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Gene Therapy of Erectile Dysfunction in the Rat with Penile Neuronal Nitric Oxide Synthase[1]

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ABSTRACT

Gene transfer to the penile corpora cavernosa of constructs of the inducible and endothelial nitric oxide synthase (NOS) cDNAs ameliorates erectile dysfunction in aged rats. In this study, we investigated whether the neuronal NOS (nNOS) variant responsible for erection, penile nNOS (PnNOS), can exert a similar effect, and whether the combination of electroporation with a helper-dependent adenovirus (AdV) improves gene transfer. PnNOS and β-galactosidase cDNAs were cloned in plasmid (pCMV-PnNOS; pCMV-β-gal) and "gutless" AdV (AdV-CMV-PnNOS; AdV-CMV-β-gal) vectors, and injected into the penis of adult (β-gal) or aged (PnNOS) rats, with or without electroporation. Penile erection was measured at different times after PnNOS cDNA injection, by electrical field stimulation of the cavernosal nerve. The expression of β-galactosidase or PnNOS was estimated in penile tissue by either histochemistry and luminometry or Western blot, and the effects of AdV-CMV-PnNOS on mRNA expression were examined by a DNA microarray. We found that electroporation increased pCMV-β-gal uptake, and its expression was detectable at 56 days. In the aged rats treated with pCMV-PnNOS and electroporation, the maximal intracavernosal:mean arterial pressure ratios were elevated for 11 and 18 days when compared with those in controls. Electroporation intensified penile uptake of as few as 10^6 viral particles (vp) of AdV-CMV-β-gal, and with 10^7 vp β-galactosidase was still detectable at 60 days. Electroporated AdV-CMV-PnNOS (10^7 vp) was effective at 18 days in stimulating the erection of aged rats, without inducing the expression of cytotoxic genes. In conclusion, intracavernosal gene therapy with PnNOS cDNA corrected the aging-related erectile dysfunction for at least 18 days when given by electroporation in a helper-dependent AdV at low viral loads.

aging, gene regulation, male sexual function, nitric oxide, penis

INTRODUCTION

Erectile dysfunction, the inability of a man to attain and/or sustain an erection of sufficient rigidity for penetration, affects an estimated 15 million men in the United States [1]. The most common cause of this condition is vasculogenic, presumably due to an inability of the cavernosal smooth muscle to relax sufficiently to achieve compression of the subtunical veins. Cavernosal smooth muscle relaxation is primarily a nitric oxide (NO)-cGMP-mediated response, which includes NO synthesized by the neuronal nitric oxide synthase (nNOS; NOS1) within the nerve terminals of the nonadrenergic, noncholinergic cavernosal nerve [2, 3]. Therefore, the inability of the cavernosal smooth muscle to relax normally may be due to a reduction in NO production and/or synthesis [3] or due to damage or dysfunction of the corporal smooth muscle tissue in which endogenous NO levels may be insufficient to compensate for the loss of compliance of the damaged tissue [4, 5].

A direct and stable pharmacologic increase in NO production in the penis during sexual stimulation is therefore an attractive alternative or complement to current medical treatments for erectile dysfunction [6, 7]. One of the most promising approaches is gene transfer of NOS cDNA constructs to the corpora cavernosa to increase NOS concentration in the penis [3, 8, 9]. Previously, it was shown that a single injection of a plasmid construct of the inducible NOS (iNOS; NOS II) cDNA corrects for at least 10 days the defective erectile response to electrical field stimulation (EFS) of the cavernosal nerve in the aging rat without any detectable side effects [10]. However, this NOS isoform is not normally involved in penile erection, and iNOS may have secondary effects on homeostatic mechanisms within the cavernosa [11]. A similar up-regulation of the erectile response has been obtained with an adenoviral (AdV) construct of another isoform of NOS, endothelial NOS (eNOS; NOS III), which is not normally involved in the nitrergic neurotransmission necessary for penile erection [3, 8]. The measurement of the therapeutic effect was performed for shorter periods [12, 13].

The efficacy of gene therapy to ameliorate erectile dysfunction has also been extended to other genes related to either cavernosal relaxation, such as maxi K^+ channels (hSlo) [14], and to trophic factors, such as vascular endothelial growth factor [15] or brain-derived neurotrophic factor (BDNF) [16]. In the latter cases, the adeno-associated virus (AAV) was used as a vehicle for introducing the gene. Although AAV constructs can have prolonged effects, their preparation is cumbersome, and they are not particularly well suited for the cloning of large cDNAs, such as those of the NOS isoforms [17]. Alternatively, AdV can be used and is relatively easy to produce, but this virus can suffer from rapid clearance that can severely affect the duration of the response [17, 18].

The penile nNOS (PnNOS) is a potential candidate for gene transfer because it is considered one of the NOS isoforms responsible for penile erection [3, 19]. It is present in the nerve terminals of the corpora cavernosa, in the pelvic ganglion, and in hypothalamic and spinal cord regions involved in the control of reproductive function [20, 21].

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PnNOS has been cloned from the rat [19] and human penis (unpublished data). This alternatively spliced form has a specific sequence insert with potential modulatory function that is absent in the brain counterpart [19, 22]. It is very similar, if not identical to, nNOSu an alternatively spliced form present in skeletal muscle [23].

It may be possible to both increase the tissue uptake and prolong the expression of PnNOS cDNA constructs in the penis by taking advantage of replication-defective (''gutless'') AdV vectors [24–26], in which the majority of viral genes are deleted, except for the inverted terminal repeats and packaging signal (ψ). They are generally thought to be much less immunogenic than previous versions, and hence infected cells will not be cleared as rapidly. Despite this improvement, it would be convenient, particularly for human gene therapy, to reduce the viral construct load required to obtain the desired tissue uptake and biological response. One delivery procedure that may achieve this aim for the AdV constructs is electroporation to organs in live animals, a procedure that increases the efficiency of plasmid gene therapy in vivo in skeletal muscle [27, 28] and testis [29]. Electroporation also facilitates the penetration of viral particles in cultured cells with a low or negligible number of viral receptors [30].

In this study, we examined whether PnNOS cDNA in plasmid and a helper-dependent AdV vector injected into the penile corpora cavernosa can ameliorate erectile dysfunction in the aging rat model, and whether electroporation can enhance the uptake of these constructs and hence decrease the viral construct load needed to stimulate the erectile response.

MATERIALS AND METHODS

Preparation of Plasmid cDNA Constructs

The cloning and expression of the rat PnNOS cDNA in the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), now referred to as pCMV-PnNOS, has been reported previously [19, 20]. Cloning into pcDNA3 allows for strong constitutive expression from the cytomegalovirus (CMV) immediate-early promoter. For cloning into the AdV shuttle vector, pSTK120 (generous gift from Merck Co., Rahway, NJ), pCMV-PnNOS was digested with *Bph*I and *Avr*II restriction enzymes to release the expression cassette containing the CMV promoter, PnNOS gene, and bovine growth hormone poly(A) signal. The DNA fragment was agarose gel purified and blunted by end-filling with DNA polymerase Klenow fragment. pSTK120 was digested with *Swa*I restriction enzyme (blunt end) and ligated with the PnNOS containing DNA fragment, and the resulting plasmid is referred to as pSTK120-PnNOS.

The plasmid construct for the reporter gene β-galactosidase was p-Sport-β-gal (Invitrogen). The AdV shuttle vector construct containing the β-galactosidase gene, cloned in a manner similar to that for the pSTK120-PnNOS construct, was prepared as described [24] and named pSTK120-β-gal.

