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Gene transfer of endothelial nitric oxide synthase to pulmonary allografts: impact on acute rejection

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Abstract Experiments were designed to study whether overexpression of nitric oxide (NO) from endothelial nitric oxide synthase (eNOS) affects acute rejection. Allogenic, orthotopic single-lung transplantation was performed after transbronchial adenoviral-mediated gene transfer (3×10^8 pfu) of either of eNOS or β-galactosidase to donor lungs of rats ($n = 6$ each). No immunosuppression was used. After 4 days, transplanted lungs were prepared for enzyme activity, cGMP and histology. Calcium-dependent NOS activity, reflecting eNOS, was greater in eNOS-transduced lungs (587 ± 97 vs 2.1 ± 1.4 pmol/mg protein per h, $P < 0.001$). In contrast, calcium-independent NOS activity, reflecting iNOS, was comparable. Concentrations of cGMP were higher in eNOS-transduced lungs (13.2 ± 2.3 vs 4.9 ± 0.5 pmol/mg protein). Positive immunostaining for eNOS was present in pneumocytes only in eNOS-transduced lungs. No difference in histological grade of rejection was observed. eNOS gene transfer to pulmonary allografts results in a functionally active transgene product and increased NO production. Increasing NO from eNOS does not affect histogically identified acute rejection.

Key words Lung transplantation · Acute rejection · Nitric oxide synthase · Gene transfer

Introduction

Lung transplantation is an accepted treatment for selected patients with endstage pulmonary disease. In spite of modern immunosuppressive drugs, acute rejection is still a clinical problem, affecting more than 50% of recipients after lung transplantation [4, 9]. Furthermore, episodes of acute rejection are a significant risk factor for the development of obliterative bronchiolitis [9, 20].

Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes, NO synthases (NOS) [14]. There are two isoforms of NOS in lung tissue, endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is expressed in endothelial and bronchial epithelial cells of the lung and generates small amounts of NO in response to physiological stimuli [15]. iNOS produces larger amounts of NO from macrophages in response to pathophysiological stimuli such as acute rejection and infection [15].

NO increases in plasma and in exhaled air during acute rejection after experimental lung transplantation [13, 24]. NO is probably produced from iNOS, as both iNOS gene expression and iNOS enzyme activity are increased during acute lung rejection [25]. The critical role of iNOS and NO in acute rejection is further supported by the finding that treatment with aminoguanidine, an iNOS-blocking agent, ameliorates experimental acute rejection [26].

Exogenous NO reduces iNOS expression and enzyme activity in vitro by a negative feedback mechanism [3, 21]. This indicates that exogenous NO could possibly be used to reduce iNOS activity in acute rejection. One method of increasing NO in lung tissue is overexpression of genes encoding for NOS. Gene transfer of

eNOS to the lung increases NO production and reduces acute-hypoxia-induced pulmonary hypertension in the normal, nontransplanted lung [6]. Our group has recently demonstrated the feasibility of eNOS gene transfer to the transplanted lung [8]. In the present study, we tested the hypothesis that eNOS gene transfer to the acute rejecting lung would increase NO production and reduce iNOS activity, resulting in reduced histologically identified acute rejection.

Materials and methods

Generation of adenoviral vectors

A replication-incompetent adenoviral vector encoding eNOS gene (AdeNOS), driven by the cytomegalovirus promoter, was generated through homologous recombination in 293 cells [19]. The generation, propagation, purification, and evaluation of adenoviral vector containing eNOS gene have been described in detail previously elsewhere [2]. Briefly, bovine eNOS cDNA (provided by Dr. David G. Harrison, Emory University, Atlanta, Ga.) was cloned into the pACCMVpLpA vector (provided by Dr Robert Gerard, University of Texas Southwestern Medical Center, Dallas, Tex.). The resulting plasmid was linearized with NruI and cotransfected with dl309 into 293 cells by calcium phosphate/DNA coprecipitation. The recombinant adenovirus encoding an *Escherichia coli* β-galactosidase (β-gal) reporter gene (Adβ Gal), used in the experiments as a control vector, was a generous gift from Dr. James M. Wilson, Institute for Human Gene Therapy, University of Pennsylvania. The viral vector was stored at –70 °C in dialysis solution containing 10% glycerol.

