Massachusetts Male Aging Study (MMAS), a substantial epidemiological survey that quantified the prevalence of ED in a non-institutionalized population of men in the Boston suburbs (3), revealed that 52% of 1,290 men aged 40 to 70 years had some degree of ED; with almost 10% exhibiting a total absence of erectile function. It has been estimated that 25 to 30 million men in the United States have partial to complete ED (6). Extrapolation of this data estimates that the worldwide incidence of ED will increase from 152 million men in 1995 to 322 million men by the year 2025 (7). Ongoing calculations from the participants in the MMAS reveal the overall incidence of ED after an average follow-up of 8.8 years to increase to 26 cases per 1,000 man-years (8).

Basic science research on erectile physiology has focused on the pathogenesis of ED and has provided convincing evidence that ED is predominately a disease of vascular etiology. Sildenafil (Viagra®, Pfizer, New York, NY), vardenafil (Levitra®, Bayer/GSK, Raritan, NJ), and tadalafil (Cialis®, Lilly-Icos, Indianapolis, Ind.), oral selective type 5 phosphodiesterase (PDE5) inhibitors, are the agents to be recommended as a first-line therapy because of their convenience and high rate of efficacy in a diverse population of ED patients. Although all three selective PDE5 inhibitors are effective in the majority of ED cases, these oral medications have failed in certain disease states, such as diabetic ED, postprostatectomy ED, and severe veno-occlusive dysfunction. Only about 50% to 60% of these cases could benefit from PDE5 inhibitor therapy. This caused the development of new approaches including gene and cell-based therapies for the treatment of ED. This communication reviewed gene and cell-based therapy approaches for ED in light of current advancements.

3. GENE THERAPY FOR ED

Initially, gene therapy has been reserved for the treatment of life-threatening disorders including cancer, hereditary and acquired diseases. Although there have been some concerns and regulatory changes due to the loss of a young volunteer who had been treated with the intrahepatic administration of a recombinant adenoviral vector containing the ornithine transcarbamylase in 1999, there are still approximately 500 gene therapy trials being conducted worldwide (9, 10). Gene therapy is an attractive therapeutic possibility for the treatment of ED. A simple concept about ED is that in most men only a very small alteration in the balance between contracting and relaxing stimuli can cause significant effects on cavernosal and penile vascular smooth muscle tone (11, 12). The penis is also a convenient tissue target for gene therapy because of its external location, the ubiquity of endothelial lined spaces, and low level of blood flow.

Somatic gene therapy is defined as the inability to introduce genetic material (DNA or RNA) into an appropriate cell type *in vitro* and *in vivo*, thus altering gene expression of that cell in order to produce a therapeutic effect (13). For this goal, a number of consecutive procedures should be carried out: the administration of a desired gene into the body, delivery of the gene to a targeted cell that is subsequently transported into the nucleus, and expression of the therapeutic product (14). (Figure 1)

Gene therapy strategies have been divided into two categories: (15) 1) to correct the erectile deficit by increasing the supply/strength of the erectile stimulus, increasing the expression of a relevant endogenous vasomodulator of the erectile function, 2) to alter the tissue or end organ demand for a given erectile stimulus, making the corporeal tissue more sensitive to relaxatory stimuli (Table 1). Thus, erectile function can be modulated and potentially restored by altering the physiological supply and demand of the erectile apparatus in theory and in practice. Gene therapy for ED is a relatively straightforward concept that simply restores the normal balance between contracting and relaxing stimuli in the cavernosal smooth muscle.

Since the conduction of first preclinical study in an *in vivo* rat model using inducible nitric oxide synthase (iNOS) (16), a variety of gene therapy trials has been tried for the treatment of ED. Due to its significant role in the physiology of normal erectile function, gene therapy strategies have focused on the nitric oxide (NO)/guanylate cyclase/cyclic guanosine monophosphate (cGMP) pathway. All three NOS isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS and penile NOS [PnNOS, the penile specific variant of nNOS]), and iNOS, have been used for gene therapy so as to modulate erectile response. The