Preparation of AdV cDNA Constructs

The construct AdV-CMV-PnNOS containing the CMV-PnNOS-poly(A) signal cassette in the replication-defective AdV vector used in this work was generated by cotransfection of pSTK120-PnNOS with the AdV helper virus AdLC8cluc, which contains a packaging signal sequence flanked by loxP recognition sites [31]. The replication-permissive cell line used for AdV production, HEK293Cre4, has an integrated Cre4 recombinase sequence that is constitutively expressed. Infection of the AdLC8cluc virus into these cells results in cleavage and recombination of the region between the loxP sites, thereby eliminating the packaging signal needed for propagation of the helper virus but allowing for synthesis of viral proteins needed for a coinfected replication-deficient AdV.

Briefly, the viral packaging procedure was as follows: pSTK120-PnNOS was linearized by digestion with *Pme*I and cleaned up (Wizard DNA Clean-Up column; Promega, Madison, WI). HEK293Cre4 cells cultured on a 6-cm dish to 80% confluence in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) were transfected with 10 μg of the linearized plasmid using the calcium phosphate procedure [32]. Twenty-four hours posttransfection, the cells were infected for 1 h at room temperature with AdLC8cluc helper virus at a multiplicity of infection (MOI) of 5 plaque-forming units per cell, and then DMEM-5% horse serum was added. At 48 h, cells (showing complete cytopathic effect by microscopy) were harvested, washed with PBS^{2+} (1× PBS + 0.68 mM $CaCl_2$ + 0.5 mM $MgCl_2$), and resuspended in PBS^{2+}-10% glycerol. Viral particles were released by four rounds of freezing and thawing, and the viral supernatant after brief centrifugation was frozen at −70°C and designated passage 0. The crude viral stock was next amplified by multiple passages through HEK293Cre4 cells. For early passages, one half of each passage supernatant was used to infect HEK293Cre4 cells as described previously at an MOI of 1. For later passages, on 10- and 15-cm plates, the titer of each passage was determined, and cells were infected as described previously, with harvesting at 3–4 days when complete cytopathic effects were observed. The AdV-CMV-PnNOS for experiments in animals was band purified by CsCl centrifugation followed by dialysis in PBS^{2+} [33]. Because of the similar size and density of our construct and the helper virus, a single band was usually obtained. Purified viral aliquots were stored at −70°C in PBS^{2+}-10% glycerol. A similar procedure of packaging and CsCl purification was used for the preparation of the AdV construct of β-galactosidase (AdV-CMV-β-gal) from the plasmid pSTK120-β-gal.

Titration and In Vitro Test of cDNA Constructs

The purity and concentration of the plasmid cDNA constructs was assessed by agarose gel electrophoresis and spectrophotometry. Their expression ability was tested by lipofectamine transfection into cultures of HEK293 cells [20] on six-well plates and detection of the expected proteins 2–3 days after transfection. Transfection with the empty pcDNA3 vector alone was used as a control.

In the case of PnNOS, cells were lysed on the plates with an SDS-containing buffer [34], and protein was measured in the clarified supernatants with the BCA Protein Assay procedure (Pierce, Rockford, IL). Equal amounts of protein (30 μg) were run on 7.5% polyacrylamide gels and subjected to Western blot immunodetection with a custom-made polyclonal anti-rat PnNOS antibody immunoglobulin (Ig) G (1:500) directed against the 34-amino acid insert [20] or a monoclonal anti-human nNOS antibody IgG (1:500) directed against the carboxy end (Transduction Laboratories, Lexington, KY), followed by a secondary polyclonal goat anti-rabbit IgG or horse anti-mouse IgG, respectively, linked to horseradish peroxidase (Transduction Laboratories) [20]. Detection was made by a luminol reaction and x-ray scintillography. Negative controls were performed without primary antibody. In the case of β-galactosidase, cells were stained with X-gal [35], and the blue cells were counted under an inverted microscope.

The titration of viral passages and purified AdV-CMV-PnNOS was done by polymerase chain reaction (PCR) using a 5′ primer (CGAAATTAATACGACTCACT) anchored in the pCDNA3 sequence upstream of the CMV-PnNOS cassette, and a 3′ primer (AGGTCTGTCCACCTGGATTC; nucleotides 796–816; GenBank accession No. U67309) in PnNOS. Viral preparations were serially diluted, allowed to infect HEK293 cells on six-well plates for 1 h at room temperature, washed with PBS^{2+}, and incubated in DMEM-10% FBS for 3 h. Cells were then lysed on the plates and treated with phenol-chloroform, and the supernatants were subjected to PCR. The band intensities on ethidium bromide-stained agarose gels were compared with the corresponding bands generated from serial dilutions of known amounts of pSTK120-PnNOS. Titers of CsCl-purified virus were also measured by spectrophotometry (1 A_{260} = 10^9 AdV particles per microliter) [26] and were essentially similar to those obtained by PCR titration.

To verify the proper expression of the PnNOS and β-galactosidase from the AdV constructs, HEK293 cells [26] were infected at an MOI of 1 on six-well plates for 1 h at room temperature, and then DMEM-10% FBS was added, and the cells were grown for 2 days. Noninfected cells were used as controls. The detection of expressed proteins was performed as described previously by Western blotting.

Animal Handling and Treatments

Male Fisher 344 rats were purchased from the National Institutes of Health (NIH)/National Institute on Aging colony (Harlan Sprague-Dawley, Inc., Bethesda, MD), maintained under controlled temperature and lighting, and treated according to NIH regulations. The protocol was approved by the Research and Education Institute's Animal Care and Use Review

Committee. Retired breeders (9–11 mo old) were used for all experiments involving the uptake and distribution of constructs expressing β-galactosidase (n = 3 for luminometry and 2 for histochemistry), whereas aged rats (24 mo old) were used for all EFS experiments involving the effects of constructs expressing PnNOS, and young rats (5 mo old) were used as untreated controls for the aged series (n = 6–8; except for the 30- and 60-day time points, at which n = 4). Animals were anesthetized with ketamine-xylazine mix (50 mg ketamine/7 mg ketamine per kg body weight), the penis was exposed, and a ligature was placed at the base of the penis. The animals were injected in the corpora cavernosa at the middle of the penile shaft with 100 μl of either saline alone or the appropriate cDNA construct in saline, as indicated in each experiment, and the ligature was removed 2 min later. When electroporation was applied, it was given with 0.5-cm platinum electrodes spanning longitudinally the site of injection, using the Electro Square Porator ECM 830 (BTX, San Diego, CA). Settings were 100 V (voltage); 40 msec (duration); 8 pulses per sec (frequency); 1 sec (interval); unipolar (polarity), as optimized in preliminary experiments, adapted from a protocol devised for the rat skeletal muscle [27]. At the indicated periods, varying from 1 to 60 days, animals were either used for EFS determinations, or killed directly for organ collection.

Persistence of cDNA Construct Expression and Histologic Assessment

Animals not subjected to EFS were killed and used for organ excision, either after perfusion through the left ventricle with saline followed by 4% formalin [11] for histology and histochemistry examination, or without fixation for luminometry. The penises were excised, the skin was denuded removing the glans and adhering noncrural tissue, and the penile shaft was separated from the crura. In addition, the liver, heart, lungs, and kidney were excised. The excised tissues from perfused animals were postfixed overnight in 4% formalin, washed in PBS, and stored at 4°C. For β-galactosidase histochemistry [36], the whole penis including shaft and crura was incubated overnight at 4°C in a PBS containing 2 mM $MgCl_2$, 0.01% deoxycholate, and 0.02% NP-40. Staining for β-galactosidase was performed with 0.1% X-gal in permeabilization solution supplemented with 5 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ for 15 h at 37°C with agitation. After staining, the penises were embedded in O.C.T. compound (Sakura, Torrance, CA) and frozen. Four to five longitudinal sections (30 μm) were taken and stored, and the remaining blocks were photographed using a Nikon camera (Melville, NY). Sagittal and transverse sections (15–30 μm) were also used for microscopic localization of the staining. In some animals, tissue excised from lung, liver, heart, and kidney, was processed as described previously. The expression of β-galactosidase was also detected by immunostaining using a primary monoclonal antibody (Neomarkers, Inc., Fremont, CA) against the bacterial enzyme β-galactosidase and a secondary anti-mouse IgG antibody linked to peroxidase essentially as described [21].