Animals

Brown-Norway (donors) and Lewis (recipients) rats (Harlan Sprague-Dawley), weighing 250–320 g were used in the experiments. Animal care was conducted in accordance with the "*Principles of Laboratory Animal Care*" formulated by the National Society for Medical Research and the "*Guide For the Care and Use of Laboratory Animals*" prepared by the National Institutes of Health (NIH Publication no. 86–23, revised 1985).

Experimental groups

The animals were divided into two groups. In the first group ($n = 6$), donor lungs were transduced before transplantation with 3×10^8 pfu of AdeNOS. In the second group ($n = 6$), donor lungs were transduced before transplantation with 3×10^8 pfu of Adβ Gal. The virus was diluted in 100 μl of standard viral medium (2% fetal calf serum in 199 medium). No immunosuppressive drugs were used.

Gene transfer and transplantation

Transbronchial gene transfer and orthotopic single lung transplantation were performed as previously described [7, 10]. The donor rat was intubated and ventilated (Harvard Rodent Ventilator). A median sternotomy was performed to expose the lungs. Following dissection of the hilum, the rat was heparinized with 150 U of aqueous heparin injected into the inferior vena cava. The tidal volume was reduced to 50% and the right bronchus occluded. The viral solution was instilled in the left bronchus. The clamp on the right bronchus was released and the lung was ventilated for 5 min to allow distribution of virus. The left atrium was opened and 20 ml of pneumoplegia (University of Wisconsin solution) was infused into the main pulmonary artery and the heart–lung block explanted. The lung was stored for 1 h in University of Wisconsin solution at 4 °C before implantation. In the recipient, a left thoracotomy was performed and the left lung dissected and removed. The preserved, transduced donor left lung was then implanted orthotopically into the recipient by anastomosing the pulmonary vein and artery. The lung was reperfused, after which the bronchus was anastomosed. All anastomoses were performed with 10–0 monofilament sutures. The chest wall was closed with a small chest tube in situ, which was removed during recovery from anesthesia.

Tissue sampling

Four days after transplantation, native and transplanted lungs were removed and cut into equally sized pieces from the upper, middle and lower sections of the lung. Tissue sections were snap-frozen in liquid nitrogen.

NOS activity

NOS activity was determined by measuring the conversion of [^{3}H]-*l*-arginine to [^{3}H]-*l*-citrulline by methods originally described by Myatt [16] and modified by Miller and Barber [12]. In brief, tissue homogenates from all sections of the transplanted lungs were prepared and eluted through 10-DG desalting columns. To quantitate NOS activity, duplicate reactions were carried out in the presence of calcium (total activity), in the absence of calcium plus EGTA (calcium-independent activity) and in the absence of calcium plus EGTA in the presence of NG-monomethyl-L-arginine (L-NMMA; nonspecific activity). Reactions were started by adding 150 μl of protein homogenate to 150 μl of cofactor mix. The reaction was incubated on a shaker at 27 °C for 1 h and terminated by the addition of ice-cold stop buffer. Separation of [3H]-*l*-arginine from [^{3}H]-*l*-citrulline was accomplished with the aid of affinity columns containing AG 50W-X8 Na + form 200–400 mesh resin (Bio-Rad Laboratories, Hercules, Calif.). Calcium-dependent activity equals total activity minus calcium-independent activity after correction for nonspecific activity.

Cyclic GMP

Frozen pieces from all sections of the transplanted lungs were pulverized, homogenized in 1 ml of 6% trichloroacetic acid with a glass-glass potter and centrifuged at 10,000 *g* for 20 min. The supernatant was extracted four times with four volumes of water-saturated ether, lyophilized, and stored at –70 °C for subsequent determination of cGMP levels. The content of the cyclic nucleotide was determined using RIA kits purchased from Biomedical Technology (Stoughton, Mass.). The amount of protein was determined using a protein reagent assay (Pierce, Rockford, Ill.)

