Local Adenovirus-Mediated Transfer of Human Endothelial Nitric Oxide Synthase Reduces Luminal Narrowing After Coronary Angioplasty in Pigs

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Background—Nitric oxide, synthesized from L-arginine by nitric oxide synthase (NOS), is a vasodilator and inhibits vascular smooth muscle cell (SMC) proliferation and migration. The effects of local NOS gene transfer on restenosis after experimental balloon angioplasty were investigated.

Methods and Results—Left anterior descending coronary artery angioplasty was performed in 25 pigs. Animals received an intramuscular injection of adenovirus (1.5×10⁹ pfu) carrying either the NOS cDNA (AdCMVceNOS) or no cDNA (AdRR5) via the Infiltrator. Local gene transfer efficiency and bioactivity of recombinant protein were assessed after 4 days. Indices of restenosis were evaluated by computerized planimetry on coronary artery sections prepared 28 days after angioplasty. Adenoviral vectors permitted efficient gene delivery to medial SMCs and adventitial cells of coronary arteries. Vascular cGMP levels were depressed after angioplasty from 1.30±0.42 to 0.33±0.20 pmol/mg protein (P<0.05) but were restored after constitutive endothelial (ce) NOS gene transfer to 1.82±0.98 pmol/mg (P<0.05 versus injured group and P=NS versus control). The ratio of the neointimal area to the internal elastic lamina fracture length, maximal neointimal thickness, and percent stenosis were all reduced in AdCMVceNOS- versus AdRR5-transduced pigs (0.59±0.14 versus 0.80±0.19 mm, P=0.02; 0.75±0.21 versus 1.04±0.25 mm, P=0.019; and 53±15% versus 75±11%, P=0.006, respectively). Lumen area was significantly larger (0.70±0.35 mm² in AdCMVceNOS versus 0.32±0.18 mm² in AdRR5, P=0.007).

Conclusions—Percutaneous adenovirus-mediated NOS gene transfer resulted in efficient local overexpression of functional NOS after angioplasty in coronary arteries. Restored NO production in injured coronary arteries significantly reduced luminal narrowing, most likely through a combined effect on neointima formation and on vessel remodeling after angioplasty. (Circulation. 1998;98:919-926.)

Key Words: genes ■ vessels ■ remodeling ■ restenosis ■ nitric oxide

Percutaneous transluminal coronary angioplasty (PTCA) has significantly altered the management of symptomatic coronary artery disease. Despite its overall value in achieving immediate symptomatic relief, arterial restenosis still occurs in 20% to 50% of patients within 6 months.¹ Restenosis after PTCA is characterized by progressive arterial remodeling, extracellular matrix formation, and intimal hyperplasia at the site of angioplasty. Most pharmacological agents have failed to demonstrate a beneficial effect on restenosis in randomized clinical trials.¹⁵ Arterial restenosis is a complex biological process initiated by platelet adhesion and aggregation at the site of arterial injury.²⁻¹¹ Platelet activation results in the release of a variety of vasoactive and mitogenic factors that stimulate vascular smooth muscle cell (SMC) proliferation and migration, matrix formation, and the late fibroproliferative response.⁶⁻⁷ Local transfer of genes encoding antiproliferative proteins has been effective in animal models of neointima formation.¹⁴⁻¹⁶ In rat carotid and in rabbit and porcine iliac arteries, local adenovirus-mediated transfer of genes encoding herpes simplex virus thymidine kinase,¹⁷⁻¹⁹ p21,²⁰ a constitutively active form of the retinoblastoma gene product,²¹ and hirudin²² significantly reduced neointima formation after arterial injury.²³ PTCA-induced injury to the endothelial protective barrier also results in the loss of constitutively expressed endothelium-derived vasoactive factors, including NO, prostacyclin, and bradykinin, which play an important role in vascular homeostasis.²⁴ Loss of endothelial NO production after PTCA and subsequent loss of guanylate cyclase stimulation in medial SMCs is most likely the predominant factor responsible for the loss of vascular cGMP production. In animal...
models, l-arginine supplementation increases vascular NO production and has been found to reduce neointima formation at the site of injury.\textsuperscript{25,26}