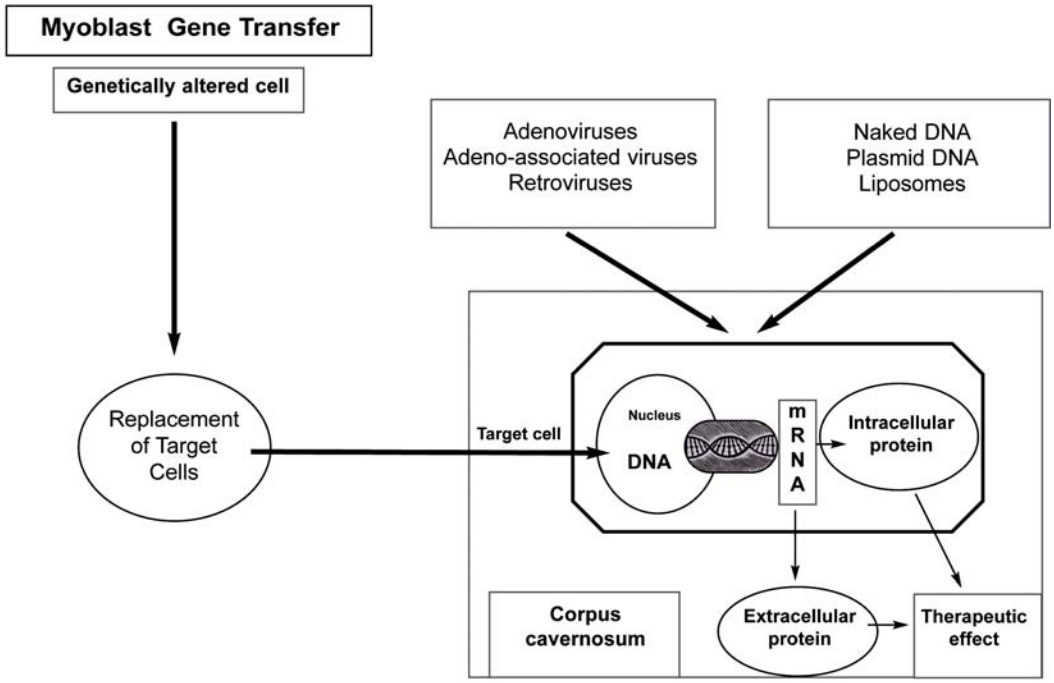

Figure 1. General schematic of gene-therapy approaches to replace a targeted cell or over expression of a therapeutic gene for the treatment of erectile dysfunction. This figure demonstrates the administration of a desired gene or cell into the penile corpora cavernosa and subsequent transportation into the nucleus and alteration of cellular function. Adapted from Ref. 14.

rationale for this target is that overexpression of important endogenous cavernosal smooth muscle relaxants and vasodilators could improve erectile response in ED. Newer approaches suggest other means of genetic manipulation: nerve and vascular growth factors, (e.g., brain-derived nerve growth factor [BDNF], vascular endothelial growth factor [VEGF]), the cyclic adenosine monophosphate (cAMP) cascade (i.e., calcitonine gene-related peptide [CGRP] receptor), the calcium sensitization pathway, and K^+ channel gene expression (a cellular convergence point for mediating the effects of all of the above). Cell-based therapy approaches are also becoming more popular.

3.1. Vectors for gene transfer

The ideal vector for gene transfer is one that allows for efficient transduction and long-term stable transgene expression and has few or no adverse effects, such as risk of infection, immunogenicity, or host-cell mutagenesis. Various vectors, such as viral vectors (adenovirus, adeno-associated virus, adeno-myoblast, and retrovirus) and nonviral vectors (naked DNA, plasmid DNA, liposomes, and myoblast-mediated) have been used to date for the transfer of genetic material to the target cell or tissue[14]. Each has advantages and disadvantages in regards to safety, efficiency, and immunogenity.

Adenovirus vectors have been most widely used for gene transfer because it has a significant number of advantages. It provides high cellular transduction efficiency in a variety of cell types and tissues (17). Produced at high titers, small volumes of virus could be efficient for gene transfer to the target. Of importance, it transfects both dividing and nondividing cell types and does not enter the cell's genome (18). Major disadvantages of adenoviral vectors are the possibility of triggering a host inflammatory and immune response due to the expression of viral proteins in infected cells. Recently, second generation or "gutless" (helper-dependent) adenovirus vectors have been developed in order to reduce cellular toxicity and immune response, while increasing efficient gene expression (19, 20). As gene treatment of ED necessitates repeated injections to the penis, these vectors may provide long-term efficiency without immune responses.

With its ability to be maintained in targeted cells as integrated proviruses, the adeno-associated virus may be an attractive vector for gene therapy. These include low immunogenity, high efficiency, ability to infect a variety of cell types, no known pathogenicity, and the ability to transduce nondividing cells as well as dividing cells (21). The possibility of being co-infected with adenoviruses is one of the major disadvantages of the adeno-associated virus vector, making it difficult to prepare large quantities of pure vectors. Another potential problem is the possibility of promoting insertional mutagenesis (22).

