Gene transfer of calcitonin gene-related peptide to cerebral arteries

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Toyoda, Kazunori, Frank M. Faraci, Andrew F. Russo, Beverly L. Davidson, and Donald D. Heistad. Gene transfer of calcitonin gene-related peptide to cerebral arteries. Am. J. Physiol. Heart Circ. Physiol. 278: H586–H594, 2000.—Overexpression of calcitonin gene-related peptide (CGRP), an extremely potent vasodilator, to blood vessels is a possible strategy for prevention of vasospasm. We constructed an adenoviral vector that encodes prepro-CGRP (Adprepro-CGRP) and examined the effects of gene transfer on cerebral arteries. Transfection of Adprepro-CGRP to Cos-7 and NIH-3T3 cells increased CGRP-like immunoreactivity in media and produced an increase in cAMP in recipient cells. Five days after injection of Adprepro-CGRP into the cisterna magna of rabbits, the concentration of CGRP-like immunoreactivity increased by 93-fold in cerebrospinal fluid. In basilar artery, CAMP increased by 2.3-fold after Adprepro-CGRP compared with a control adenovirus. After transfection of Adprepro-CGRP, contraction of basilar artery in vitro to histamine and serotonin was attenuated, and relaxation to an inhibitor of cyclic nucleotide phosphodiesterase 3-isobutyl-1-methylxanthine was augmented compared with nontransduced arteries or arteries transfected with a control gene. Altered vascular responses were restored to normal by pretreatment with a CGRP1 receptor antagonist (CGRP-(8–37)). Thus gene transfer of prepro-CGRP in vivo overexpresses CGRP in cerebral fluid and perivascular tissues and modulates vascular tone. We speculate that this approach may be useful in prevention of vasospasm after subarachnoid hemorrhage.

cerebral circulation; adenovirus; adenosine 3′,5′-cyclic monophosphate; basilar artery; gene expression

CALCITONIN GENE-RELATED PEPTIDE (CGRP), initially discovered as an alternative RNA splicing product from the calcitonin gene (2), is abundant in perivascular nerve fibers surrounding cerebral and some peripheral blood vessels (26, 36, 42). Release of the neuropeptide from trigeminal sensory fibers (25) is an extremely potent vasodilator mechanism (4), especially in the cerebral circulation (44). CGRP may have functional effects in the cerebral circulation under some disease states, including subarachnoid hemorrhage (SAH). Several studies have demonstrated depletion of CGRP in perivascular nerve terminals after SAH (11, 28), which may correlate with the onset of vasospasm. Transduction of genes with dilator potency to blood vessels is a possible strategy for the prevention and treatment of cardiovascular and cerebrovascular disorders. For example, Ooboshi et al. (30, 32) and others (6, 7) demonstrated that transduction of recombinant endothelial nitric oxide synthase (eNOS) using adenoviral vectors ex vivo and in vivo alters reactivity of carotid and cerebral arteries. Thus we speculated that overexpression of CGRP in blood vessels by adenovirus-mediated gene transfer may also alter vascular tone.

The goal of this study was to determine whether gene transfer of CGRP produces a vasoactive product that has functional effects in cerebral arteries. We chose CGRP because of its potency as a vasodilator and because it is readily diffusible. For this strategy, we constructed a recombinant adenovirus containing the cDNA for human prepro-CGRP. We chose to prepare a virus that produces prepro-CGRP, rather than CGRP, because we anticipated that the precursor would facilitate the required modification and release of the peptide from the cell.

First, we determined whether biologically active CGRP is processed and released in cell cultures following gene transfer with this virus. We examined release of CGRP and accumulation of intracellular cAMP after gene transfer in two mammalian cell lines. Although there is not a consensus regarding receptor-effector coupling mechanisms for the vasodilator response to CGRP, cAMP increases when CGRP receptors are stimulated (22, 39), and there is a correlation between CGRP-induced vasorelaxation and elevation of intracellular cAMP (13). Thus cAMP appears to be an important second messenger of the coupling mechanism, in part by cAMP-mediated activation of ATP-sensitive potassium channels (21). Second, we determined whether perivascular gene transfer using the virus in vivo expresses CGRP in the adventitia and alters cerebral vascular reactivity. Perivascular delivery of transgenes produces overexpression of transgene products in vascular adventitia and perivascular tissues (7, 9, 27, 33, 34). This mode of delivery does not require interruption of blood flow, which is potentially useful for cerebral circulation. Adventitial expression of transduced eNOS can augment NO-mediated vasorelaxation (7). Thus we anticipated that expression of CGRP in...
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METHODS