Gene-based strategies may provide an attractive alternative to selectively increase NO production at the site of injury and restore vascular cGMP production, which in turn is believed to modulate important vascular functions, including relaxation, migration, and proliferation. However, the majority of studies demonstrating successful gene therapy strategies to date have been performed either in rodents or in surgically exposed peripheral arteries in pigs and rabbits, and the results cannot readily be extrapolated to patients undergoing coronary angioplasty. Therefore, the present study was carried out in a porcine coronary artery injury model, the morphology of which is closer to postangioplasty restenosis in humans.\textsuperscript{27,28}

The effects of catheater-based human constitutive endothelial NO synthase (ceNOS) gene transfer on local NO generation and neointima formation were evaluated.

Methods

Construction and Purification of Recombinant Adenovirus

Recombinant adenoviruses containing human ceNOS cDNA under the control of the cytomegalovirus (CMV) promoter/enhancer (AdCMVceNOS) was constructed, amplified, and purified as previously described.\textsuperscript{29} For all in vivo studies, viral titers were adjusted to $5 \times 10^5$ pfu/mL. Recombinant adenoviruses carrying the Escherichia coli lacZ gene encoding a nuclear-localized variant of b-galactosidase (AdCMVBgal) or no cDNA (AdRR5) were used to evaluate gene transfer efficiency or to serve as control virus, respectively.

Animal Preparation

All animal care and handling were performed in accordance with the guidelines specified by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Leuven. Juvenile domestic pigs weighing 20 to 25 kg were treated with aspirin 300 mg PO 5 days per week starting the day of the procedure. The pigs were anesthetized with pentobarbital 10 mg/kg IV bolus and ketamine 5 mg/kg IV bolus followed by a 10-mg·kg$^{-1}·$h$^{-1}$ IV infusion, intubated, and ventilated with O$_2$-enriched room air. ECG and arterial pressure were continuously monitored throughout the experiment. Heparin was given as a bolus of 15 000 IU intra-arterially. A 8F left Judkins guiding catheter was introduced via the right carotid artery to engage the left main coronary ostium. A 3.0-mm balloon dilatation catheter was advanced over a standard 0.014-in flexible wire into the left anterior descending coronary artery (LAD) and positioned distal to the first diagonal branch. The artery was injured by 3 successive 30-second inflations at 1 atm with a 1-minute reflow after each inflation. Coronary arteriography was realized before and after the angioplasty, and balloon-to–uninjured-artery ratio was obtained by quantitative coronary angiography by use of the AWOS angiographic workstation for digital quantitative coronary angiography (AWOS version 3.0, Siemens AG). Angiography was not systematically performed at 28 days because previous studies failed to show significant differences in vessel dimensions.\textsuperscript{30,31} The balloon was deflated and the Infiltrator catheter (InterVentional Technology Inc) advanced to the site of injury for adenoviral gene transfer. The Infiltrator is a triple-lumen balloon catheter allowing intramural drug delivery via 3 longitudinal strips of 6 or 7 low-profile 0.254-mm injector ports capable of penetrating the internal elastic lamina (IEL) (Figure 1). The catheter allows local drug delivery without perforation, dissection, or hemorrhage of the arterial segment.\textsuperscript{32} The inflation of the 3.0-mm balloon at 2 atm was followed by manual injection of 0.3 mL of the viral solution over a period of 15 seconds. A total of 25 pigs were studied (AdCMVceNOS, n = 12 and AdRR5, n = 13). After gene transfer, the Infiltrator was deflated and withdrawn, and the right carotid artery was ligated. After surgical repair of the neck cutdown, the animals were allowed to recover, and enrofloxacin (5 mg/kg IM) was administered for the first 3 days. After 4 weeks, the animals were killed by injection of saturated KCl, and the injured coronary arteries were harvested and prepared for analysis.