Although having the ability to remove all viral genes and replace those with the therapeutic gene, retroviral vectors have major disadvantages which restrict their therapeutic applicability. Unlike adenovirus and adeno-associated virus, it is difficult to produce high titers with retroviruses. Additionally, successful retroviral gene transfer necessitates cell proliferation [23], which potentially limits its use for ED gene therapy because the endothelial

Table 1. Gene-therapy approaches for erectile dysfunction

Supply/strength side	**Increase NO**
	• eNOS
	• nNOS
	• Superoxide dismutase
	Increase nerve supply
	• BDNF
	Increase vascular supply
	• VEGF
	Increase CGRP
Demand side	
	Decrease amount of stimulus required for relaxation
	• Alter potassium/calcium channels (via hSlo)
	• Alter calcium sensitization (via RhoA)

cells and smooth muscle of the penile corpus cavernosum cannot actively proliferate. It also carries the risk of insertional mutagenesis.

Use of both naked and plasmid DNA vectors has been limited to low efficiencies of transduction *in vivo* (24, 25). In order to increase the transgene expression, DNA has been incorporated into liposomes that facilitate increased stability of the desired DNA and increase cellular entry by promoting fusion with the plasma membrane. Although this approach has been demonstrated to increase transgene expression *in vitro*, *in vivo* applications were documented as less successful (26, 27). Myoblast-mediated gene transfer provides stable and safe delivery of a specific genetically altered cell into a tissue. In this technique, cells are removed from the body, genetically engineered in culture and subsequently reintroduced into a particular tissue, in which they become integrated into a pre-existing tissue (28, 29).

Various vectors used for potential gene therapy of ED are summarized in Figure 2.

3.2. Endothelial nitric oxide synthase (eNOS)

eNOS localizes to the endothelial layers of the dorsal arteries and veins of the penis and the cavernosal sinusoidal spaces (30). NOS activity reduces with aging, as well as in diabetic states (31, 32). Therefore, methods to enhance local NO delivery is a clever target for the treatment of ED. Once again, an attractive approach to increase local NO delivery is the use of gene transfer techniques.

Champion *et al* first demonstrated that adenovirus-mediated transfer of the eNOS gene in the rat penis could enhance eNOS protein level and activity, and elevate cGMP levels (33). In this study, the authors used an adenovirus which had limited activity of expression of the eNOS gene, requiring repeated injections into the penis. This issue is important as it could injure the vascular smooth muscle cells of the penis, and more importantly, induce a host immune response. These investigators utilized the cytomegalovirus (CMV) and Rous sarcoma viruses (RSV) and noted expression of eNOS for at least 1 month after intracavernosal injection. They also documented that 5 days after transfection with adenoviral eNOS, aged rats demonstrated a significant increase in erectile function as determined by cavernosal nerve stimulation and pharmacological injection with the endothelium-dependent vasodilator, acetylcholine, and the PDE5 inhibitors, zaprinast and sildenafil (33-35). Subsequently, using different promoters, these researchers showed that the effect of adenoviral gene transfer of eNOS could exhibit longer durations of expression of eNOS (36). These data also proved that adenoviral gene transfer of eNOS could cause physiological changes in erectile responses in aged rats. Future studies will need to focus on the development of vectors with greater efficiency and stability of transduction, with fewer unwanted side effects.

Most observations with adenoviral eNOS vectors have focused on the physiological responses to nerve-stimulated intracavernous pressure changes. The pharmacological responses to intracavernous injection of zaprinast or acetylcholine also improved, suggesting that combinations with gene therapy might be efficacious in capturing nonresponders to traditional single modality therapy. Recent studies have shown that adenoviral-mediated gene transfer of eNOS to streptozotocin-induced diabetic rat penises increased erectile responses *in vivo* as a consequence of an increase in cGMP formation (35). Moreover, the combination of eNOS gene therapy and sildenafil in similar diabetic rats induced an erectile response that was greater than those resulting from either monotherapy.

3.3. Neuronal nitric oxide synthase (nNOS)

NO is synthesized from the substrate L-arginine by the catalytic activity of nNOS and mediates the relaxation of corpus cavernosum smooth muscle (37, 38). Previous studies had demonstrated that knockout mice that are devoid of nNOS expression still possess erectile function sufficient for copulation and reproduction (39, 40). This finding contradicts the notion that the primary source of NO involved in the erectile response is derived from nNOS (41, 42), and suggests that either nitrergic or non-nitrergic compensatory mechanisms can maintain erectile function in the absence of nNOS. Relaxation of human corpus cavernosum by transmural electrical stimulation does not necessarily require a functional endothelium (43), as NOS is also expressed in corporal smooth muscle (44, 45).

