Adenoviral Gene Delivery of Pigment Epithelium-Derived Factor Protects Striatal Neurons from Quinolinic Acid–Induced Excitotoxicity

Tomomi Sanagi, PhD, Takeshi Yabe, PhD, and Haruki Yamada, PhD

Abstract
The 50-kDa secreted glycoprotein pigment epithelium-derived factor (PEDF) is neuroprotective for various types of cultured neurons, but whether it is neuroprotective for neurons in vivo is not known. We examined the effects of adenovirus-mediated gene transfer of PEDF on quinolinic acid (QA)–induced neurotoxicity in rats. Adenoviral vector containing the human PEDF gene (Ad.PEDF) or Escherichia coli β-galactosidase gene (Ad.LacZ) was directly injected into the right striatum 7 days before the injection of QA. Immunohistochemical analysis using antibodies specific for the neuronal markers dopamine and cyclic adenosine monophosphate–regulated phosphoprotein of 32 kDa, neuronal nuclei, and choline acetyltransferase revealed that the QA-induced striatal damage was significantly reduced in Ad.PEDF-treated rats. Overexpression of PEDF also reduced the expression of the inflammation-related genes for interleukin 1β, tumor necrosis factor α, and macrophage inflammatory protein 1α 1 day after QA injection. Deletion analysis of human PEDF protein demonstrated that overexpression of PEDFΔ44-121 failed to protect neurons against QA-induced excitotoxicity, whereas PEDFΔ78-121 retained the neuroprotective activity, suggesting that amino acid residues 44–77 of the PEDF sequence are essential for PEDF-mediated neuroprotection in vivo. These results provide the first evidence that PEDF and its deletion mutant PEDFΔ78-121 are effective in protecting CNS neurons against excitotoxicity in vivo.

Key Words: Adenoviral gene transfer, Glia, Huntington disease, Neuroprotection, Pigment epithelium-derived factor, Quinolinic acid.

INTRODUCTION
Glutamate, the major excitatory neurotransmitter in the brain, is a potent excitotoxin to many neurons under a variety of experimental conditions. Excessive activation of glutamate receptors in the mammalian brain causes stimulation of receptor-gated channels, leading to a large and sustained rise in intracellular calcium that is followed by other (as yet not fully understood) events that result in neuronal death. Glutamate-mediated excitotoxicity has been proposed to contribute to neuronal death due to brain ischemia and traumatic brain injury and in a variety of neurodegenerative diseases, including Alzheimer disease (AD), Parkinson disease, amyotrophic lateral sclerosis, and Huntington disease (HD) (1–3). These effects are in particular thought to be due to excessive activation of the N-methyl-d-aspartate (NMDA) receptor (4, 5). Quinolinic acid (QA), an endogenous NMDA receptor agonist, has been shown to evoke degenerative events in nerve tissue via NMDA receptor overactivation and oxidative stress (6, 7). Striatal injection of QA has been used extensively to replicate the pathologic and neurochemical alterations that occur in HD (8).

Pigment epithelium-derived factor (PEDF) is a member of the serine protease inhibitor gene family (9) that exhibits a neuroprotective effect in various types of cultured neurons (10–12). PEDF is also a potent anti-angiogenic factor that inhibits new vessel formation in the eye and in tumors (13, 14). Previous reports have shown that human PEDF is expressed in a variety of cells and tissues both during development and in the adult (10). PEDF expression in rats is observed in almost all brain regions; in particular, intense PEDF immunoreactivity has been seen in the cerebral cortex, striatum, and cerebellum (15). In the injured rat brain after kainic acid injection or transient brain ischemia, PEDF expression was markedly induced in reactive astrocytes (15, 16). In clinical studies, elevated PEDF expression has been found in cerebrospinal fluid of patients with amyotrophic lateral sclerosis (17), AD (18, 19), and frontotemporal dementia (20). Thus, brain injury seems to regulate PEDF expression in the central nervous system (CNS). Given that PEDF has a neuroprotective effect on cultured neurons against various stimuli (10), we speculated that an increase in PEDF expression in the injured brain may form part of a compensation mechanism against neuronal degeneration. Consistent with this hypothesis, adenovirus-mediated PEDF gene transfer protected the cerebral cortex and striatum from ischemic injury in vivo (16). Although a complicated cascade is involved in neuronal damage after ischemic stimuli, excitotoxicity via NMDA receptors is considered to be critical to ischemic neuronal death.
Here, we tested whether PEDF could directly protect striatal neurons against NMDA-mediated excitotoxicity in vivo. We also sought to identify the functional domains of the PEDF protein relevant to its in vivo neuroprotective effects.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan), weighing 280 to 320 g were used in this study. The rats were housed individually under conditions of constant temperature (23 ± 2°C) and humidity (55 ± 10%) with food and water available ad libitum, unless otherwise specified, and a 12-hour light/dark cycle (light at 08:00 hours and dark at 20:00 hours). All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals at Kitasato University and conformed to the US National Institutes of Health guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering.