Adenovirus-Mediated ceNOS Gene Transduction In Vitro and In Vivo

Porcine SMCs were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies), 50 U/mL penicillin, and 50 \mu g/mL streptomycin. The cells were grown in chamber slides to 60% confluence and infected with AdCMVceNOS or AdRR5 at a multiplicity of infection (MOI) of 2, 20, and 200. After 24 hours, the viral suspension was removed, and the cells were maintained in culture for another 24 hours and fixed for 20 minutes in 4% formaldehyde. The presence of the ceNOS gene product was detected by immunostaining. Cells were preincubated with rabbit preimmune serum (dilation 1:5) for 45 minutes and incubated overnight with a monoclonal mouse anti-human ceNOS antibody (1:125) (Transduction Laboratories), followed by incubation for 1 hour with a rabbit anti-mouse IgG peroxidase complex (dilution 1:50). Antibody binding was visualized with 3,3-diaminobenzidine tetrahydrochloride substrate in 0.1 mol/L Tris buffer, pH 7.2, containing 0.03% H$_2$O$_2$. Slides were counterstained with Harris’ hematoxylin, dehydrated, and mounted with D.P.X. compound (Prosan) for light microscopy.

In vivo, ceNOS expression was evaluated by immunohistochemistry on frozen arterial sections. Coronary arterial rings of 4 pigs infected with AdCMVceNOS were harvested at day 4, embedded in O.C.T. compound (Sakura Finetek Europe BN), and frozen in liquid nitrogen. Sections (5 \mu m) were fixed for 20 minutes in ice-cold methanol, washed, preincubated with a rabbit preimmune serum, and incubated overnight with a monoclonal mouse anti-human ceNOS antibody (dilation 1:125), followed by incubation for 30 minutes in phosphate buffer containing 1% H$_2$O$_2$. Biotinylated rabbit anti-mouse antibody (1:200) was used as secondary antibody (Vector Laboratories Inc), and antibody binding was visualized by the avidin-biotin complex method (ABC kit, Vector Laboratories, Inc).

Vascular cGMP Measurements

To investigate the production of NO by recombinant ceNOS, cGMP levels were measured in frozen segments from uninfected (Inj, n = 8) and AdCMVceNOS-infected (Inj + AdCMVceNOS, n = 4) balloon-injured coronary arteries, as well as from uninjured coronary arteries (Con, n = 8). Vessels from the ceNOS-transduced animals were
removed 5 days after PTCA and gene transfer. All frozen segments were homogenized in 1 mL ice-cold 6% trichloroacetic acid, pH 4.0, and centrifuged at 10 000g for 15 minutes at 4°C. The supernatant was transferred to a glass centrifuge tube, and trichloroacetic acid was extracted 4 times with H2O-saturated ether. A 0.5-mL aliquot of the sample was then lyophilized, resuspended in 0.05 mol/L sodium acetate buffer (pH 5.8), and assayed for cGMP with a commercial enzyme immunoassay (Amersham Life Science).

**Efficiency of Adenovirus-Mediated Gene Transfer In Vivo**

To assess gene transfer efficiency with the Infiltrator catheter in the coronary vessel wall, AdCMVβgal gene transfer was performed in native (n=4) and balloon-injured (n=4) arteries at day 0. After the animals were killed at day 4, the LAD was cannulated, perfused with 4% formaldehyde at 100 cm H2O pressure for 2 hours, and washed with PBS for 24 hours. To identify transduced cells expressing the transgene, arteries were cut into 5-mm rings and incubated in X-Gal for 8 hours at 37°C, and paraffin sections (5 μm) were prepared and counterstained with nuclear fast red. The entire vessel length was scanned, and the medial and adventitial cells expressing the transgene were identified by the dark blue coloration of the nucleus.

**Morphometric Analysis**

Twenty-eight days after gene transfer, the injured segment of the LAD was carefully dissected from the epicardial surface, sectioned transversely into 2- to 3-mm rings, washed, and embedded in paraffin. Transverse sections 5 μm thick were cut every 200 μm and stained with hematoxylin and eosin. Cross-sectional areas of the intimamedia (IEL), intima-external elastic lamina (EEL), and internal layer of the adventitia (IEL) were identified by the dark blue coloration of the nucleus. Vessel remodeling was assessed by measuring the ratio (EEL (IEL fracture length)/IEL circumference).