Immunostaining and histology

A mid-section of the transplanted lung was embedded in OCT compound (Miles, Elkhart, Ind.) and quick-frozen in a liquid nitrogen-cooled 2-methylbutane bath. Five cryostat sections (5 μm)

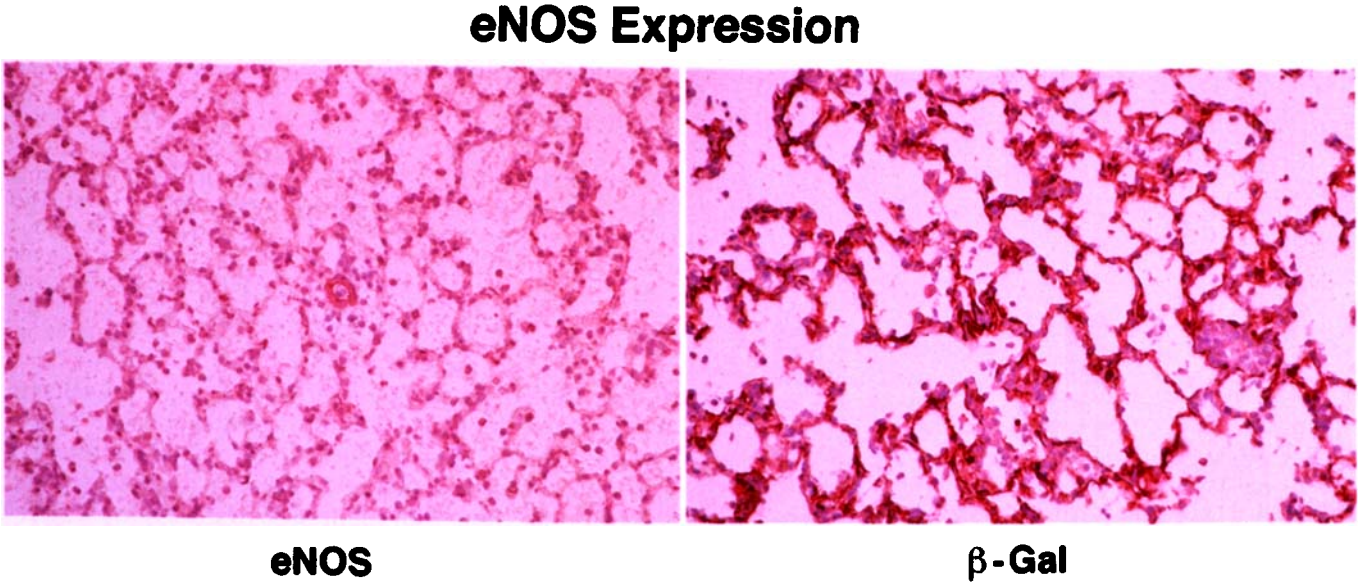

Fig. 1 Immunostaining for endothelial nitric oxide synthase (*eNOS*) **A** in eNOS-transduced, transplanted lungs and **B** in β-galactosidase (βGal)-transduced, transplanted lungs. In pneumocytes, positive staining for eNOS was observed in eNOS-transduced animals only. (× 100)

were cut, fixed for 10 min in cold acetone (4 °C), fan-dried for 10 min and further fixed in 1% paraformaldehyde/EDTA for 3 min. After rinsing, endogenous peroxidase activity was blocked by incubating sections in 0.1% sodium azide/0.3% H_2O_2 for 10 min. Nonspecific protein-binding sites were blocked by incubating sections with 5% goat serum/PBS-Tween 20 and then drained off. Monoclonal mouse anti-eNOS 5 μg/ml (Transduction Laboratories, Lexington, Ky.) was added and the sections incubated for 60 min at room temperature. They were then rinsed in tapwater, incubated with biotinylated rabbit anti-mouse F(ab')2 (1:300) for 20 min and re-rinsed. They were further incubated for 20 min with peroxidase-conjugated streptavidin (1:300) and rinsed. Sections were then incubated for 30 s in 0.1 M sodium acetate buffer, pH 5.2, after which slides were placed in AEC (3-amino-9-ethylcarbazole) substrate solution and incubated for 15 min at room temperature. After rinsing in tapwater, the slides were counterstained in mercury-free hematoxylin for 1 min and further rinsed for 3 min in cold running tapwater before being mounted. Midsections of the βGal-transduced lungs were stained in a solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase (X-Gal; Boehringer Mannheim, Indianapolis, Ind.) and counterstained with eosin. Ten fields in each section were counted and the percentage of positively stained cells was calculated.