Construction of the Adenoviral Vector

Replication-defective adenovirus vector expressing human PEDF (Ad.PEDF) or Escherichia coli LacZ (Ad.LacZ) under the transcriptional control of the cytomegalovirus immediate-early promoter was constructed using the Vira-power Adenoviral Gateway Expression System (Invitrogen, Carlsbad, CA), as previously described (16). The deletion mutant human PEDF cDNA, PEDFΔ78-121, and PEDFΔ44-121 were prepared using polymerase chain reaction (PCR)–based strategy with full-length human PEDF cDNA (GenBank: M76979.1) as the template. Purified PCR products were ligated and assembled by PCR and subcloned into the adenoviral vector plasmid pAd/cytomegalovirus/V5-DEST (Invitrogen). The sequences of the resultant mutants were confirmed by DNA sequencing using the dideoxynucleotide chain termination method (21). PEDFΔ78-121 lacks amino acid residues 78-121, and PEDFΔ44-121 lacks amino acid residues 44-121. Adenoviral vectors were amplified in 293A cells and purified by ultracentrifugation through a CsCl gradient. Experiments using recombinant adenoviruses were approved by the Recombinant DNA Committee of Kitasato University and performed according to institutional guidelines. Primers used for mutated PEDF cDNA construction were as follows:

PEDF1 forward, 5'-ATGCAGGCCCCTGGTGCTACT CCTCTGC-3';
PEDF43 reverse, 5'-CTCCTCCTCCACCAGCAGCCC TGTGCT-3';
PEDF122 forward, 5'-TATAAGGAGCTCCTTGACA CGGTCACT-3';
PEDF77 reverse, 5'-GTTGGTCGTGGGGCTCATGC TGGATCG-3';
PEDF418 reverse, 5'-TTAGTGATGGTGATGGTGAT GGGGGCCCCTGGGGT-3'.

In Vivo Adenoviral Gene Transfer

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Using a stereotactic frame (Narishige, Tokyo, Japan), $1 \times 10^8$ pfu adenoviruses in 10 µL were injected into the right striatum at the following coordinates: anteroposterior, 0 mm; mediolateral, −3.0 mm; and dorsoventral, −6.0 mm. Injections were performed at a rate of 1 µL/minute using a Hamilton syringe equipped with a 26-gauge needle. The needle was left in place for at least 5 minutes before being withdrawn at a rate of 1 mm/minute. Although the expression of glial fibrillary acidic protein (GFAP) was slightly increased in adenovirus-injected striatum, the morphology of neurons and microglial cells was not changed 7 days after Ad.PEDF.
A. 7 days after QA injection

Control  
Ad.LacZ  
Ad.PEDF

NeuN

B. 7 days after QA injection

Control  
Ad.LacZ  
Ad.PEDF

DARPP-32

C. 7 days after QA injection

Control  
Ad.LacZ  
Ad.PEDF

ChAT

D. NeuN

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or Ad.LacZ injection (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A84). Furthermore, Reverse-transcriptase–PCR analysis revealed that mRNA expression of proinflammatory molecules such as interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNFα), and inductible nitric oxide synthase was not detectable 7 days after virus injection (data not shown), suggesting that the concentration of adenovirus vectors used in this study did not induce inflammatory or toxicity events at the 7-day time point.