**Statistics**

Results are presented as mean±SD. A Mann-Whitney rank sum test was used to compare EEL area, (neointima area)/(IEL fracture length), (IEL fracture length)/IEL, maximal intimal thickness, and percentage area stenosis. Differences are considered significant at P<0.05. Vascular cGMP levels were compared by Kruskal-Wallis 1-way ANOVA with Dunn’s correction for multiple comparison.

**Results**

**Transgene Expression In Vitro in Porcine SMCs Infected With AdCMVβgal and AdCMVceNOS**

Porcine SMCs were infected with AdCMVβgal or AdCMVceNOS at various MOIs. After fixation, transgene expression was revealed by X-Gal chemical staining and a monoclonal anti-ceNOS antibody, respectively. After 4 days, specific nucleus-localized blue staining was observed in 92±2% of the AdCMVβgal-infected SMCs at an MOI of 20 (Figure 2a). Abundant cytoplasmic ceNOS immunoreactivity was observed in AdCMVceNOS-infected SMCs (Figure 2b) but not in SMCs infected with AdRR5 (Figure 2c). The number of ceNOS-positive SMCs was proportional to the MOI applied (45±2% at MOI 2, 88±4% at MOI 20, and 100±1% at MOI 200).

**Transgene Expression in Normal and Injured Porcine Coronary Arteries**

Adenovirus-mediated gene transfer was achieved in vivo with a triple-lumen, intramural catheter (Infiltrator). In uninjured coronary arteries, segments harvested 4 days after infection with AdCMVβgal showed marked nuclear β-galactosidase activity in the media and the adventitia (Figure 2d). Analysis of sections spanning the area of gene transfer showed a circumferential nuclear staining pattern in which transduced cells were present primarily in the outer layers of the media and in the internal layer of the adventitia. Numerous blue cells were also observed adjacent to dissection planes, as previously reported in other models.33 Only occasional blue staining was observed in some adjacent cardiomyocytes, as previously reported.34 No nuclear blue staining was observed in segments from coronary arteries infected with AdRR5 (Figure 2e) or AdCMVceNOS (data not shown).

The intensity and distribution of ceNOS expression after intramural AdCMVceNOS delivery was studied by immunohistochemistry 4 days after balloon injury and gene transfer. Marked ceNOS immunoreactivity was also observed throughout the adventitia and in the outer cell layers of the media (Figure 2f). Endogenous ceNOS immunoreactivity was detected in endothelial cells of adventitial vasa vasorum in both AdCMVceNOS- and AdRR5-infected arteries. In AdRR5- and AdCMVβgal-infected arteries, no ceNOS immunoreactivity was detected in medial SMCs (Figure 2g).

**Recombinant Protein Bioactivity**

NO generated by ceNOS in endothelial cells diffuses to underlying SMCs and binds to the heme group of soluble guanylate cyclase, which catalyzes the conversion of GTP to cGMP. Vascular cGMP levels were significantly decreased after coronary angioplasty compared with uninjured controls from 1.30±0.42 to 0.33±0.20 pmol/mg protein (P<0.05) (Figure 3). Five days after balloon injury, ceNOS overexpression increased vascular cGMP to 1.82±0.98 pmol/mg protein, compared with 0.33±0.20 pmol/mg protein in the injured group (P<0.05). These levels were equal to those observed in uninjured control arteries (1.30±0.42 pmol/mg protein, P=NS versus AdCMVceNOS).