Adjacent sections were stained with hematoxylin and eosin for routine histopathological examination. Rejection was graded according to ISHLT's current recommendations [27] by an experienced pathologist blinded to the origin of the slides.

Statistical analysis

The nonparametric Mann-Whitney test was used to compare the groups. Correlation between NOS enzyme activity and cGMP levels was determined with Spearman's rank sum test, the using pooled data from both groups being used. A *P*-value of less than 0.05 was considered significant.

Results

Expression of eNOS in transplanted lungs

Endogenous eNOS expression was detected by positive immunohistochemical staining in endothelial cells and airway epithelial cells in lungs from both groups of animals and was not different in eNOS- and βGal-transduced lungs (Fig. 1 a,b). In contrast, pneumocytes stained positively for eNOS only in eNOS-transduced lungs (Fig. 1 b). More than 60% of the pneumocytes in every field stained positively in the eNOS-transduced group. In the βGal-transduced lungs, the distribution of βGal paralleled the transgene eNOS distribution in eNOS transduced lungs (data not shown).

NOS activity

Calcium-dependent NOS activity, reflecting eNOS, in eNOS-transduced lungs was 300 times than in βGal-transduced lungs (Fig. 2). In contrast, no difference in calcium-independent NOS activity, reflecting iNOS, was observed between eNOS-transduced and βGal-transduced lungs (Fig. 2). Total NOS activity (calcium-dependent + calcium-independent) in eNOS-transduced lungs (729 ± 91 pmol/mg protein per h) was 7-fold than in βGal-transduced lungs (105 ± 34 pmol/mg protein per h, $P = 0.01$).

In the βGal group, calcium-dependent NOS activity was lower in the transplanted lungs than in the native lungs (2.2 ± 1.4 vs 12.0 ± 0.8 pmol/mg protein per h,

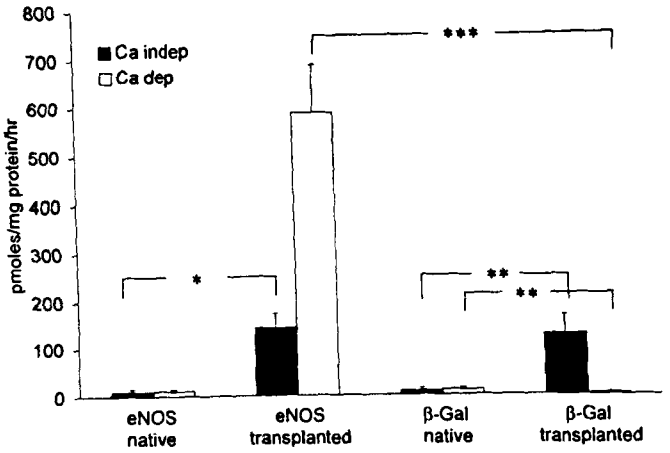

Fig.2 Calcium-dependent (*white bars*) and calcium-independent (*black bars*) nitric oxide synthase activity in native and transduced, transplanted lungs. Statistically significant differences are indicated: $*P<0.05$, $**P<0.01$, $***P<0.001$. Data are shown as mean ± SEM, $n = 6$ in each group