For determination of β-galactosidase activity [13], shafts were homogenized in TMTC (50 mM Tris HCl [pH 7.4], 5 mM $MgCl_2$, 100 mM NaCl, and 4% [w/v] CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate]). Protein levels were estimated as described previously and 20-μl aliquots were assayed using β-galactosidase chemiluminescent reporter assay Galacto-light PLUS (Tropix, Bedford, MA). After 1 h, luminescent signals were recorded in a TD20/20 luminometer (Turner Design, Sunny Vale, CA) for 10 sec with a delay period of 2 sec. The results were expressed as relative luminescent units per milligram of protein.

For histologic assessment, transverse paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin. Pictures were taken using a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI).

Measurement of Erectile Response to EFS

EFS was performed as described previously [5, 10]. Briefly, after induction of general anesthesia, a midline surgical approach was used to obtain exposure of the cavernosal nerve. Platinum electrodes were applied to the cavernous nerve, and arterial and intracavernosal pressure measurements were obtained by simultaneous direct intrafemoral artery and cavernosal catheterization, respectively. EFS was applied at 10 V and a frequency of 15 Hz for pulses of 30 sec, separated by 2-min intervals, with a Grass Stimulator (Grass Instruments Co., Quincy, MA). A data acquisition system (Biopac Systems, Santa Barbara, CA) connected to a personal computer simultaneously recorded arterial blood pressure (femoral artery) and intracavernosal pressure, and values were expressed in millimeters of mercury (mean ± SEM). The ratio between the maximal intracavernosal pressure (MIP) and the mean arterial pressure (MAP) obtained at the peak of erectile response was calculated to normalize for variations in blood pressure.

Assays of Gene Expression after EFS

For the determination of PnNOS protein expression after AdV-CMV-PnNOS administration, the penile shafts were excised immediately after EFS determinations and homogenized in an SDS-containing buffer with protease inhibitors, and the supernatant was obtained by centrifugation at 15 000 × *g* for 5 min [20]. Protein concentration was estimated and extracts were analyzed by Western blot as described previously. The 155-kDa bands for PnNOS were computer scanned, and their relative intensities were determined by densitometry [20].

For the analysis of multiple gene expression, total RNA was isolated from the penis of rats subjected to EFS using Trizol (Gibco-BRL, Grand Island, NY) and estimated by spectrophotometry, and the total RNA quality was verified by analytic gel electrophoresis. Total RNA was digested with RNase-free DNase I (Epicentre Technologies, Madison, WI) to eliminate residual DNA and reverse transcribed. Complementary DNAs were labeled with [^{32}P]dATP using a primer mix specific for the genes represented in the microarray assay (Clontech Rat Atlas array kit; Clontech, Palo Alto, CA) [37]. The labeled cDNAs were separately hybridized against a pair of identical nylon membranes containing 588 cDNA probes. Each cDNA is represented as single spots distributed in panels of well-characterized genes grouped in functional or biochemical pathways. The resulting radioactive signals were visualized by both autoradiography and phosphorimaging at 24- and 72-h exposure, and a comparison of the intensities of each signal was performed between the plaque and the control RNAs using the manufacturer's software (Atlas Image 1.0; Clontech). Values were corrected for differences in hybridization efficiency between the 2 membranes by dividing the average expression of all genes in the respective arrays (''global normalization''). Only adjusted values with relative differences in gene expression greater than 2.0 up or down were considered significant.

Statistical Analysis

Values were expressed as mean ± SEM. The normality distribution of the data was established using the Wilk-Shapiro test, and the outcome measures between two groups were compared by the *t*-test. Multiple comparisons among the different groups were analyzed by a single-factor ANOVA, followed by post hoc comparisons with the Student-Newman Keuls test, according to the Graph Pad prism v. 3.0 (GraphPad Software, San Diego, CA). Differences among groups were considered significant at $P < 0.05$.

RESULTS

Effect of Electroporation on the Uptake of Plasmid cDNA Constructs in the Corpora Cavernosa

To test the ability of electroporation to increase the uptake of plasmid cDNA constructs into the penile corpora cavernosa, increasing amounts of a reporter plasmid expressing β-galactosidase under the control of the CMV promoter (pSPORT-β-gal; referred to as pCMV-β-gal in this study) were injected into the penis of retired breeder rats (9–11 mo old), with or without electroporation. Ten days later, β-galactosidase expression in the shaft tissue from nonelectroporated penis as measured by luminometry in tissue homogenates showed a significant dose-dependent increase, whereas the application of electroporation stimulated the uptake/expression of the cDNA construct by 10-fold when 30 μg plasmid were administered (Fig. 1A). This effect was visible as a uniform staining along the shaft and glans by X-gal histochemistry in fixed sagittal blocks of the whole penis, contrasting with staining in the crura restricted to the proximal area. Virtually no endogenous β-galactosidase activity was observed in animals injected with saline, but even 10 μg of pCMV-β-gal induced a visible staining in the distal shaft and crura (Fig. 1B). Similar luminometry and histochemical determinations were conducted at several time points after electroporation of 30 μg of pCMV-β-gal.

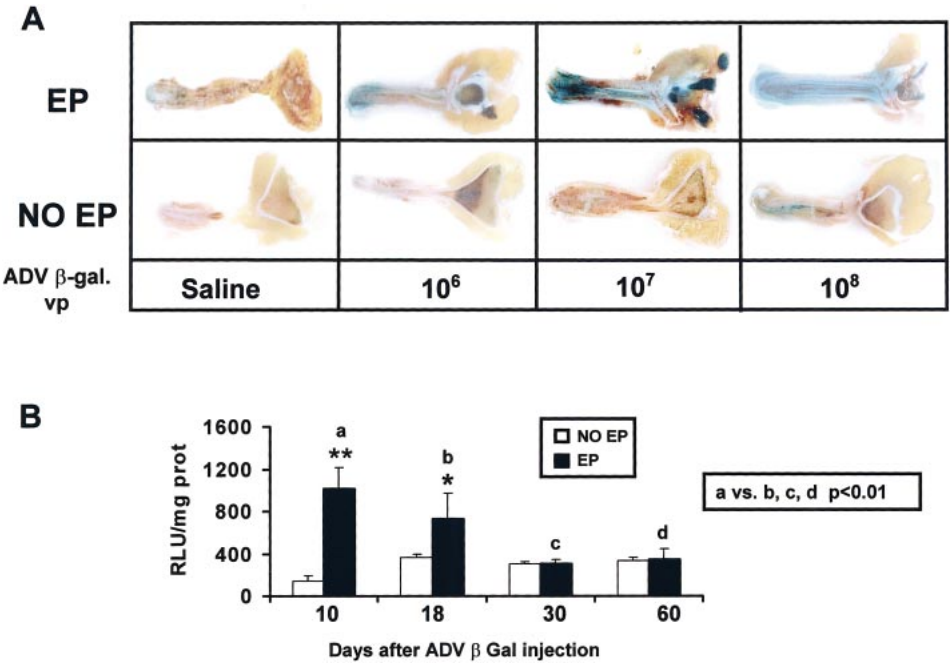

FIG. 1. Effect of electroporation on the dose curve and time course of expression of β-galactosidase plasmid cDNA injected into the rat corpora cavernosa. The expression of pCMV-β-gal given to the penis at different doses was determined by either luminometry (**A**) or histochemistry (**B**) 10 days after injection. $*P < 0.05$ for EP versus no EP. The time course was characterized with a dose of 30 μg and by luminometry (**C**).