**Effect of Gene Transfer on Neointima Formation and Vessel Remodeling**

Relevant characteristics of the AdRR5- and AdCMVceNOS-treated groups were similar (Table 1). Two AdRR5-infected pigs had an occlusive thrombus at 28 days and were excluded from the final analysis. One additional artery from a AdCMVceNOS-infected pig was lost during processing/embedding. Morphometric analysis was performed 28 days after PTCA in coronary arteries infected with AdRR5 (n=10) or AdCMVceNOS (n=10) (Table 2). Arteries showed a marked neointimal lesion that consisted mostly of stellate and spindle-shaped cells in a loose extracellular matrix. In this model, neointimal response is proportional to the degree of injury.6,35,36 The degrees of injury estimated by both the injury score defined by Schwartz et al6 and...
Figure 2. a through c, Histological analysis of transgene expression in vitro. Porcine SMCs were infected with either AdCMVβgal (a), AdCMVceNOS (b), or AdRR5 (c) at an MOI of 20 for 24 hours. β-Galactosidase staining (a) and ceNOS immunostaining (b, c) were performed as described. Marked, nucleus-localized, dark blue staining is found in AdCMVβgal-infected cells (a) (×200). Abundant cytoplasmic ceNOS immunoreactivity is observed in AdCMVceNOS-infected cells (b) but not in AdRR5-infected cells (c) (×200). d through g, Distribution of adenoviral transgene expression after adenoviral delivery with the Infiltrator (×100). Medial (m) and adventitial (ad) cells layers are labeled. Porcine coronary arteries were infected with adenoviruses carrying the \textit{E. coli lacZ} gene encoding a nucleus-localized variant of β-galactosidase AdCMVβgal (d) or no cDNA (AdRR5) (e) and stained for β-galactosidase expression. Marked β-galactosidase activity, as assessed by blue staining of nuclei of infected cells, was detected in media and adventitia of AdCMVβgal-
angioplastied coronary arteries after percutaneous, catheter-based, local gene transfer. This study shows that (1) the Infiltrator catheter allows highly efficient percutaneous adenovirus-mediated gene transfer into medial and adventitial cells of noninjured and balloon-injured coronary arteries, (2) intramural gene transfer results in expression of significant levels of functional recombinant protein, and (3) adenovirus-mediated ceNOS gene transfer restores vascular cGMP levels and reduces luminal narrowing 28 days after PTCA, probably mediated by a combined effect on neointima formation and vascular remodeling.

In endothelial cells, ceNOS catalyzes the formation of NO from L-arginine.\(^\text{38}\) NO plays an important role in normal cardiovascular homeostasis through pleiotropic effects on the vessel wall. Endothelium-derived NO induces cGMP-dependent vasorelaxation and inhibits SMC proliferation, migration, and extracellular matrix formation,\(^\text{59,40}\) although the molecular mechanisms involved remain unclear. Given its capacity to inhibit platelet adhesion and aggregation,\(^\text{41,42}\) to modulate leukocyte adhesion,\(^\text{43,44}\) and to induce SMC apoptosis,\(^\text{45}\) NO is recognized as an important endogenous inhibitor of vascular lesion formation in vivo. Indeed, strategies aimed at increasing local NO production, including oral administration of L-arginine, the precursor of NO, inhibited neointimal thickening 4 weeks after balloon denudation of normocholesterolic rabbit iliac arteries.\(^\text{26}\) Similarly, intracoronary administration of NO-donor compounds significantly reduced platelet-induced cyclic flow variations and the response to injury in an open-chest canine model.\(^\text{46}\) Systemic administration of NO-donor compounds, however, may be associated with systemic hypotensive side effects, limiting their use to modify the vascular response to local injury. AdCMVceNOS infection after angioplasty led to local cGMP levels after 5 days that were comparable to baseline levels, indicating that intravascular NO production was restored. Increased NO
concentrations at the site of injury may modulate different pathophysiological processes contributing to restenosis, such that the primary intervention and the passivation of the lesion results in significant reduction of neointima formation and vascular remodeling after several weeks.