Despite the commercial success of the novel oral PDE5 inhibitors, new medical treatments using gene therapy are warranted. PDE5 inhibitors rely on sexual stimulation to produce sufficient NO to activate guanylate cyclase and synthesize cGMP, causing penile erection. Cashen *et al* have demonstrated that at least one nNOS isoform is required for the sildenafil-induced facilitation of erectile responses *in vivo* in mice (46). Penile nNOS is a potential candidate for gene transfer (42, 47). It is present

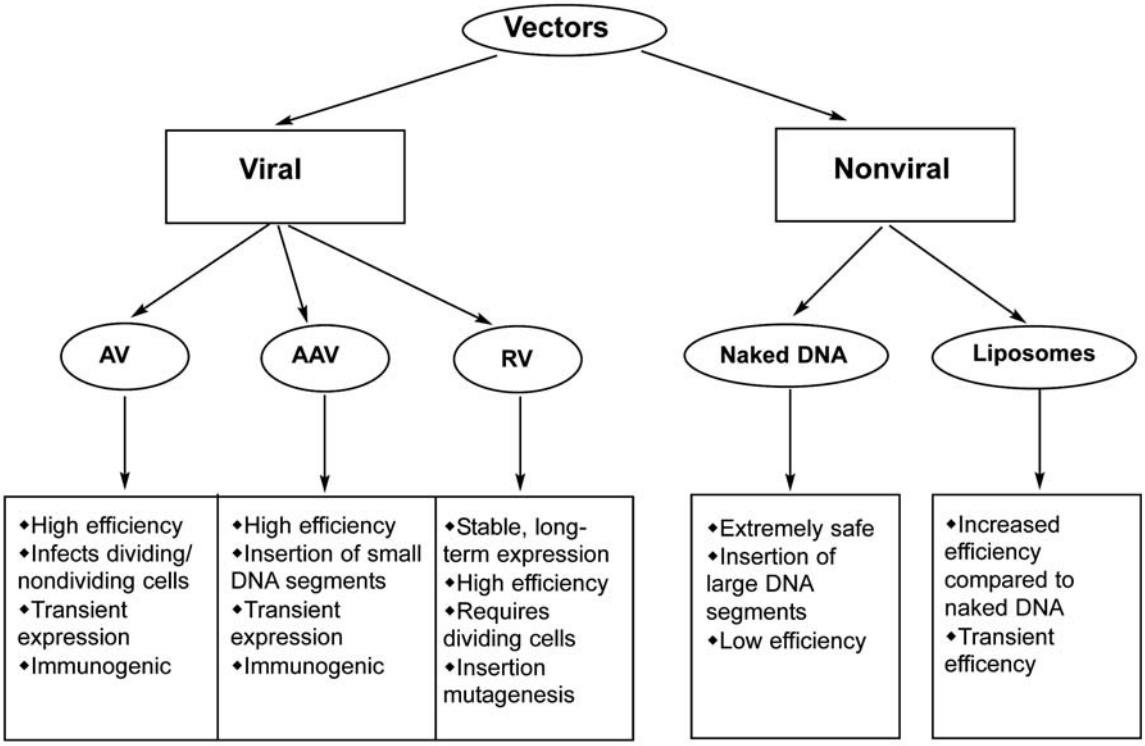

Figure 2. Various vectors used for gene transfer and their potential advantages/disadvantages. AV: Adenovirus, AAV: Adeno-associated virus, RV: Retrovirus

in the nerve terminals of the corpora cavernosa, in the pelvic ganglion, and in the hypothalamic and spinal cord regions involved in the control of erectile function (48). It may be possible to both increase the tissue uptake and prolong the expression of nNOScDNA constructs in the penis by taking advantage of replication-defective (gutless) adenoviruses (19, 49, 50).

A pharmacologic increase in NO formation in the penis during sexual stimulation may become an attractive complement to current medical treatments for ED (51, 52). One of the most promising approaches is gene transfer of nNOScDNA constructs into the corpora cavernosa to increase nNOS concentrations (14, 42). Magee *et al* demonstrated that intracavernosal gene therapy with nNOScDNA corrected age-related ED for at least 18 days when administered by electroporation, application of electrical current across the injection site, in a helper-dependent adenovirus at low viral loads (53).