The highest expression seen was at the earliest time point assayed (14 days), and expression was still detectable by 56 days (Fig. 1C). β-Galactosidase was not detected in the liver, heart, and kidney, but some weak staining was detected in the lung of both saline-injected and pCMV-β-gal injected rats (data not shown).

Intracavernosal injection in conjunction with electroporation initially caused per se moderate inflammation, as shown 1 day after administration of 100 μl vehicle (saline) followed by electroporation. Histochemical staining detected a cell infiltrate, primarily consisting of monocytes, neutrophils, and eosinophils, in corporal tissue, and the collapse of some cavernosal spaces (not shown). This process was seen all along the penile shaft and extended uniformly within the injected side of the penis. Inflammation was also observed, although to a lesser degree, with saline injection only without electroporation. The inflammation had disappeared completely at 8 and 17 days after electroporation. The appearance and activity of the animals and the external appearance of the penis was similar at all times in the electroporated and nonelectroporated rats (not shown).

Effects of Plasmid PnNOS on Erectile Function

After confirmation of the efficacy and safety of electroporation, aged rats (24 mo old) were injected with a cDNA construct similar to the one expressing β-galactosidase, but in this case driving the expression of the rat PnNOS cDNA (pCMV-PnNOS). This construct has been previously shown to express the 155-kDa PnNOS protein when transfected into HEK293 cells and assayed by Western blot with a PnNOS antibody [20]. The MIP elicited by EFS of the cavernosal nerve was measured after injection of pCMV-PnNOS or saline. To account for possible variations in the MAP, the systolic and diastolic values were continuously recorded, and MAP was calculated. The erectile response was then expressed as MIP:MAP. No significant changes ($P > 0.05$) occurred in MAP in the various treatment groups when compared with that in the controls, as shown for some indicated treatments and periods: 5-mo-old adult untreated rats: 108.8 ± 3.7 mm Hg; 24-mo-old untreated rats, 94.8 ± 1.5 mm Hg; 24-mo-old rats treated with pCMV-PnNOS, 11 days: 102.5 ± 4.2 mm Hg; 24-mo-old rats treated with AdV-CMV-PnNOS, 18 days: 98.4 ± 1.7 mm Hg; and 24-mo-old rats treated with AdV-CMV-PnNOS, 30 days: 102.2 ± 13.6 mm Hg. The MIP:MAP ratios of the control 24-mo-old animals not subjected to injection and/or electroporation, were 40% lower than those of the control 5-mo-old animals (Fig. 2, saline). Both the magnitude of the erectile dysfunction in the old rats and the MIP:MAP ratios for the 2 groups were in close agreement with the values that we have previously obtained (e.g., [5, 10, 38, 39]). When pCMV-PnNOS (30 μg) was given without electroporation to the aged rats, there was a very small, nonsignificant increase in the MIP:MAP ratio after 11 days (Fig. 2), a time frame selected on the basis of our previous results with pCMV-iNOS [10]. Addition of electroporation, however, led to a significant MIP:MAP increase in the aged rats to a response comparable to the levels found in the

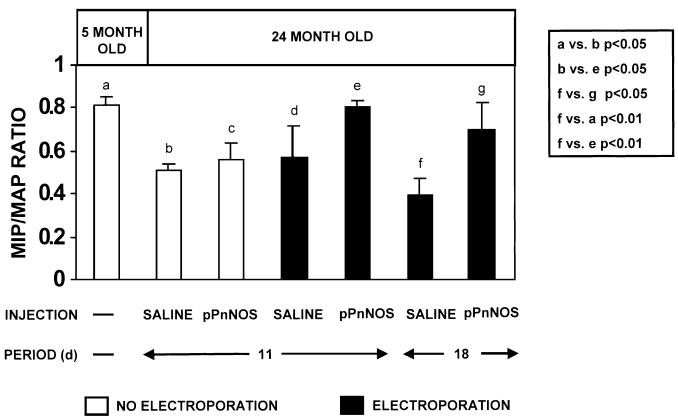

FIG. 2. Effect of electroporation on the erectile response to EFS of aging rats injected with a plasmid construct of PnNOS. Adult and aged rats were submitted to EFS of the cavernosal nerve, and MIP and MAP values were obtained either without (adult: 5 mo old) or with and without (old: 24 mo old) previous electroporation. After the indicated treatments, erectile function was assayed at either 11 or 18 days after injection.

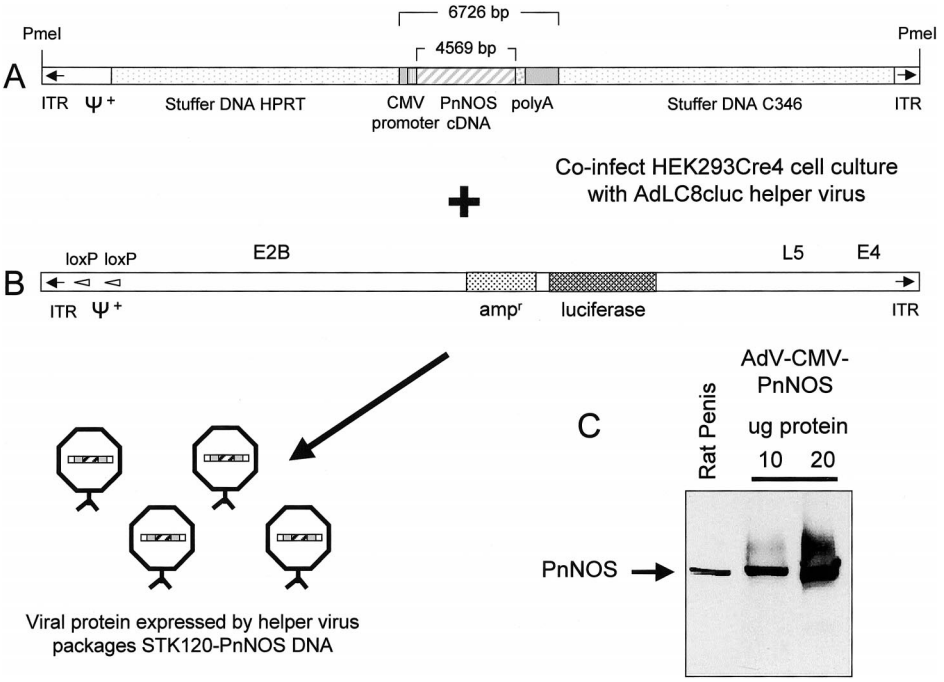

FIG. 3. Schematic representation and in vitro expression of the CMV-PnNOS construct in a helper-dependent AdV vector. The cassette containing the CMV promoter and rat PnNOS cDNA was subcloned into linearized plasmid pSTK120 at the *Swa*I site (**A**). This plasmid was linearized with *Pme*I and coinfected with the AdLC8cluc helper virus into HEK293Cre4, and as a result of the viral proteins expressed by the helper virus, a replication-defective AdV construct was packaged (AdV-CMV-PnNOS). The helper virus is prevented from packaging itself into viral capsids because of cleavage/recombination at loxP sites (**B**). The expression of PnNOS protein was determined by Western blot analysis in lysates from regular HEK293 cells infected with AdV-CMV-PnNOS at an MOI of 1. A homogenate from the rat penile shaft was used as a positive control (**C**).

control 5-mo-old adult animals. The response was nonsignificant, however, when compared with that in the control aged rats injected with saline and electroporated because of the high variability of the response in those animals at 11 days. In contrast, at 18 days posttransfection, the pCMV-PnNOS plasmid given by electroporation to aged rats induced a significant increase in the MIP:MAP ratio to a value that exceeded that of the saline-injected controls.