Adenoviral vectors. A replication-deficient adenovirus encoding the human prepro-CGRP gene (Adprepro-CGRP) expressed from a long terminal repeat of Rous sarcoma virus (RSV-LTR) as a promotor was generated using standard methods by the University of Iowa Gene Transfer Vector Core (Iowa City, IA) (30). Briefly, the entire coding sequence of human prepro-CGRP was cloned by blunt end ligation into pAdRSV4. The resultant plasmid and adenovirus backbone sequences restricted of E1 were transfected into human embryonic kidney (HEK) 293 cells, and plaques were isolated and amplified for analysis of CGRP expression. A recombinant adenovirus encoding a nuclear-targeted bacterial β-galactosidase expressed from the RSV-LTR promotor (Adβgal) was used as a control virus. Recombinant adenoviruses were triple plaque purified, and virus titer was determined by plaque assay on HEK-293 cells. Purified viruses were stored in phosphate-buffered saline (PBS) with 3% sucrose and kept at −80°C until use.

Gene transfer to cultured cells. Cos-7 cells (African green monkey kidney cells transduced with simian virus 40 (SV40) T-antigen) and NIH-3T3 cells (mouse fibroblast cells) were cultured on 35-mm dishes in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at 3 × 10^5 cells/cm^2 and infected 18–24 h after seeding when cells were ~70% confluent. The medium was replaced with 1 ml of DMEM containing Adβgal [500 plaque-forming units (pfu)/cell], Adprepro-CGRP (10, 100, and 500 pfu/cell), or vehicle (20 µl of PBS with 3% sucrose). For 3T3 cells, a noncovalent complex of Adβgal or Adprepro-CGRP with poly-L-lysine at a 1:100 ratio of adenovirus particles to poly-L-lysine molecules was used to enhance the efficiency of gene transfer (15). The complex was formed as previously described (15, 40). Cells were incubated for 2 h in a 5% CO_2 humidified atmosphere at 37°C, the infection solution was removed, fresh serum-containing DMEM was added, and the cells were incubated for an additional 72 h. The culture medium was replaced every 24 h. The infection solution and each 24-h culture medium were stored at −80°C for measurement of CGRP-like immunoreactivity.

Gene transfer to rabbits in vivo. All animal procedures were approved by the Animal Care and Use Review Committee at the University of Iowa. Male New Zealand White rabbits weighing 2.4–3.0 kg were anesthetized with 5 mg/kg im of xylazine and 50 mg/kg im of ketamine. A 25-gauge needle was aseptically inserted into the cisterna magna as previously described (23). 250 µl of cerebrospinal fluid (CSF) were withdrawn for measurement of white blood cell count and CGRP-like immunoreactivity, and 250 µl of viral suspension (PBS with 3% sucrose with Adβgal or Adprepro-CGRP) or vehicle (PBS with 3% sucrose) were infused. The adenoviral titer in suspension varied from 1 × 10^9 to 3 × 10^10 pfu/ml.

Five days after injection, rabbits were anesthetized with 50 mg/kg iv of pentobarbital sodium and exsanguinated from the common carotid artery after collection of CSF from the cisterna magna. Basilar arteries were removed and cut into four rings, each 3 mm in length, for assays of β-galactosidase activity and cAMP, immunohistochemistry for CGRP, and recording of isometric tension. The brain was also removed for immunohistochemistry for CGRP. Rectal temperature was measured just before injection and before exsanguination. Some rabbits were perfused transcardially with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS without exsanguination.
15 min before exposure to CGRP (100 nmol/l). The rings were frozen and later assayed for cAMP levels.