**Quinolinic Acid Injection**

Quinolinic acid (2,3-pyridinedicarboxylic acid; Sigma, St. Louis, MO) was dissolved in 2 mol/L of NaOH. The pH was adjusted to 7.4 and the volume completed with PBS (pH 7.4). Under pentobarbital anesthesia (50 mg/kg, i.p.), the rats received an intrastriatal injection of 4 μL of QA (100 nmol) at a rate of 1 μL/minute at the following coordinates: anteroposterior, 0 mm; mediolateral, −3.0 mm; dorsoventral, −6.0 mm. The needle was left in place for at least 5 minutes before being withdrawn at a rate of 1 mm/minute.

**Perfusion and Tissue Processing**

Rats were deeply anesthetized with ether and then perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS. The brains were removed and stored in 4% paraformaldehyde at 4°C for a minimum of 24 hours. Fifty-micrometer coronal sections were then prepared on a microslicer (Dosaka EM, Kyoto, Japan) and used for immunohistochemical analysis.

**Immunohistochemistry**

Endogenous peroxidase activity was removed with a 20-minute incubation period in 80% methanol containing 3% H2O2. After 3 washes with PBS containing 0.3% Triton X-100 (PBST), background staining was blocked by incubation in 1% bovine serum albumin (Vector Laboratories, Burlingame, CA) containing 0.3% Triton X-100 and 0.1% BSA. After 2 hours in biotinylated anti-rabbit immunoglobulin (Ig) G or anti-mouse IgG secondary antibodies at a dilution of 1:200 (Vector Laboratories). Images were collected using a fluorescent microscope BioZero (Keyence, Osaka, Japan).

**Behavioral Analysis**

Apomorphine (APO)-induced rotational asymmetry was measured 8 days after QA administration. Rats were injected with APO (1.0 mg/kg, i.p.) and placed in a test cage for a 5-minute habituation period, followed by a 15-minute test session. Rotations were defined as complete 360-degree ipsilateral turns and were reported as the net difference between ipsilateral and contralateral rotations. Results are expressed as the net difference between the total numbers of turns after the administration of QA versus control. Ipsilateral turns were counted as positive turns, and contralateral turns were counted as negative turns.

**Western Blotting**

Human PEDF levels were determined in striatal lysates from rats injected with Ad.LacZ, Ad.PEDF, Ad.PEDFΔ44-121, or Ad.PEDFΔ78-121. Rats were killed by ether 1 week after the injection of adenoviruses. The brains were dissected and immediately frozen using liquid N2. Lysates from frozen tissues were prepared in lysis buffer (Celllytic MT Mammalian Tissue Lysis/Extraction Reagent; Cell Signaling Technologies). Samples were sonicated and centrifuged (10 minutes at 4°C, 15,000 × g). The supernatants were collected and protein concentration evaluated using the Bradford reagent assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ) for Western blot analysis. Human PEDF was detected using an anti-human PEDF monoclonal antibody (Vector Laboratories) for 1.5 to 3 hours. After 3 washes, bound antibody was visualized with 3, 3′-diaminobenzidine tetrahydrochloride (Sigma). The sections were mounted on silane-coated glass slides, dehydrated through graded alcohols and xylene, and coverslipped with Vecta Mount (Vector Laboratories). Images were collected using a fluorescent microscope BioZero (Keyence, Osaka, Japan).

**Image Analysis**

Image analysis was performed on blind-coded slides in 3 predesignated fields of striatum. The average number of NeuN- and ChAT-positive neurons was counted using an image analysis program (NIH image version 1.62; National Institutes of Health, Bethesda, MD) and expressed as the ratio of lesioned versus nonlesioned side. The QA lesions were analyzed by digitizing DARPP-32–immunostained sections with a scanner and by quantifying the lesion area with an image analysis program (NIH image version 1.62). Data are expressed as the ratio of DARPP-32–immunopositive area in QA-treated over control.