Effect of Electroporation on the Uptake of AdV cDNA Constructs in the Corpora Cavernosa

Since the preceding experiments suggested that electroporation improved plasmid cDNA uptake in the rat corpora cavernosa and demonstrated the effectiveness of PnNOS in ameliorating erectile dysfunction in the aged rat, we investigated whether electroporation would enhance the uptake of a helper-dependent, AdV vector expressing PnNOS. It was hypothesized that electroporation would possibly increase the number of viral particles entering cells and reduce the viral load required for an efficient PnNOS transfer to the corpora cavernosa, thus decreasing further the risk of a residual immune response against the circulating vector. A construct of the PnNOS cDNA driven by the CMV promoter was inserted into the AdV plasmid vector pSTK120. This construct was then transfected into HEK293Cre4 cells followed by infection with a helper virus. Helper virus allows the efficient packaging of the PnNOS containing DNA into viral particles (AdV-CMV-PnNOS). Figure 3, A and B, show a schematic of the main features of this construct and the AdLC8cluc helper virus. The AdV-CMV-PnNOS preparations were obtained at 10^8–10^9 vp/ml, and when infected into HEK293 cells, expressed PnNOS as visualized by the 155-kDa band detected on Western blots of the cell lysates with an antibody specific for PnNOS (Fig. 3C). Nontransfected cells do not express PnNOS [20]. A parallel construct using the same AdV-CMV vector for expressing the β-galactosidase gene (AdV-CMV-β-gal) was also prepared at titers of 10^9–10^{10} vp/ml and tested by HEK293 transfection and X-gal staining (data not shown).

Increasing viral loads of the AdV-CMV-β-gal construct, within the very low range selected (10^6–10^8 vp), were injected into the corpora cavernosa of the retired breeder rats with or without electroporation, and the animals were killed at 10 days. X-gal staining of sagittal penile sections revealed that with electroporation, 10^7 vp was virtually as effective as 10^8 vp in eliciting a substantial β-galactosidase expression all along the shaft and glans (Fig. 4A). In the absence of electroporation, the uptake of AdV-CMV-β-gal at low viral loads was very low even at the highest dose tested. When expression was examined for increasing time periods, the mean β-galactosidase expression in rats subjected to electroporation, measured by luminometry in shaft homogenates, was initially much higher than that in the nonelectroporated animals (Fig. 4B). Reporter gene expression then decreased with time and by 30 and 60 days, differences between electroporated and nonelectroporated animals were virtually indistinguishable. Frozen cross-sections from the penis of rats injected for 7 days with the AdV construct of the reporter β-galactosidase protein were used to determine its cellular distribution and compare it with the one obtained with the corresponding plasmid construct (Fig. 5). The staining was spread among different cell types, mainly tunical fibroblasts and cavernosal/spongiosum smooth muscle cells, as well as endothelial cells lining the cavernosal spaces, and some nerve terminals. This pattern was established by X-gal staining, in both corpora cavernosa (Fig. 5, bottom left) and in nerve bundles (Fig. 5, bottom right). The distribution of the plasmid construct, as judged by X-gal staining, paralleled that with the AdV vector (Fig. 5, top left), and staining it was also found in nerve terminals (Fig. 5, top right). The more restricted immunodetection with an anti-β-galactosidase antibody confirmed the X-gal results (data not shown).

Effects of AdV PnNOS on Erectile Response

To determine if age-associated erectile dysfunction can be ameliorated by AdV gene transfer and whether electroporation enhances the effect, AdV-CMV-PnNOS was injected at a low viral load (10^7 vp), and the effects on the

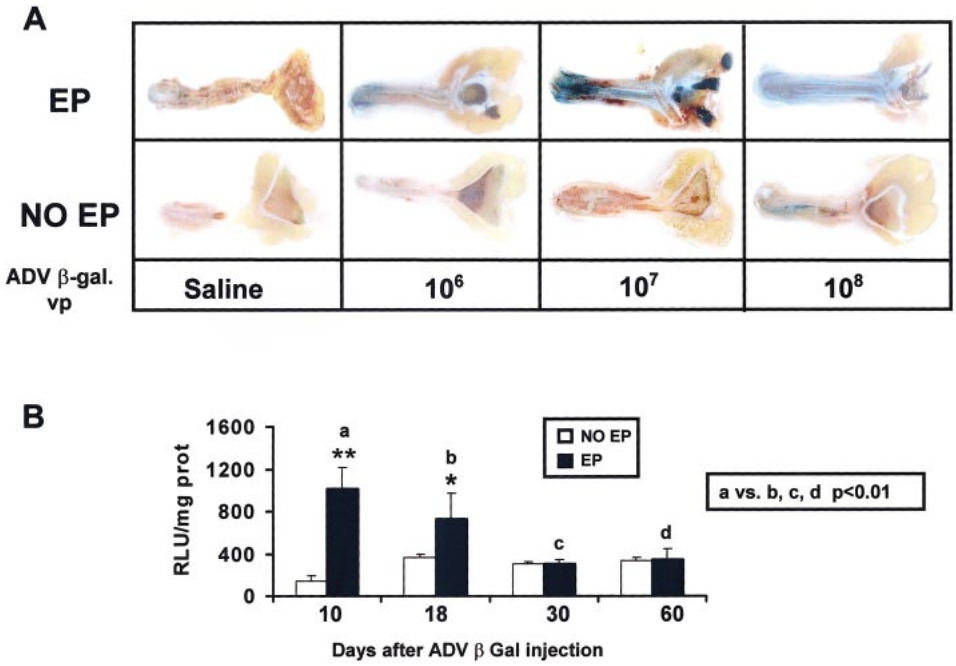

FIG. 4. Effect of electroporation on the dose curve and time course of expression of β-galactosidase from AdV-CMV-β-gal cDNA injected into the rat corpora cavernosa. The expression of β-galactosidase from AdV-CMV-β-gal given at different viral loads to retired breeder rats, with or without electroporation, was estimated by histochemistry 11 days after injection (**A**). The time course of expression was assessed at the indicated time points with 10^7 vp and by luminometry (**B**). Significant differences between no EP and EP are denoted by asterisks ($*P < 0.05$ and $**P < 0.01$).

erectile response were examined at 18, 30, and 60 days after injection and/or electroporation. Figure 6, top panel, shows that treatment of the 24-mo-old rats with the AdV-CMV-PnNOS construct for 18 days achieved a significant increase in the MIP/MAP ratio over that in the saline-injected and electroporated aged animals (0.92 vs. 0.40). The erectile response in the aged animals after electroporation was even higher than that seen for the untreated 5-mo-old rats (0.81) although the values were not statistically different. MIP values in some individual aged animals treated with AdV-CMV-PnNOS and electroporation were above 100 mm Hg. However, in the absence of electroporation, there was no stimulation of the erectile response (data not shown), in agreement with the difference in β-galactosidase staining between electroporated and nonelectroporated animals at 18 days. In parallel with the AdV-CMV-β-gal expression findings, AdV-CMV-PnNOS plus electroporation-enhanced erectile function was decreased at 30 and 60 days as compared with that at 18 days. The effects of the AdV-CMV-PnNOS in the aged rats were paralleled by an increased level of PnNOS gene expression in the penile shaft, as shown by autoradiography of Western blots (Fig. 6, bottom left) and densitometry (Fig. 6, bottom right). The increase disappeared at later time points (30 and 60 days), in agreement with the EFS and β-galactosidase expression results (data not shown).