Measurement of vascular reactivity. Arterial rings were suspended in organ baths filled with Krebs bicarbonate solution maintained at 37°C and bubbled with 95% O2-5% CO2 and connected to force transducers. Resting tension of vessels was increased to 500 mg, and the preparations were allowed to equilibrate for 60 min. Concentration-response curves were determined for three different vasoconstrictive agents: KCl (40 mmol/l), histamine (0.01–3 µmol/l), and serotonin (0.01–3 µmol/l), measured as percent contraction of response to 40 mmol/l KCl. Increase in calcium concentration in the cell culture medium from Cos-7 cells treated with vehicle or Adβ-gal (Fig. 1, A and B). Treatment with Adprepro-CGRP increased levels of CGRP-like immunoreactivity in both Cos-7 and 3T3 cells in a dose-dependent manner (P < 0.0001 vs. vehicle or Adβ-gal), which indicates effective expression of CGRP following gene transfer.

After adenoviral infection, we transferred culture medium from Cos-7 cells to BHK-21 cells and measured cAMP levels in the recipient cells. Application of medium from Cos-7 cells treated with vehicle and Adβ-gal produced similar low levels of cAMP generation in BHK-21 cells (Fig. 1C). Medium, which contained 12.6 ± 2.4 nmol/l CGRP-like immunoreactivity, from Cos-7 cells treated with Adprepro-CGRP increased cAMP levels in BHK-21 cells by threefold (P < 0.0005). This increase in cAMP in recipient cells was abolished by pretreatment of the cells with CGRP-(8–37). The findings indicate that medium from Adprepro-CGRP-transduced cells is biologically active in cell culture.

Effects of synthetic CGRP on rabbit basilar artery. Synthetic CGRP produced an increase in cAMP levels in rabbit basilar arteries and relaxation of the arteries (Fig. 2, A and B). For example, 1 nmol/l CGRP increased cAMP levels by 2.3-fold and relaxed the arterioles in vitro.

**RESULTS**

Gene transfer in vitro. To test whether adenovirus-mediated gene transfer of prepro-CGRP resulted in transgene expression in mammalian cells, levels of CGRP-like immunoreactivity were determined in cell culture media following infection. Both Cos-7 and NIH-3T3 cell lines expressed minimal levels of CGRP-like immunoreactivity after treatment with vehicle or Adβ-gal (Fig. 1A and B). Treatment with Adprepro-CGRP increased levels of CGRP-like immunoreactivity in both Cos-7 and 3T3 cells in a dose-dependent manner (P < 0.0001 vs. vehicle or Adβ-gal), which indicates effective expression of CGRP following gene transfer.

**Histochemistry**

Histochemical staining for detection of β-galactosidase was performed as described previously (33) to examine localization of transgene product after gene transfer in vivo. The number of blue-stained nuclei and total nuclei of adventitial cells were counted in consecutive cross sections of the basilar artery as described previously (31).

Measurement of vascular reactivity. Arterial rings were suspended in organ baths filled with Krebs bicarbonate solution maintained at 37°C and bubbled with 95% O2-5% CO2 and connected to force transducers. Resting tension of vessels was increased to 500 mg, and the preparations were allowed to equilibrate for 60 min. Concentration-response curves were determined for three different vasoconstrictive agents: KCl (40 mmol/l), histamine (0.01–3 µmol/l), and serotonin (0.01–3 µmol/l), measured as percent contraction of response to 40 mmol/l KCl. Increase in calcium concentration in the cell culture medium from Cos-7 cells treated with vehicle or Adβ-gal (Fig. 1, A and B). Treatment with Adprepro-CGRP increased levels of CGRP-like immunoreactivity in both Cos-7 and 3T3 cells in a dose-dependent manner (P < 0.0001 vs. vehicle or Adβ-gal), which indicates effective expression of CGRP following gene transfer.

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ies by 49 ± 10% relative to the baseline value. Thus both cAMP levels and vascular reactivity are indicators of bioactivity of CGRP. Pretreatment of the basilar artery with 10 or 100 nmol/l of synthetic CGRP also attenuated vasoconstriction to 0.03–0.3 µmol/l of histamine (Fig. 2C).

Gene transfer in vivo. We injected Adprepro-CGRP into CSF of rabbits to examine expression of CGRP in basilar arteries and effects on vascular reactivity. All rabbits tolerated the injection of adenoviral vectors without alteration of rectal temperature (39.0 ± 0.1°C before injection and 39.1 ± 0.1°C 5 days after injection) or detectable neurological deficits. Leukocyte count in CSF increased from 3 ± 1 to 249 ± 100 cells/mm³ 5 days after Adβgal injection and 193 ± 68 cells/mm³ after Adprepro-CGRP. Leukocyte count did not increase 5 days after injection of vehicle (5 ± 2 cells/mm³).