Recently, penile NOS (PnNOS, the penile specific variant of nNOS) was transfected into the aged rat corpus cavernosum using a "gutless" (e.g. replication incompetent) adenovirus vector, as well as a plasmid.[53] The main goal of this study was to increase transfection efficiency and reduce the immunogenic potential by using a modified adenovirus vector, and to reduce required viral load, by increasing the transfection efficiency with electroporation techniques. Penile erection was measured at different times after PnNOS cDNA injection, by electrical field stimulation of the cavernosal nerve. These investigators found that electroporation increased pCMV--Gal uptake and its expression was detectable up to 56 and 60 days after transfection with plasmid and adenovirus, respectively. In the aged rats treated with pCMV-PnNOS and electroporation, the maximal intracavernosal/mean arterial pressure ratios (ICP/MAP) were elevated for 11 and 18 days when compared with those in controls. They suggested that intracavernosal gene therapy with PnNOS cDNA corrected aging-related ED for at least 18 days when given by electroporation in a helper-dependent adenovirus at low viral loads. Electroporation, caused an increase in transfection efficiency and duration of effect of both plasmid and adenoviral-mediated gene transfer techniques. The main limitation of these studies with nNOS gene transfer is the relatively short duration of physiological effect, although the *in vivo* effects may be more significant at latter time points. The reason for the short duration of efficacy is undetermined, but may be related to the presence of the "gutless" viral vector, or cellular regulation of the gene product. Although no immunologic, histologic or circulatory adverse effects were observed, the potential long-term side effects of over-expression of the product are not known.

3.4. Inducible nitric oxide synthase (iNOS)

Animal studies have demonstrated that aging is associated with a reduction of penile smooth muscle compliance and a decrease in penile NOS, resulting in ED. Garban *et al* used iNOS gene therapy in an aging rat model (16). These investigators found that the expression of iNOS after transfection was observed for up to 10 days. Of importance, gene therapy exhibited a significant increase in the cavernous nerve-stimulated erectile responses. Those

results open the way for the possible use of NOS isoforms for gene therapy of ED. Tirney *et al* studied iNOS gene therapy using a solution of plasmid, adenovirus, or adenovirus-transduced myoblast cells (adeno-myoblast) (54). They reported the best results with adeno-myoblast followed by adenovirus and then plasmid. Cavernous nerve-mediated erectile responses showed a 2-fold increase in ICP compared to the basal level of the iNOS-treated animals. These researchers concluded that myoblast-mediated gene therapy was more successful in delivering iNOS into the corpus cavernosum than were the direct adenovirus or plasmid transfection methods.

3.5. Brain-derived neurotropic factor (BDNF)

BDNF was initially characterized as a protein present in brain extracts that is capable of increasing the survival of dorsal ganglia cells (55). BDNF is a member of the neurotrophin family and plays a distinct role in neuron regeneration (56). When axonal communication within the cell body is interrupted by injury, Schwann cells produce neurotrophic factors. *In vitro* studies have shown that BDNF can enhance the survival and differentiation of several classes of neurons (57). BDNF is not restricted to neuronal target fields and thus may act in autocrine and paracrine fashions on neurons (58). The cavernosal nerves of the penis that originate along the posterolateral location of the prostate are vulnerable to injury during radical prostatectomy or cystoprostatectomy. Recovery of erectile function may depend in part on re-growth of nerves from the remaining neural tissues (59). In experimental studies, Bakircioglu *et al* transfected penile tissue by injecting AAV-BDNF into the corpus cavernosum to increase the amount of BDNF for several weeks and reported a beneficial effect on erectile function in animals with freeze-injured cavernosal nerves (60). Their results revealed that intracavernous injection of AAV-BDNF improved recovery of erectile function, enhanced regeneration of the intracavernous and dorsal nerves, and prevented neuronal degeneration in the major pelvic ganglia. The authors hypothesized that the production of BDNF protein in penile tissue was transported in a retrograde manner to the major pelvic ganglia to prevent neuronal damage and preserve nNOS activity. To enhance neurotrophic activity BDNF was combined with VEGF in a rat nerve-crush model to augment nerve regeneration and prevent degeneration. Another study using this combination of BDNF and VEGF intracavernosally, immediately and one month after experimental nerve injury in rats, caused a significant recovery in erectile function and the morphology of cavernosal nerve fibers in the corpus cavernosum (61). Further experiments with BDNF are planned; namely, to extend the treatment period to allow for complete regeneration and increase the AAV-BDNF titer in this animal model.