The MAP values in the aged rats receiving the AdV-CMV-PnNOS by electroporation were similar to those in

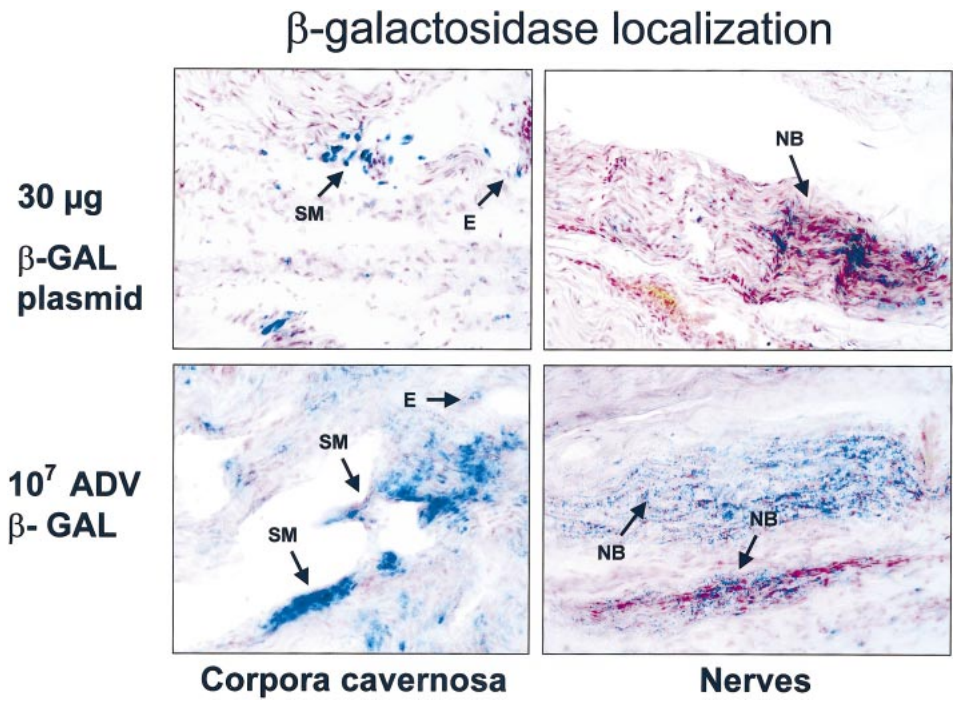

FIG. 5. Localization of β-galactosidase in different cell types after injection of pCMV-β-gal and AdV-CMV-β-gal into the rat corpora cavernosa and nerves. Both constructs were given (30 µg for the plasmid and 10^7 for the AdV) with electroporation, and the animals were killed at 7 days. Frozen transverse sections were fixed and assayed with the X-gal histochemical procedure. Tissues were counterstained with nuclear fast red. Left panels: arrows denote positive smooth muscle cells (SM) and endothelial cells (E); right panels: arrows denote nerve bundles (NB). Magnification ×200.

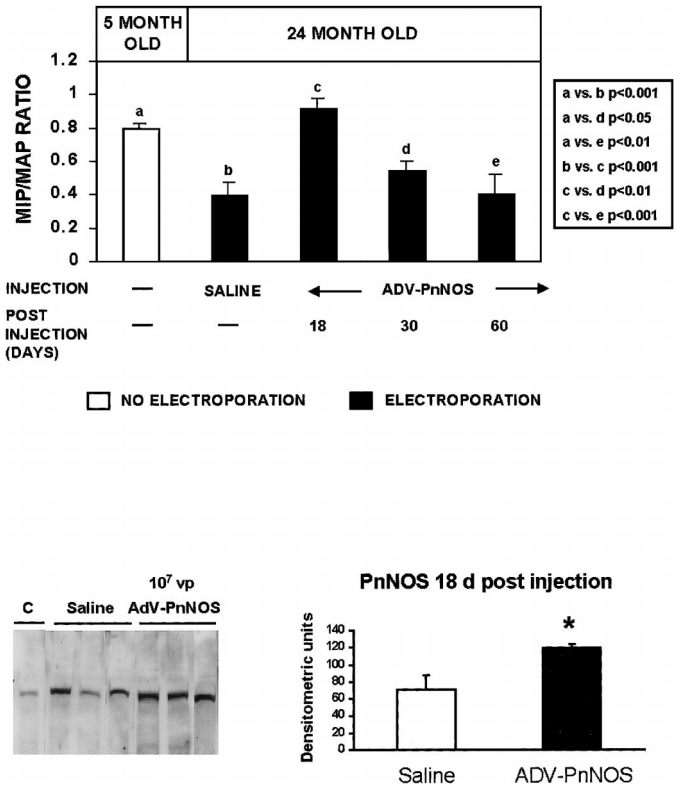

FIG. 6. Improvement of the erectile response to EFS in aging rats by injection and electroporation of a helper-dependent AdV construct of PnNOS. Top panel: adult and aged rats were treated as indicated, either without (adult) or with (old) electroporation, and were submitted to EFS of the cavernosal nerve, and the MIP and MAP values were obtained. Bottom panels: the penile shafts were excised after completing the EFS from one untreated rat, three rats injected with saline, and 3 rats treated with AdV-CMV-PnNOS. PnNOS protein was detected by Western blot (left). The relative expression was estimated by quantitative densitometry (right).

the control (saline plus electroporation) animals, and the general appearance of the virus/electroporation-treated rat cavernosal tissue appeared to be indistinguishable from that of the control rats based on light microscopy (data not shown). To corroborate this visual impression, the effect of the AdV-mediated gene transfer on the overall gene expression profile in the penile corpora cavernosa was evaluated. We used a DNA expression microarray, the Clontech Atlas Rat 1.2 array, to determine whether there were significant changes in the expression of genes involved in a variety of deleterious pathways such as cell death, fibrosis, inflammation, proteolysis, and others [37]. Virtually no changes were seen when total RNA of an AdV-CMV-PnNOS-treated penis versus a control saline-injected penis were compared (not shown). Only seven genes had higher than 2-fold changes in gene expression, namely the genes for insulinlike growth factor binding protein 6 (up-regulation), and L-type amino acid transporter, neuroglycan c, interleukin 6 receptor, cytochrome oxidase, and ribosome proteins L12 and L13 (down-regulation). None of these genes is considered to be indicative of a toxic or deleterious effect in the cavernosal tissue.

DISCUSSION

In this study, we have demonstrated that gene therapy of the corpora cavernosa with the penile NOS isoform, PnNOS, can ameliorate age-dependent erectile dysfunction in the rat animal model and that the correction is more prolonged than that in previous studies using the two isoforms expressed in the smooth muscle and endothelium, iNOS and eNOS, respectively [10, 12, 13], Additionally, electroporation can reduce substantially the amount of helper-dependent AdV-CMV-PnNOS that is required to restore normal erectile response. These observations confirm the proposal that up-regulation of NOS expression, and presumably of NO production, within the penis is feasible as a form of gene therapy for the correction of erectile dysfunction. Furthermore, this effect can be achieved with low viral loads and may reduce the risks associated with the use of a viral vector and also facilitates the laboratory preparation of the construct.

In aged rats, gene therapy for the correction of erectile response has previously used rat penile iNOS in a plasmid/liposome preparation [10] or human eNOS in an AdV vector [12, 13], and these constructs gave an MIP for the aged rats of 97 and 72 mm Hg, respectively, when measured between 5 and 10 days after injection. In the current work, using rat PnNOS, we have obtained a higher MIP (102 mm Hg) for a longer time period (at least 18 days). This suggests not only that PnNOS improves erectile response but also that the normally expressed penile form or its variants may prove to be more efficacious than other NOS isoforms for gene therapy. First, the presence of a 34-amino acid insert between exons 16 and 17 expressed in PnNOS [19, 20, 22] may have a regulatory role that could be exploited for selective manipulation of PnNOS enzyme activity without affecting neurons containing the brain-type nNOS. In addition, the exon 2 deleted splicing variant of PnNOS (PnNOSβ [20]) may increase the enzyme activity of the nNOS protein because of a lack of a region binding to the protein inhibitor of NOS (PIN), and therefore could be more active in producing NO [3, 20].