Recently, transfer of ceNOS cDNA by use of a Sendai virus–liposome complex was shown to restore local NO production and demonstrated a 70% reduction of neointimal mass in balloon-injured rat carotid arteries without significant side effects. ceNOS gene transfer in the rat model reduces neointima formation at least in part via an antiproliferative effect on SMCs. Porcine coronary artery SMCs infected in vitro with AdCMVceNOS show an inhibition of in vitro proliferation similar to that reported for rat SMCs (data not shown). Although our study was not designed to investigate the degree and time of reendothelialization after PTCA, part of the inhibition of neointima formation may also be associated with differences in endothelial cell regrowth, as has been observed in balloon-injured rat carotid arteries. It remains to be determined to what extent ceNOS gene transfer affects SMC migration and apoptosis or platelet and neutrophil adhesion in the porcine model. As the role and contribution of the adventitia in restenosis and chronic remodeling after PTCA become better appreciated, the efficient transduction of particular genes into the adventitial cell layers will be an important investigative tool. Several indices of vessel size suggested a potentially beneficial effect of AdCMVceNOS gene transfer on remodeling. The reduction in luminal narrowing cannot be accounted for by an effect of ceNOS gene transfer on neointima formation alone. Indeed, lumen area increased by 0.38 mm², whereas a 0.16-mm² reduction in neointima area was observed. These quantitative differences may reflect a positive effect on constructive remodeling by ceNOS gene transfer that did not reach statistical significance. The lack of significance may result from the loss of transgene expression 28 days after adenoviral gene transfer. To further assess constructive vascular remodeling, serial invasive investigations using intravascular ultrasound or digital angiography during maximal transgene expression would be required.

Although the anatomy of coronary arteries and the progression and morphology of neointima formation in the porcine model is similar to that in humans, extrapolation of the data to patients requires caution. One potential confounding factor may be the use of recombinant adenoviral vectors. Control pigs in this study received a recombinant adenovirus carrying no transgene to exclude the possibility that the adenovirus itself, independently of the expression of ceNOS, may modulate the vascular response to angioplasty. Intravascular adenoviral infection might be associated with species-specific toxicity leading to neointimal hyperplasia, as described in rabbit iliac arteries. In contrast, adenoviral infection of rat carotid and porcine coronary arteries does not induce cytotoxic effects. As is characteristic for this model, inflammatory cell infiltrates were occasionally observed in vascular sections of both uninfected and infected angioplastied arteries but not in unrelated coronary arteries or the livers of pigs that received recombinant virus. It is therefore unlikely that the adenoviral vectors per se modulate the vascular response to injury.

In conclusion, intracoronary ceNOS gene transfer restores local NO production after injury and significantly reduces neointima formation and luminal narrowing 28 days after PTCA. Intramural gene transfer constitutes a useful tool to study key molecular events in the injured vessel wall and may lead to potential new therapeutic approaches for restenosis after PTCA.

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**TABLE 2. Morphometric Analysis of LADs**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AdRR5 (n=10)</th>
<th>AdCMVceNOS (n=10)</th>
<th>P</th>
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<tr>
<td>EEL area, mm²</td>
<td>2.27±0.52</td>
<td>2.55±0.79</td>
<td>NS</td>
</tr>
<tr>
<td>EEL/EELref*</td>
<td>1.11±0.28</td>
<td>1.34±0.39</td>
<td>NS</td>
</tr>
<tr>
<td>IEL area, mm²</td>
<td>1.25±0.30</td>
<td>1.52±0.60</td>
<td>NS</td>
</tr>
<tr>
<td>IEL fracture length, mm</td>
<td>1.78±0.59</td>
<td>1.98±0.74</td>
<td>NS</td>
</tr>
<tr>
<td>IEL fracture length/IELp</td>
<td>0.40±0.10</td>
<td>0.43±0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Injury score</td>
<td>2.30±0.48</td>
<td>2.20±0.47</td>
<td>NS</td>
</tr>
<tr>
<td>Maximal neointimal thickness, mm</td>
<td>1.04±0.25</td>
<td>0.75±0.21</td>
<td>0.019</td>
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<tr>
<td>Neointimal area, mm²</td>
<td>1.37±0.40</td>
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<td>NS</td>
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<tr>
<td>Neointimal area/IEL fracture length, mm</td>
<td>0.80±0.19</td>
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<tr>
<td>Lumen area, mm²</td>
<td>0.32±0.18</td>
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<td>Stenosis,%†</td>
<td>75±11</td>
<td>53±15</td>
<td>0.006</td>
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</table>

*IELp indicates internal elastic lamina perimeter.*

*†The ratio EEL/EELref is defined as the ratio of the EEL area at the site of maximal stenosis (EELref) to the EEL area in the noninjured proximal LAD (EELp).*
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