3.6. Vascular endothelial growth factor (VEGF)

The development and growth of the vascular system—angiogenesis and vasculogenesis—are regulated by angiogenic factors (62). VEGF, a potent angiogenic stimulator, was initially purified from tumor cell-lines as a specific mitogen for endothelial cells *in vitro* (63). VEGF stimulates endothelial cell growth and angiogenesis. An insufficient vascular supply is thought to be responsible for a large component of ED. While sufficient cavernosal smooth muscle relaxation can overcome this deficit to a certain degree, there is a biological rationale for increasing blood flow to the cavernosal tissue to promote recovery of erectile function. Previous studies have shown that all VEGF isoforms are abundantly expressed in rat and human penile tissues (64-67). On the supply side method of gene transfer techniques, direct intracavernosal injection of VEGF in a rat model of vascular insufficiency demonstrated restoration of the veno-occlusive mechanism as documented by nerve-stimulated intracavernous pressure response (68).

Penile smooth muscle cells possess VEGF receptors and VEGF treatment enhanced penile smooth muscle cell proliferation and migration in culture (69). Additionally, VEGF has also been reported to have neuroprotective and neurotrophic effects (70, 71). Local intracavernous delivery of VEGF isoforms has been documented to lead to recovery from ED induced by castration (72), traumatized iliac arteries (68) or hyperlipidemia (67, 73, 74). In a recent study, Rogers *et al* demonstrated that intracavernous VEGF injection of protein and the VEGF gene prevented veno-occlusive dysfunction in castrated rats with venogenic ED (72). In rats with venous leakage, VEGF gene treatment reversed the cavernosometric findings of venous leakage. It has been suggested that intracavernous injection of either the VEGF protein or VEGF gene might become the preferred treatment to preserve erectile function in patients in whom testosterone therapy is contraindicated, and may hold the key to the prevention and cure of many other forms of ED (72).

In another related study, Hsieh *et al* demonstrated that intracavernosal injection of BDNF and VEGF prevented degeneration and facilitated regeneration of nNOS-containing neurons in the major pelvic ganglion, dorsal nerve, and intracavernosal tissue (61). Gholami *et al* examined neurogenic and vasculogenic ED associated with hypercholestrolemia and evaluated VEGF and AAV-BDNF for the potential treatment of ED (73). They noted that a high-fat diet caused ED with accompanying neurological and vascular changes, and VEGF and AAV-BDNF treatment ostensibly prevented these problems.

Jesmin *et al* demonstrated that expression of VEGF, its receptors (Flk-1 and Flt-1), and its signaling pathway, Akt, are markedly diminished in penile tissues in type-II diabetic rats (75). They hypothesized that these abnormalities might play an important role in the reduced expression of penile eNOS and impaired cavernosal nerves containing reduced levels of nNOS seem to be involved in diabetic ED. Any decrease in the activity of VEGF in diabetic rats could contribute to the loss of cavernosal smooth muscle cells by apoptosis. Recent data provides a molecular explanation for VEGF's stimulatory effects on penile erection via phosphorylation of eNOS (76). In this study, animals were injected intracavernosally with a replication-deficient adenovirus expressing human VEGF145, thereby establishing a specific mechanism whereby VEGF promotes erectile function.

3.7. Calcitonine gene-related peptide (CGRP)

CGRP is an effector of erectile capacity with its ability to produce a receptor-mediated increase in intracellular cAMP levels, resulting in cellular hyperpolarization through increased K^+ channel activity. Using an adenoviral-mediated transfer of CGRP in aged rats, Bivalacqua *et al* documented overexpression of CGRP reversed the age-related reduction of CGRP and cAMP levels in the corpora cavernosa (77). The investigators observed a significant increase in neurogenic-mediated erectile responses in CGRP-transfected aged rats compared to young rats with duration of efficiency of up to 5 days. Moreover, any detectable effects of CGRP overexpression on resting ICP or blood pressure was not observed. These data suggest that *in vivo* adenoviral gene transfer of CGRP can physiologically improve erectile function in the aged rat.

3.8. K^+-Ca^{+2} system via *hSlo*

Although there are at least four distinct types of potassium channels present in human corporeal smooth muscle, the maxi-K^+ channel (Ca^{2+} sensitive potassium channel or K_{Ca} channel) is the most important in corporal smooth muscle physiology. Alterations in the function and regulation of the maxi-K^+ channel are likely to be important in the genesis of organic ED (78-80). Activation of the maxi-K^+ channel in corporal smooth muscle represents an important and attractive mechanism for control of corporal smooth muscle function (78-80). Given the central role of the maxi-K^+ channel in modulating intracellular Ca^{2+} levels and transmembrane Ca^{2+} flux in corporal smooth muscle, modification of channel function is a logical target for molecular and pharmacological intervention in the treatment of ED.