β-Galactosidase activity in basilar arterial rings from rabbits treated with 1 × 10⁹, 3 × 10⁹, 1 × 10¹⁰, and 3 × 10¹⁰ pfu/ml of Adβgal was 14 ± 4, 523 ± 56, 413 ± 149, and 3 ± 2 mU/mg protein, respectively (n = 3 for all viral titer), suggesting that injection of 3 × 10⁹ pfu/ml is optimal for adenoviral vectors. Thus we performed the following assays using 250 µl of viral suspension with 3 × 10⁹ pfu/ml of Adβgal or AdCGRP for gene transfer in vivo. Because the total volume of freely flowing CSF in the rabbit is >2 ml (23), the final adenoviral titer in CSF was estimated to be <4 × 10⁹ pfu/ml.

Overexpression of CGRP after gene transfer in vivo. Five days after injection of Adprepro-CGRP in vivo, histochemistry for β-galactosidase revealed blue staining extensively on the ventral surface of the brain and in ventricles in rabbits treated with Adβgal as we previously demonstrated in other species (9, 27, 33). The percentage of blue-stained cells in the adventitia of the basilar artery was 34 ± 3% (n = 4 rabbits).

Release of CGRP into CSF was evaluated by RIA. Levels of CGRP-like immunoreactivity in CSF were similar before and 5 days after injection of Adβgal or vehicle (Fig. 3A). Five days after injection of Adprepro-CGRP, however, CGRP-like immunoreactivity was 93-fold greater than baseline values (P < 0.0005).

Intracellular cAMP was measured in basilar arteries to verify bioactivity of transduced CGRP following gene transfer in vivo. Five days after injection of AdCGRP, but not Adβgal, cAMP in basilar arteries was greater than after injection of vehicle (P < 0.01, Fig. 3B). Increased cAMP was restored to baseline levels by pretreatment of the harvested basilar artery with CGRP-(8–37).
CGRP-(8–37) before assay. Thus gene transfer of prepro-CGRP resulted in overexpression of CGRP in adventitia of basilar arteries, on the ventral brain stem and ventricles, and in CSF, and expressed CGRP was bioactive.

Effects of gene transfer on vascular reactivity. Contraction to 40 mmol/l KCl was similar in arteries treated with vehicle (1.55 ± 0.07 g), Adβgal (1.56 ± 0.18 g), and Adprepro-CGRP (1.43 ± 0.12 g). Contractile responses to histamine (0.01–3 µmol/l) were not significantly different in arteries treated with vehicle or Adβgal (Fig. 4A). In arteries treated with Adprepro-CGRP, however, contractile responses to histamine were attenuated (P < 0.02 vs. vehicle-treated arteries, and P < 0.01 vs. Adβgal-treated arteries). EC50 [log(mol/l)] (−6.16 ± 0.11) and maximal contraction (1.40 ± 0.14 g, 98 ± 10% of response to 40 mmol/l KCl) for Adprepro-CGRP were significantly different from those for vehicle (EC50, −6.46 ± 0.07, P < 0.05; Rmax 1.91 ± 0.08 g, 123 ± 5%, P < 0.01) or Adβgal (EC50, −6.45 ± 0.07, P < 0.05; Rmax 1.92 ± 0.12 g, 124 ± 8%, P < 0.005).

Contractile responses to serotonin (0.01–3 µmol/l) were not significantly different in arteries treated with vehicle or Adβgal (Fig. 4C). In arteries treated with Adprepro-CGRP, contractile responses to serotonin were attenuated (P < 0.01 vs. vehicle-treated arteries, and P < 0.02 vs. Adβgal-treated arteries). EC50 (−6.91 ± 0.09) and maximal contraction (0.79 ± 0.12 g, 55 ± 9% of response to 40 mmol/l KCl) for Adprepro-CGRP were significantly different from those for vehicle (EC50, −7.23 ± 0.15, P < 0.05; Rmax 1.24 ± 0.03 g, 80 ± 2%, P < 0.01) or Adβgal (EC50, −7.40 ± 0.12, P < 0.05; Rmax 1.18 ± 0.11 g, 76 ± 8%, P < 0.05).