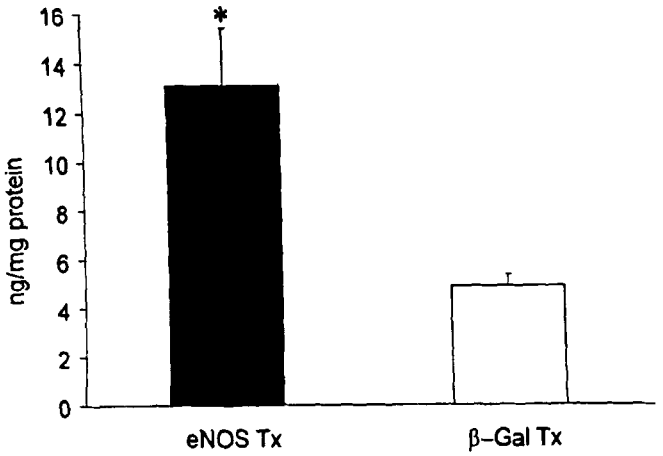

Fig.3 Cyclic cGMP levels in eNOS- and βGal-transduced, transplanted lungs. A statistically significant difference was observed. Data are shown as mean ± SEM, with $n = 6$ in each group. *Asterisk* denotes statistically significant differences ($P<0.01$)

$P = 0.01$; Fig. 2). Calcium-independent activity was higher in the transplanted lungs than in the native lungs in both groups (eNOS group: 143 ± 34 vs 10 ± 5 pmol/mg protein per h, $P = 0.01$; βGal group: 127 ± 44 vs 9 ± 5 pmol/mg protein per h, $P = 0.05$; Fig. 2).

Cyclic GMP

Concentrations of cGMP were higher in transplanted lungs of animals in the eNOS-transduced than in lungs of the βGal-transduced group ($P<0.05$; Fig. 3). cGMP levels (independent of group) correlated positively with calcium-dependent NOS activity ($r = 0.88$, $P<0.001$).

Histology

No difference in histological grade of acute rejection was observed between groups (2.5 ± 0.2 vs 2.8 ± 0.2, $P = 0.20$; Fig. 4).

Discussion

The results of this study confirm that gene transfer to transplanted lungs via the transbronchial route results predominantly in transduction of pneumocytes. We have previously demonstrated this in a syngeneic model of orthotopic lung transplantation in the rat [8]. The results of the current study extend these observation to the allogeneic setting. Functional activity of recombinant eNOS was suggested by positive immunostaining and the marked increase in calcium-dependent NOS activity in the transduced lungs. Overexpression of eNOS in this model did not influence histologically identified acute rejection.

The present study confirms that iNOS enzyme activity is up-regulated during acute allograft rejection. The calcium-independent NOS activity levels were approximately 3–4 times as high as we had previously found in similarly transplanted syngeneic lungs [8]. This indicates that the increase in iNOS activity in the allogeneic animals is not an effect of the surgical trauma per se. In contrast, the reduced calcium-dependent NOS activity in the rejecting β-Gal transduced control lungs suggests that acute rejection reduces eNOS enzyme activity. This confirms the previously demonstrated reciprocal relationship of eNOS and iNOS during acute rejection [23]. As NO may down-regulate iNOS [3, 21] and we have previously demonstrated increased NOS activity in transplanted lungs after eNOS gene transfer [8], we set up the hypothesis that overexpression of eNOS by gene transfer might down-regulate iNOS activity in the allografted lung and impact on acute rejection.

While eNOS overexpression resulted in increased NOS activity and increased NO production was demonstrated by increased cGMP levels in transplanted lung allografts, no effects on acute rejection were observed. The reason for this is unclear. The timing of the transfection and expression of transgene product may be important. Transfection occurred simultaneously with transplantation in the absence of immunosuppressants. Therefore, inducible allogenic processes may have begun prior to the expression of transgene product. To avoid this, the donor lungs may be needed to be transduced 12–24 h before transplantation. This is of particular importance if gene transfer is to be used to counter-