The strategy underlying ion channel gene therapy is based on the tight link among K^+ channel activity, transmembrane Ca^{2+} flux through voltage-dependent Ca^{2+} channels, and corporal smooth muscle tone (15). Christ and colleagues demonstrated that injection of *hSlo* cDNA, which encodes for the large conductance Ca^{2+} sensitive maxi-K^+ channels, into the rat corpora cavernosa could increase gap-junction formation and enhance erectile responses to nerve stimulation in aged and diabetic rats (81, 82). Christ *et al* reported that naked pcDNA/*hSlo* DNA was easily incorporated and the expression was sustained in rat corporal smooth muscle *in vivo* for at least 4 months (83). This prolonged expression of *hSlo* cDNA was capable of altering erectile responses as measured by the intracavernous pressure response to stimulation of the cavernous nerve. Thus, increasing the expression of maxi-K^+ channels influences the overall function of cavernosal smooth muscle cells.

The group in New York (Drs. Melman and Christ) recently made a successful presentation to the NIH Recombinant Advisory Committee for the pilot study of the use of the human hSlo/maxi-K gene to treat ED (15).

3.9. Calcium sensitization via RhoA

The contracted state of the penile vasculature is hypothesized to be mediated by the release of norepinephrine, endothelin-1 (ET-1) and a host of other vasoconstrictors (52). Vasoconstrictor agents operate by elevating intracellular calcium and activating myosin light-chain kinase (MLCK), causing myosin phosphorylation and cross-bridge activation. In addition, calcium sensitization is activated through an agonist of heterotrimeric G-protein-coupled receptors, activation of RhoA through the exchange of GTP for GDP. Activated RhoA, in turn, activates Rho-kinase, which inhibits myosin light chain phosphatase (MLCP), resulting in a net increase in myosin phosphorylation and force at a constant calcium level (84, 85).

Chitaley *et al* examined the role of Rho-kinase on cavernosal tone based on the hypothesis that antagonism of Rho-kinase caused cavernosal smooth muscle relaxation, initiating the erectile response independent of NO (86). The fact that Rho-kinase antagonism stimulates penile erection in rats independently from the NO pathway introduces a potential alternative avenue for the treatment of ED. Wingard *et al* demonstrated that cavernosal tissues exhibited increased RhoA/Rho-kinase protein levels after castration (87), suggesting that an active RhoA/Rho-kinase pathway contributes to a reduced erectile response following castration due to upregulation of RhoA/Rho-kinase protein levels and that inhibition of this pathway may potentially serve as an effective treatment for ED. The increased responsiveness to α-adrenergic stimulation in this castrated rat model may be associated with an increase in Rho-kinase activity through lowered NO production.

Another study, employing corpus cavernosum smooth muscle cells from diabetic rabbits, revealed that overexpression of endothelin receptors and increased sensitivity to ET-1-induced higher force generation (88). Studies using RT-PCR on cavernosal tissues from diabetic and normal rabbits (using primers that specifically amplify Rho-kinase-α and Rho-kinase-β; the two known isoforms of Rho-kinase) revealed that the expression of Rho-kinase-α was not altered, but the expression of Rho-kinase-β increased 3-fold in diabetic corpora cavernosa. Similarly, Western blot analysis revealed a 2.5-fold up-regulation of Rho-kinase-β at the protein level. This evidence showed that ET-1-induced contractions of corpus cavernosum smooth muscle cell are largely mediated by Rho-kinase and that, specifically, the Rho-kinase-β isoform is selectively upregulated in the diabetic corpus cavernosum. Their commentary suggests that sensitization of corpus cavernosum smooth muscle to ET-1 may be a key factor involved in the pathogenesis of ED in diabetics and could, at least, partly explain the decreased efficacy of sildenafil in treating ED in diabetic patients (88).