In arteries treated with Adprepro-CGRP, responses to histamine and serotonin were restored to those of Adβgal-treated arteries by pretreatment with CGRP-(8–37) (Fig. 4B and D). Concentration-contraction curves to histamine and serotonin were similar when contraction was expressed using absolute values for tension. These data indicate that after gene transfer of CGRP in vivo, contractile responses of the basilar artery are attenuated by an effect on CGRP1 receptors.

Relaxation to IBMX (0.1–3 µmol/l) was significantly augmented in arteries treated with Adprepro-CGRP compared with vehicle (P < 0.0005) or Adβgal (P < 0.0005) (Fig. 5A). EC50 for Adprepro-CGRP (−6.28 ± 0.05) was significantly different from that for vehicle (−6.05 ± 0.03, P < 0.0005) or Adβgal (−6.08 ± 0.06, P < .
0.02). Maximal relaxation for Adprepro-CGRP (94 ± 4%) tended to be greater than that for vehicle (83 ± 4%, P < 0.1) or Adβgal (79 ± 8%, P < 0.1). Augmented relaxation to IBMX was blocked by CGRP-(8–37) (Fig. 5B). Responses to acetylcholine and nitroprusside were virtually identical among the three groups (Fig. 5, C and D). Concentration-relaxation curves were similar when precontraction and relaxation were expressed using absolute values for tension. Thus gene transfer of prepro-CGRP enhanced relaxation of basilar arteries to an inhibitor of phosphodiesterase but not to other vasorelaxant stimuli.

DISCUSSION

In this study, we have described successful overexpression of vasoactive CGRP after gene transfer of prepro-CGRP. There are three major new findings. First, treatment with Adprepro-CGRP greatly increased levels of CGRP-like immunoreactivity in mammalian cell lines and enhanced levels of cAMP. Thus the recombinant adenoviral vector produces biologically active CGRP, which is released from the cell. Second, gene transfer with Adprepro-CGRP in vivo increased CGRP-like immunoreactivity in the CSF and increased cAMP in the basilar artery. Thus biologically active CGRP can be expressed in vivo by adenovirus-mediated gene transfer. Third, gene transfer of prepro-CGRP in vivo altered reactivity of the basilar artery in vitro with inhibition of contraction and selective augmentation of relaxation.

Gene transfer in vitro. CGRP is biosynthesized as a precursor propeptide that is processed posttranslationally before secretion. The processing of pre-pro-CGRP begins in the rough endoplasmic reticulum with the removal of the signal peptide, then continues in the secretory granules with cleavage at basic residues and COOH-terminal amidation. We prepared an adenovirus that expresses a precursor propeptide, which had a potential disadvantage that the propeptide may not be processed correctly.

We demonstrated an enormous increase in CGRP-like immunoreactivity in cell culture media after gene transfer. This RIA has no cross-reaction to calcitonin or other peptides but may cross-react with pro-CGRP. We measured cAMP to assess bioactivity of the viral product, because cAMP is coupled to CGRP1 receptors (22, 39) and is a second messenger in CGRP-induced physiological actions (12, 43). Enhanced accumulation of cAMP in the recipient cells after gene transfer suggested effective processing of the propeptide to bioactive CGRP. Thus an important finding of this study is that adenovirus-mediated gene transfer of a precursor propeptide can overexpress a bioactive product.

CGRP-like immunoreactivity in the medium of Cos-7 cells treated with Adprepro-CGRP was >10 nmol/l. Medium from cells treated with Adprepro-CGRP pro-
duced a significant increase in cAMP, which indicates that the peptide was biologically active, but cAMP levels in the recipient cells were smaller after application of this medium than after application of 1 nmol/l synthetic CGRP (Fig. 1C). Thus it is possible that biologically inactive CGRP (perhaps unprocessed pro-CGRP) was also detected by radioimmunoassay in the medium of Cos-7 cells. We speculated that because Cos-7 cells have relatively low activity of the amidation enzyme peptidyl-glycine α-amidating monoxygenase, which is required for CGRP bioactivity (38), amidation may be less in Cos-7 cells than in other tissues. These findings nevertheless provided evidence in tissue culture that the recombinant adenovirus produces an active product and led us to examine effects of the virus in vivo.