Although this study shows that gene therapy using the PnNOS cDNA is effective at ameliorating age-related erectile dysfunction, the functional distribution of the PnNOS cDNA in the penis is unclear. The homogeneous distribution of the corresponding reporter gene constructs along the penile shaft suggests that a significant portion is taken up by the corpora cavernosa and corpus spongiosum smooth muscle, and to a much lesser degree by the cisternae and vascular endothelium, with only a minor fraction going into the sparse nerve terminals of the penis. Because of the difficulty in designing vectors with target selectivity and promoter specificity, our CMV-driven PnNOS constructs are, like all of those previously used [10, 12–16], expressed predominantly in nonneural tissues in the penis, instead of being restricted to the neuronal endings responsible for the synthesis of NO as a neurotransmitter [2, 3]. However, since the channel-dependent, Ca^{2+} flow linked to synaptic transmission and responsible for nNOS activation [3] occurs mainly in the nerves, there may be some tissue specificity in the case of the NO synthesis catalyzed by the recombinant PnNOS. The fraction that goes to the nonneural tissues may be activated by other mechanisms, such as shear responses to blood flow for eNOS [40]. The AdV is an efficient vector for gene transfer not only to neuronal cells in culture, but also to brain regions and to the peripheral nervous system, administered intramuscularly, systemically, or directly into the neural tissue [41–43], and AdV receptor proteins have been identified in the brain and may be present in nerves [44]. However, since no improvement in vector expression in nerves could be seen as compared with that of plasmid vectors, the problem of targeting neu-

ral tissue expression may be addressed by using AdV driven by neuronal-specific promoters [41].

This study demonstrated that electroporation can greatly facilitate cell uptake of AdV and may reduce the effective viral dosage for gene therapies. Electroporation increased tissue uptake (with gene reporter constructs) and erectile stimulation (with PnNOS constructs) at 18 days with a very low AdV load, 10^7 vp. This is equivalent to roughly 0.1 ng of PnNOS cDNA, or about 10^5-fold lower than the PnNOS cDNA present in 30 μg of pCMV-PnNOS construct used for the DNA gene therapy experiments. In addition, 10^7 vp is about 1/2000 of the AdV dose used previously in aged rats with eNOS [13]. It should be noted that the rats used in our work are much older (24 versus 14 mo old), and theoretically more refractory to correction of the erectile dysfunction, than the ones used by other authors [13, 14]. In the case of AAV-human BDNF [16], the dose required for increasing the MIP from 20 to 44 mm Hg in an animal model of neurogenic impotence was 1000-fold higher than that used in our study with electroporation.

Although electroporation has been used extensively to facilitate plasmid gene transfer to the skeletal muscle of laboratory animals [27, 28] and also to reproductive organs such as the testis [29], to our knowledge, there are no publications on the application of this procedure to mediate the entry of viral constructs into tissues. In cell culture, there is a report on the improvement of AAV production based on membrane permeabilization by electroporation [30]. This mechanism may be similar to another finding that AdV uptake is increased by surfactants and liposomes that enhance cell porosity [45, 46]. Increased membrane permeabilization is, therefore, a likely mechanism that would enhance the binding and internalization of low viral loads, particularly if the densities of AdV or AAV receptors in the target cell are also low, as in several tissues such as vascular smooth muscle and endothelium [47, 48]. Electroporation combined with AdV therapy, in our study, was safe and free from undesirable adverse effects, other than an initial short-lived moderate inflammation. However, further studies are needed on the effect of repeated injections, since this would be the modality most likely to be applied in human gene therapy

Despite the success of the novel oral inhibitors of phosphodiesterase 5 (PDE5) [6, 7, 49], new medical treatments using gene therapy are warranted. Sildenafil and other PDE5 inhibitors depend on sexual stimulation to produce sufficient NO to activate guanylate cyclase and synthesize cGMP, and therefore trigger or potentiate the NO-mediated neurotransmission by enhancing cGMP levels. However, if the endogenous nitrergic mechanism is insufficient, the PDE5 inhibitors may fail to raise cGMP to the required levels, and it is possible that this may explain the failure of these drugs in some forms of organic impotence [50]. NOS-based gene transfer to the corpora cavernosa may not only be beneficial in itself for the treatment of erectile dysfunction, but by acting on a different site of the nitrergic signal pathway, PnNOS gene therapy may also enhance the efficacy and scope of PDE5 inhibitors.

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REFERENCES

1. McKinlay JB. The worldwide prevalence and epidemiology of erectile dysfunction. Int J Impot Res 2000; 12:S6–S11.
2. Lue TF. Erectile dysfunction. N Engl J Med 2000; 342:1802–1813.
3. González-Cadavid NF, Ignarro L, Rajfer J. Nitric oxide and cyclic GMP in the penis. Mol Urol 1999; 3:51–59.
4. Moreland RB. Pathophysiology of erectile dysfunction: the contributions of trabecular structure to function and the role of functional antagonism. Int J Impot Res 2000; 12:S39–S46.
5. Garban H, Vernet D, Freedman A, Rajfer J, Gonzalez-Cadavid NF. Effect of aging on nitric oxide-mediated penile erection in the rat. Am J Physiol 1995; 268:H467–H475.
6. Padma-Nathan H, Giuliano F. Oral drug therapy for erectile dysfunction. Urol Clin North Am 2001; 28:321–334.
7. Andersson KE. Pharmacology of penile erection. Pharmacol Rev 2001; 53:417–450.
8. Gonzalez-Cadavid NF, Ignarro L, Rajfer J. Gene therapy of erectile dysfunction. In: Mulcahy JJ (ed.), Male Sexual Function: A Guide to Clinical Management. Totowa, NJ: Humana Press; 2001: 1–16.
9. Bivalacqua TJ, Hellstrom WJ. Potential application of gene therapy for the treatment of erectile dysfunction. J Androl 2001; 22:183–190.
10. Garban H, Marquez D, Magee T, Moody J, Rajavashisth T, Roadriguez JA, Hung A, Vernet D, Rajfer J, Gonzalez-Cadavid NF. Cloning of rat and human inducible penile nitric oxide synthase: application for gene therapy of erectile dysfunction. Biol Reprod 1996; 56:954–963.
11. Ferrini M, Magee TR, Vernet D, Rajfer J, Gonzalez-Cadavid NF. Aging-related expression of inducible nitric oxide synthase and markers of tissue damage in the rat penis. Biol Reprod 2001; 64:974–982.
12. Champion HC, Bivalacqua TJ, Hyman AL, Ignarro LJ, Hellstrom WJG, Kadowitz PJ. Gene transfer of endothelial nitric oxide synthase to the penis augments erectile responses in the aged rat. Proc Natl Acad Sci U S A 1999; 96:11648–11652.
13. Bivalacqua TJ, Champion HC, Mehta YS, Abdel-Mageed AB, Sikka SC, Ignarro LJ, Kadowitz PJ, Hellstrom WJG. Adenoviral gene transfer of endothelial nitric oxide synthase (eNOS) to the penis improves age-related erectile dysfunction in the rat. Int J Impot Res 2000; 12: S8–S17.
14. Christ GJ, Rehman J, Day N, Salkoff L, Valcic M, Melman A, Geliebter J. Intracorporeal injection of *hSlo* cDNA in rats produces physiologically relevant alterations in penile function. Am J Physiol 1998; 275:H600–H608.
15. Rodgers R, Meyer T, Lin CS, Kan YW, Lue TF. Intracavernosal VEGF and AAV-VEGF gene prevents ED in a rat model of venous leakage impotence. Int J Impot Res 2000; 12:S63 (abstract 8).
16. Bakircioglu ME, Lin C-S, Fan P, Sievert K-D, Kan YW, Lue TM. The effect of adeno-associated virus-mediated brain-derived neurotrophic factor in an animal model of neurogenic impotence. J Urol 2001; 165: 2103–2109.
17. Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. Science 1996; 270:404–410.
18. Wilson JM. Adenoviruses as gene-delivery vehicles. N Engl J Med 1996; 334:1185–1187.
19. Magee TR, Fuentes AM, Garban H, Rajavashisth T, Marquez D, Rodriguez JA, Rajfer J, Gonzalez-Cadavid NF. Cloning and sequencing of a novel neuronal nitric oxide synthase expressed in the penis and lower urogenital tract. Biochem Biophys Res Commun 1996; 226: 146–151.
20. González-Cadavid NF, Burnett AL, Magee T, Zeller CB, Vernet D, Gitter J, Smith N, Rajfer J. Expression of penile neuronal nitric oxide synthase variants in the rat and mouse penile nerves. Biol Reprod 2000; 63:704–714.
21. Ferrini MG, Vernet D, Magee TR, Hayden C, Rajfer J, Gonzalez-Cadavid NF. Penile neuronal nitric oxide synthase and its regulatory proteins are present in rat hypothalamic regions associated with the control of penile erection. Urology 2000; 163:198 (abstract 879).
22. Salerno JC, Harris DE, Irizarry K, Patel B, Morales AJ, Smith SME, Martasek P, Roman LJ, Masters BSS, Jones CL, Weissman BA, Lane P, Liu Q, Gross SS. An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. J Biol Chem 1997; 272:29769–29777.
23. Silvagno F, Xia H, Bredt DS. Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. J Biol Chem 1996; 271:11204–11208.
24. Kochanek S, Clemens PR, Mitani K, Chen H-H, Chan S, Caskey CT. A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galactosidase. Proc Natl Acad Sci U S A 1996; 93:5731–5736.
25. Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C,