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**FIGURE 2.** Effect of Ad.PEDF pretreatment 7 days after quinolinic acid (QA) injection. One week after the injection of Ad.LacZ or Ad.PEDF in the right striatum, QA (100 nmol) was injected into the ipsilateral striatum. Brains were removed 7 days later, and striatal sections were immunostained. (A–C) Neuronal nuclei (NeuN)—(A), dopamine and cyclic adenosine monophosphate–regulated phosphoprotein of 32 kDa (DARPP-32)—(B), and choline acetyltransferase (ChAT)—immunostained (C) striatal sections of control (left), Ad.LacZ—(middle), and Ad.PEDF-injected (right) rats. (D–F) Numbers of NeuN—(D) or ChAT—(F) neurons and DARPP-32–immunopositive areas of striatum—(E) are expressed as percentages of the nonlesioned side. Values are expressed as mean ± SEM (n = 4 for Ad.LacZ group, n = 6 for Ad.PEDF group). Scale bars = (A) 50 μm; (B) 1 mm; (C) 100 μm.
antibody (1:2000; Trans Genic, Kumamoto, Japan) and an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Anti-human PEDF monoclonal antibody used in this assay does not cross-react with rat endogenous PEDF protein. The density of bands was measured using ImageJ version 1.29. The amount of human PEDF was determined using a standard curve based on known concentrations of recombinant human PEDF.

**Reverse-Transcribed-PCR**

Total RNA was extracted according to the manufacturer’s instructions for the TRIZOL LS Reagent (Invitrogen). Total RNA (1 μg) was converted to first-strand cDNA using ReverTra Ace (TOYOBO, Osaka, Japan), and 1 μL of the resulting first-strand cDNA was used for each PCR reaction. The PCR amplification mixture in a final volume of 25 μL contained ThermoPol Reaction Buffer (New England BioLabs, Ipswich, MA), 0.2 mmol/L of dNTPs, 2 mmol/L of MgSO4, 0.5 μmol/L of each specific primer, and 0.625 U of Taq DNA polymerase (New England BioLabs). Cycle number was chosen such that amplification of the products was linear with respect to the amount of input RNA. Each cycle consisted of 30 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, and 60 seconds at 72°C for extension. The PCR products were analyzed on a 1.5% agarose gel in Tris-acetate-EDTA buffer and products visualized with ethidium bromide. The sequences of specific primers for IL-1α, TNFα, and macrophage inflammatory protein 1α were previously described (22). The density of bands was measured using ImageJ version 1.29.

**Statistical Analysis**

All values are expressed as mean ± SEM. Statistical analysis was performed using Student’s t-analysis or 1-way analysis of variance, followed by post hoc Bonferroni-Dunn correction. All analyses were performed with the StatView 5.0 software program (SAS Institutes, Inc., Cary, NC).

**RESULTS**

**Intrastriatal Injection of Ad.PEDF Protects Neurons Against Excitotoxicity**

Adenovirus vectors were used to direct expression of human PEDF within the adult rat striatum 7 days before QA injection (Fig. 1A). To estimate the efficacy of recombinant adenoviruses, in vivo expression of PEDF was analyzed by immunohistochemistry 7 days after Ad.PEDF injection into the right striatum. There was intense PEDF immunoreactivity only in the right hemisphere of rats injected with Ad.PEDF (Fig. 1B, left panel). Most of the immunoreactivity was seen in cells with glia-like morphology (Fig. 1B, right panel). The 50-kDa human PEDF protein was detected in striatal lysates from rats injected with Ad.PEDF (1 × 10⁸ pfu) by quantitative Western blot analysis, whereas striatal lysates from rats injected with Ad.LacZ (1 × 10⁸ pfu) showed no detectable signal (Fig. 1C). The PEDF protein level in the rat striatum injected with Ad.PEDF was 255.2 ± 97.1 ng/mg of protein (Fig. 1C).