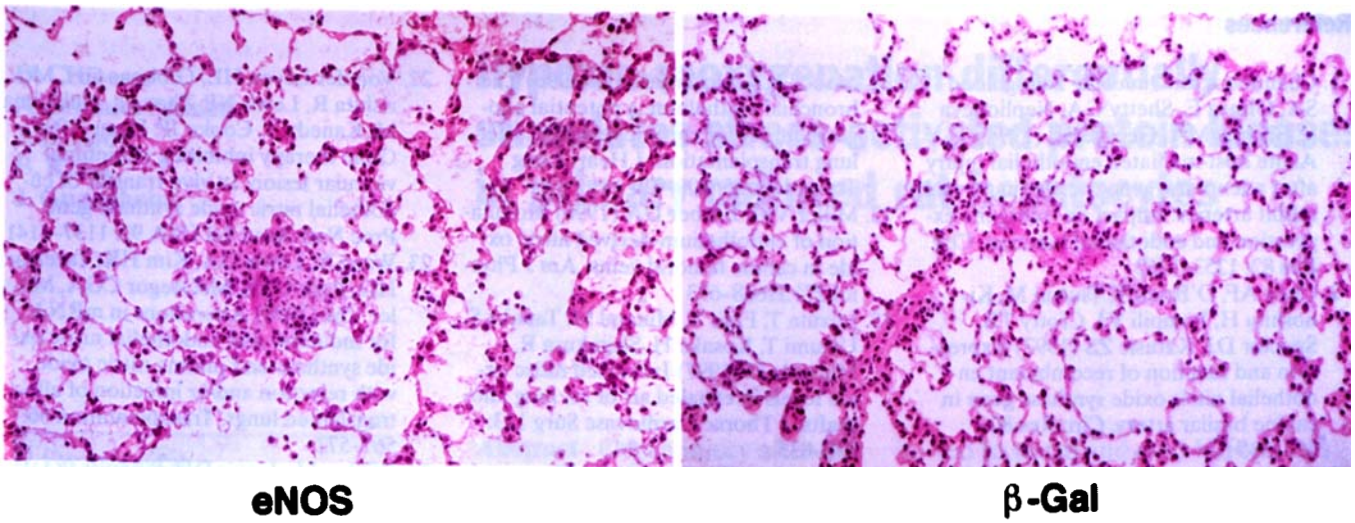

Fig. 4 Hematoxylin and eosin staining for eNOS in **A** eNOS-transduced, transplanted lungs and **B** βGal-transduced, transplanted lungs. No difference in histological grade of rejection was observed (× 100)

act ischemia-reperfusion injury immediately after transplantation.

Furthermore, the distribution of eNOS in pneumocytes may not represent an appropriate target tissue. Endothelial cells and infiltrating macrophages are the cell types directly involved in acute lung rejection. It may be that these cells were not influenced by the increased NO production. On the other hand, NO is a diffusable molecule and can probably impact on cells adjacent to the NO-producing pneumocytes.

Gene transfer of iNOS suppressed the development of atherosclerosis in rat aortic allografts [17]. This indicates that NO may reduce sclerosis-type processes associated with chronic allograft rejection and suggests that NOS gene transfer may have more of a role in prevention of chronic rejection than in acute rejection. Obliterative bronchiolitis (OB) after lung transplantation is generally believed to be the endstage of chronic rejection [11, 28]. OB is characterized by cell proliferation, fibrous scarring, and luminal obliteration of the terminal airways [18, 28]. NO may potentially influence some of the factors involved in OB, as NO promotes bronchodilation, reduces leukocyte adhesion and reduces proliferation of smooth muscle cells [14], a cell type that may be involved in OB [18]. Gene transfer with eNOS to the lung increases NO production and reduces acute hypoxia-induced hypertension in the normal nontransplanted lung [6] and can also reduce smooth muscle cell proliferation after endothelial injury in the carotid arteries [22]. Therefore, gene transfer of eNOS has been demonstrated to result in increased NO generation and beneficial biological effects, although it remains to be demonstrated that eNOS gene transfer also impacts on OB.

A major disadvantage of adenovirus-mediated gene transfer is the potential for induction of an immunological response to the vector in the transduced organ. The response may cause inflammation and limit the duration of transgene expression [1, 5]. In syngeneic animals, no differences were observed in inflammation between lungs transduced with eNOS and with βGal [8]. In the present study, inflammation due to the adenovirus could not be differentiated from processes of acute rejection.

In summary, the present study demonstrates the feasibility of gene transfer with biologically active genes to the transplanted lung in the allogenic setting and suggests that concurrent eNOS gene transfer at the time of transplantation does not affect acute rejection.

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