Graham FL, Beaudet AL, Kochanek S. Genomic DNA transfer with a high-capacity adenovirus results in improved *in vivo* gene expression and decreased toxicity. Nat Genet 1998; 18:180–183.
26. Frey A, Schneider-Rasp S, Marienfeld U, Yu JC-M, Paul M, Poller W, Schmidt HHHW. Biochemical and functional characterization of nitric oxide synthase III gene transfer using a replication-deficient adenovirus vector. Biochem Pharmacol 1999; 58:1155–1166.
27. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci U S A 1999; 96:4262–4267.
28. Maruyama H, Ataka K, Gejyo F, Higuchi N, Ito Y, Hirahara H, Imazeki I, Hirata M, Ichikawa F, Neichi T, Kikuchi H, Sugawa M, Miyazaki J. Long-term production of erythropoietin after electroporation-mediated transfer of plasmid DNA into the muscles of normal and uremic rats. Gene Ther 2001; 8:461–468.
29. Yamazaki Y, Fujimoto H, Ando H, Ohyama T, Hirota Y, Noce T. In vivo gene transfer to mouse spermatogenic cells by deoxyribonucleic acid injection into seminiferous tubules and subsequent electroporation. Biol Reprod 1998; 59:1439–1444.
30. Maxwell F, Harrison GS, Maxwell IH. Improved production of recombinant AAV by transient transfection of NB324K cells using electroporation. J Virol Methods 1997; 63:129–136.
31. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc Natl Acad Sci U S A 1996; 93:13565–13570.
32. Chen C, Okayama H. High efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987; 7:2745–2752.
33. Hitt M, Bett AJ, Addison CL, Prevec L, Graham FL. Techniques for human adenovirus vector construction and characterization. Methods Mol Genet 1995; 7:13–30.
34. Artaza JN, Bhasin S, Mallidis C, Taylor W, Ma K, Gonzalez-Cadavid NF. Endogenous expression and localization of myostatin and its relation to myosin heavy chain distribution in C2C12 skeletal muscle cells. J Cell Physiol 2002; 190:170–179.
35. Hogan B, Beddigton R, Costintini F, Lacy E. Manipulating the Mouse Embryo, 2nd ed. New York: Cold Spring Harbor Laboratory; 1994: 373.
36. Cheng G, Thompson RP, Gourdie RG. Improved detection reliability of beta-galactosidase in histological preparations. Biotechniques 1999; 27:438–440.
37. Magee TR, Qian A, Rajfer J, Sander F, Levine L, Gonzalez-Cadavid NF. Gene expression profiles in the Peyronie's disease plaque. Urology 2002; 59:451–457.
38. Garban H, Marquez D, Cai L, Rajfer J, Gonzalez-Cadavid NF. Restoration of normal penile erectile response in aged rats by long-term treatment with androgens. Biol Reprod 1995; 53:1365–1372.
39. Moody J, Vernet D, Laidlaw S, Rajfer J, González-Cadavid NF. Effect of long-term administration of L-arginine on penile erection and nitric oxide synthase in the rat. J Urol 1997; 158:942–947.
40. Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. Am J Physiol 2001; 280:F193–F206.
41. Navarro V, Millecamps S, Geoffroy MC, Robert JJ, Valin A, Mallet J, Gal La Salle GL. Efficient gene transfer and long-term expression in neurons using a recombinant adenovirus with a neuron-specific promoter. Gene Ther 1999; 6:1884–1892.
42. Millecamps S, Nicolle D, Ceballos-Picot I, Mallet J, Barkats M. Synaptic sprouting increases the uptake capacities of motoneurons in amyotrophic lateral sclerosis mice. Proc Natl Acad Sci U S A 2001; 98:7582–7587.
43. Morelli AE, Larregina AT, Smith-Arica J, Dewey RA, Southgate TD, Ambar B, Fontana A, Castro MG, Lowenstein PR. Neuronal and glial cell type-specific promoters within adenovirus recombinants restrict the expression of the apoptosis-inducing molecule Fas ligand to predetermined brain cell types, and abolish peripheral liver toxicity. J Gen Virol 1999; 80:571–583.
44. Honda T, Saitoh H, Masuko M, Katagiri-Abe T, Tominaga K, Kozakai I, Kobayashi K, Kumanishi T, Watanabe YG, Odani S, Kuwano R. The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. Brain Res Mol Brain Res 2000; 77:19–28.
45. Kukowska-Latallo JF, Chen C, Eichman J, Bielinska AU, Baker JR Jr. Enhancement of dendrimer-mediated transfection using synthetic lung surfactant exosurf neonatal *in vitro.* Biochem Biophys Res Commun 1999; 264:253–261.
46. Qiu C, De Young MB, Finn A, Dichek DA. Cationic liposomes enhance adenovirus entry via a pathway independent of the fiber receptor and alpha(v)-integrins. Hum Gene Ther 1998; 9:507–520.
47. Hay CM, De Leon H, Jafari JD, Jakubczak JL, Mech CA, Hallenbeck PL, Powell SK, Liau G, Stevenson SC. Enhanced gene transfer to rabbit jugular veins by an adenovirus containing a cyclic RGD motif in the HI loop of the fiber knob. J Vasc Res 2001; 38:315–323.
48. Kibbe MR, Murdock A, Wickham T, Lizonova A, Kovesdi I, Nie S, Shears L, Billiar TR, Tzeng E. Optimizing cardiovascular gene therapy: increased vascular gene transfer with modified adenoviral vectors. Arch Surg 2000; 135:191–197.
49. Uckert S, Kuthe A, Stief CG, Jonas U. Phosphodiesterase isoenzymes as pharmacological targets in the treatment of male erectile dysfunction. World J Urol 2001; 19:14–22.
50. Rendell MS, Rajfer J, Wicker PA, Smith MD. Sildenafil for treatment of erectile dysfunction in men with diabetes: a randomized controlled trial. Sildenafil Diabetes Study Group. JAMA 1999; 281:421–426.