Researchers have recently suggested that inhibition of RhoA/Rho-kinase can improve eNOS enzyme activity and protein expression, and help restore ED in diabetes (89). Most intriguing is evidence for a common signaling mechanism responsible for synergistic vasoconstriction induced by a combination of endothelin and phenylephrine that activates the calcium-sensitizing pathway involving RhoA Rho-kinase in cavernosal tissues. These results demonstrate that RhoA/Rho-kinase is

upregulated in cavernosal tissue isolated from the penises of diabetic rats and identifies a physiologic role for RhoA/Rho-kinase in modulating erectile function *in vivo* by reducing eNOS protein and activity, thus decreasing penile cGMP levels (89). In addition, this study demonstrates the Rho-kinase inhibitor, Y-27632, induces a greater erectile response when administered intracavernosally to the diabetic rat, illustrating that Rho-kinase activity is increased in the diabetic corpus cavernosum (89). Adeno-associated virus gene transfer of the dominant-negative Rho-mutant to the diabetic penis decreased RhoA/Rho-kinase protein expression and restored erectile function *in vivo*. These findings show how the RhoA/Rho-kinase pathway influences erectile function through the attenuation of endothelial-derived NO formation in the penis (89). RhoA/Rho-kinase plays an important role in the regulation of penile endothelial cell function and dysfunction as related to vascular diseases of the penile vasculature.

3.10. Superoxide dismutase (SOD)

Endothelial cells produce reactive oxygen species (ROS) in response to shear stress, endothelium-derived agonists including acetylcholine and bradykinin, and also in various vascular disease states. Potential sources of ROS in endothelial cells include NADP phosphate oxidase (which generates superoxide anion), lipooxygenase, cyclooxygenase, peroxidases, cytochrome P450s, xanthine oxidase, and iNOS (90). The reaction of superoxide anions and NO in the vascular endothelium or smooth muscle cells triggers the formation of the highly toxic molecule, peroxynitrite (91). Due to its toxic effects, peroxynitrite can cause direct tissue injury, alterations in vascular tone, oxidation of vascular proteins and lipids, and overall organ dysfunction (92). The antioxidants SOD, catalase, glutathione peroxidase and reductase play a pivotal role at the cellular level in protecting against ROS (93). Among the three types of SOD isoforms in the human body, Cu/Zn and EC-SOD have been identified in the penis, predominantly in the endothelial and cavernosal smooth muscle cells (94). Increased levels of superoxide anions in the endothelium and cavernosal smooth muscle cells contribute to ED by causing endothelial dysfunction and reducing cavernosal NO biosynthesis. Apart from reducing NO biosynthesis, superoxide anions cause Ca^{2+} mobilization, which results in reduced Ca^{2+} levels in the cavernosal endothelial cells. Overall, increased oxidative stress and superoxide anion production alters the penile vasculature and impairs endothelial-derived NO in the erectile tissues, resulting in ED (95, 96).

Oxidative stress is quite prominent in certain chronic disease states, including diabetes, hypercholesterolemia, and aging, and is associated with significant changes in the endothelium and smooth muscle cells in the penis (97, 98). As noted, superoxide anions react with NO and form peroxynitrite. In aging, increased production of peroxynitrite accelerates the degeneration of nerves and endothelial cells involved in the erectile process (94). An imbalance in superoxide anion generation and inactivation in the penile vasculature causes impaired endothelial-dependent smooth muscle relaxation and ED (94). In aged rats experimentally transfected with adenoCMV-EC-SOD, there was a significant increase in EC-SOD mRNA and protein expression and a significant reduction of superoxide anions. Moreover, EC-SOD gene therapy increased cGMP levels in the corpus cavernosum that was found to enhance the *in vivo* erectile response to cavernosal nerve stimulation. These observations implicate EC-SOD as beneficial in limiting superoxide anion production and preventing some of the outcomes of age-related ED (94).

4. CELL-BASED THERAPY

Another potential application for ED is the (re)-implantation of genetically modified cells into the corpus cavernosum. The aim is to seed the corpora cavernosa with cells having desired, genetically modified physiological characteristics. Endothelial cells are recognized to have the ability to adhere and self-aggregate once they are transplanted into another organ, such as the penis (99). Wessels *et al* first demonstrated that autologous endothelial cells could be transplanted into the corpus cavernosum, and undergo cell adherence and persistence in the cavernosal sinusoids for up to 2 weeks and eventually become part of the sinusoidal lining of the penis (100). This is the basis for the rationale of cell-based gene therapy for the treatment of ED. Studies have documented that muscle-cell-mediated gene therapy was even more efficacious in delivering iNOS into the corpus cavernosum (54). In this study, a significant increase in cavernosal nerve-stimulated ICP responses was documented. Transplanted cells are of considerable interest and future studies using marker genes and functional transgenes will further our knowledge about the survival, replication, and function of endothelial cells within the corpus cavernosum.

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