The effect of PEDF gene transfer on neuronal loss induced by QA injection was evaluated by immunostaining with anti-NeuN antibody, which recognizes most neuronal cell types in the CNS (23). At 7 days after QA injection, NeuN-positive neurons were markedly decreased in the striatum treated with Ad.LacZ (Fig. 2A, middle panel), whereas loss of neurons was suppressed in Ad.PEDF-treated rats (Fig. 2A, right panel). The number of the NeuN-positive neurons was calculated for each rat and expressed as a percentage of the intact side. Only 24.5 ± 5.4% of NeuN-positive neurons was preserved in Ad.LacZ-injected rats, whereas 74.2 ± 9.7% was detected in Ad.PEDF-injected rats (Fig. 2D). There was also marked loss of DARPP-32 (a marker of the striatal γ amino butyric acid [GABA]-ergic projection neurons occupying most of the striatum [24]) 7 days after QA injection of the Ad.LacZ group (Fig. 2B, middle panel); the DARPP-32-immunoreactive area of Ad.LacZ-treated striatum was 37.5 ± 4.9% (Fig. 2E). Loss of DARPP-32 immunoreactivity was significantly attenuated in Ad.PEDF-injected rats (Fig. 2B, right panel; Fig. 2E, 93.7 ± 9.8%; p < 0.005 vs. Ad.LacZ). The injection of QA into striatum also caused a profound depletion of ChAT-positive cholinergic neurons in Ad.LacZ-injected rats (Fig. 2C, middle panel; Fig. 2F, 9.1 ± 1.9%). This reduction was attenuated after striatal injection of Ad.PEDF before QA treatment (Fig. 2C, right panel; Fig. 2F, 58.0 ± 7.2%, p < 0.001 vs. Ad.LacZ). A similar neuroprotective effect was observed in rats treated with Ad.PEDF 1 day after QA injection (data not shown).

[Image: Figure 3](http://jnen.oxfordjournals.org/)

**FIGURE 3.** Effect of Ad.PEDF pretreatment on apomorphine (APO)-induced rotational behavior after quinolinic acid (QA) lesion. Histogram shows the average changes in rotational asymmetry induced by APO stimulation (1 mg/kg, i.p.) 8 days after QA lesion. Positive values correspond to turns ipsilateral to the lesion. There was a significant reduction of the APO-induced rotational behavior in Ad.PEDF-injected rats compared with PBS- or Ad.LacZ-injected rats. Values are expressed as mean ± SEM. *p < 0.05 versus PBS-injected group; **p < 0.05 versus Ad.LacZ-injected group.
Intrastriatal Injection of Ad.PEDF Ameliorates APO-Induced Rotational Behavior

To determine whether the reduction of neuronal loss corresponded to a functional improvement, we evaluated the rotational behavior induced by the dopamine agonist APO. The striatal degeneration produced by the unilateral administration of QA resulted in an APO-induced rotational asymmetry (25). Adenoviral vector containing the \( E. \ coli \) \( \beta \)-galactosidase gene injected rats displayed ipsilateral turning in response to the intraperitoneal injection of APO 8 days after QA administration (Fig. 3). In contrast, APO-induced rotational behavior was not observed in the Ad.PEDF-treated rats (Fig. 3). Similar results were observed in rats treated with Ad.PEDF 12 or 15 days after QA injection (data not shown).

Intrastriatal Injection of Ad.PEDF Attenuates Glial Reaction After QA Treatment

Glial reaction to neuron injury is a prominent feature in the course of excitotoxic lesion in the CNS (26, 27). There was a significant upregulation of GFAP-positive reactive astrocytes occupying most of the lesioned area in the Ad.LacZ-treated striatum 7 days after QA lesion (Fig. 4A, middle panel). In contrast, this reaction was attenuated by Ad.PEDF treatment (Fig. 4A, right panel). The number and intensity of ionized calcium binding adaptor molecule 1–positive microglial cells were increased in Ad.LacZ-treated striatum 7 days after QA injection (Fig. 4B, middle panel); this activation of microglia was suppressed in Ad.PEDF-pretreated striatum (Fig. 4B, right panel). Similar results were observed in rats treated with Ad.PEDF 1 day after QA injection (data not shown).