Gene transfer in vivo. Injection of an adenoviral vector into CSF, which results in overexpression of transgene products (β-galactosidase and eNOS) in the adventitia of cerebral arteries and perivascular tissue, is a useful approach for gene transfer in vivo (7, 9, 27, 33). From histochemical detection of β-galactosidase, we estimate that about one-third of the adventitial cells may be infected after gene transfer of CGRP. Adventitia consists primarily of fibroblasts, and findings in fibroblast cell culture suggest that adventitial fibroblast cells are efficiently transduced by Adprepro-CGRP. Measurement of cAMP and vascular reactivity demonstrated that vasoactive CGRP was overexpressed in the artery. Interestingly, a large amount of CGRP is released in CSF after gene transfer. Concordant with the finding of extensive histochemical staining for β-galactosidase, CGRP appears to be overexpressed extensively on the ventral regions of the brain stem surface as well as the adventitia of the arteries after gene transfer of prepro-CGRP. The transgene product on the brain surface may secrete CGRP into CSF and potentially alter vascular function.

Increases in cAMP in the basilar artery after Adprepro-CGRP demonstrated bioactivity of the transduced CGRP, as cAMP increased greater than twofold after gene transfer (Fig. 3B). The increase in cAMP in the basilar artery after gene transfer, which was associated with a concentration of 0.6 ± 0.1 nmol/l CGRP in CSF, was similar to or slightly greater than the response to 1 nmol/l synthetic CGRP (Fig. 2A). Thus overexpression of CGRP in the basilar artery, in addition to increases in cAMP in CSF, probably contributed importantly to responses of the artery in vitro. We speculate that responses of the arteries in vivo may be altered even more, because the arteries are exposed to CGRP present in the CSF in addition to CGRP that is present in the artery. Nevertheless, it is important to note that increases in cAMP in the basilar artery after gene transfer were appropriate for the concentration of CGRP that was achieved and provided clear evidence for bioactivity after gene transfer in vivo.

Effects of gene transfer on vascular reactivity. In carotid arteries after endothelial denudation, gene transfer of eNOS ex vivo enhances vasorelaxation to A-23187 (30). Perivascular delivery of eNOS by adeno-
the vascular adventitia, surrounding leptomeninges, and subarachnoid space after injection of adenoviral vectors in the cisterna magna. In this study, we found that leukocyte counts in CSF were similar after injection of Adβgal and Adprepro-CGRP, which suggests that the inflammatory response is the result of primarily the virus rather than the transduced CGRP. A key point is that reactivity of arteries from rabbits treated with vehicle and Adβgal is virtually identical, so that inflammation after viral injection does not appear to account for alteration of vascular function.

Implications. We speculate that alteration of vascular reactivity after gene transfer of prepro-CGRP may become a strategy for cardiovascular and cerebrovascular diseases associated with decreased arterial diameter. In the cerebral circulation, this approach may be useful in the prevention of vasospasm after subarachnoid hemorrhage. There is a >90% reduction of CGRP in the middle cerebral arteries of patients who die following SAH (11), which leads us to speculate that gene transfer of CGRP after SAH may produce a beneficial effect.

Intrathecal administration of CGRP increased arterial diameter in animals with vasospasm after SAH (19, 29). Intravenous administration of CGRP to patients with SAH improved neurological outcome despite hypotension (14, 20). We suggest that gene transfer of prepro-CGRP may have important advantages compared with administration of an exogenous peptide. In vivo synthesis of neurotrophic factors may be more effective than administration of exogenous neurotrophins (1, 8). By gene transfer, implantable devices or repeated injections are not needed to deliver the gene product, and peptide concentrations may remain relatively stable compared with repeated injection. Local gene transfer into CSF may avoid systemic effects of CGRP, including hypotension. Currently available adenoviral vectors have important disadvantages, especially induction of inflammation. Recent advances with adenoviral and other vectors, however, may enable greater expression of transgene products and minimal inflammation (24, 37) and increase application of gene transfer to clinical medicine.

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