Because excessive production of inflammation-related genes by activated glial cells is involved in promoting and propagating neuronal injury in many neurodegenerative disorders, we investigated whether inflammation-related gene expression after QA injection is related to the in vivo neuroprotective effect of PEDF. Reverse-transcriptase–PCR showed that expression of the inflammation-related genes IL-1\( \beta \), TNF\( \alpha \), and MIP1\( \alpha \) mRNA was significantly decreased in Ad.PEDF-treated striatum compared with that of Ad.LacZ-treated brain (Fig. 5).
Effect of Mutant PEDF on QA-Induced Excitotoxicity

To identify the functional epitopes of the PEDF amino acid sequence with regard to its neuroprotective effect, 3 deletion mutants of PEDF were prepared and expressed using adenoviral vectors (Fig. 6A). PEDF$^{78-121}$ lacks amino acid residues 78–121, and PEDF$^{44-121}$ lacks amino acid residues 44–121. The expression level of adenovirally transduced mutated PEDF genes (PEDF$^{44-121}$ or PEDF$^{78-121}$) was similar to that of full-length PEDF (Fig. 6B). Animals were injected with vectors containing the mutated genes 7 days before injection of QA, and the neuroprotective effects were evaluated by NeuN immunostaining and APO-induced rotational behavior. Ad.PEDF$^{78-121}$ protected NeuN-positive striatal neurons from QA-induced excitotoxicity (Fig. 6C; p < 0.0001 vs. Ad.LacZ-treated group), whereas Ad.PEDF$^{44-121}$ failed to protect neurons (Fig. 6C). In agreement with the result of NeuN staining, ipsilateral turning in response to injection of APO was observed in Ad.LacZ- and Ad.PEDF$^{44-121}$-treated rats, whereas there was a significant reduction of the rotational behavior in the AdLacZ- and Ad.PEDF$^{78-121}$-treated rats (Fig. 6D).

DISCUSSION

Intrastriatal injection of QA induces excitotoxicity both by elevating glutamate outflow and directly stimulating NMDA receptors (6, 28). Quinolinic acid striatal lesioning, a widely used model of HD, causes a dramatic loss of striatal GABA-ergic projection neurons and a relative sparing of cholinergic interneurons (8, 29). In the present study, pretreatment of striatum with Ad.PEDF rescued DARPP-32- and ChAT-positive cells from QA injection (Fig. 1), suggesting that the induced expression of PEDF in striatum protects GABA-ergic projection neurons and cholinergic interneurons against excitotoxicity.

The intracellular signaling pathways by which PEDF protects neurons have mainly been explored in vitro. We previously reported that the induction of prosurvival genes via the activation of NF-κB and CRE pathway is required for PEDF protection against glutamate-induced necrosis or low K+-induced apoptosis of cultured cerebellar granular cells (30, 31). More recently, Pang et al (32) demonstrated that PEDF protects cultured retinal ganglion cells from glutamate- and trophic factor withdrawal–mediated neurotoxicity via the activation of the NF-κB and ERK1/2 pathway. Indeed, the induction of manganese-superoxide dismutase, which is transcriptionally controlled by NF-κB (33), CREB (34), and ERK1/2 (35), was observed in both in vitro and in vivo (16, 30, 31). Further studies will be needed to clarify whether these pathways are associated with in vivo neuroprotective effect of PEDF.

In addition to neuronal loss, glial reactions have been implicated in the pathophysiology of excitotoxic brain injury (36, 37). Activated glial cells may be detrimental for neuronal survival by producing neurotoxic mediators such as proinflammatory cytokines and oxidative free radicals (26, 27). Inflammation and oxidative stress leading to secondary excitotoxicity have also been implicated in QA-induced neurotoxicity (38–40). We demonstrated here that intrastriatal injection of Ad.PEDF before QA treatment potently attenuated...
the morphologic changes of glial cells (Fig. 4) and the expression of inflammation-related genes (Fig. 5), suggesting that the ability to regulate glial function may account for part of the neuroprotective effect of PEDF against excitotoxic brain injury.

PEDF exhibits a variety of biologic activities, including neuroprotective, anti-angiogenic, anti-tumorigenic, and anti-oxidative effects. Although the mechanisms by which PEDF initiates these multifunctional activities remain largely unclear, distinct PEDF receptors may be involved in divergent signals that generate different biologic effects. Notari et al. (41) identified 80-kDa PEDF-R (TTS-2.2/ independent phospholipase A2 zeta and mouse desnutrin/ ATGL), a lipase-linked membrane protein with high affinity for PEDF in the retina. PEDF binding stimulates the enzymatic phospholipase A2 activity of PEDF-R, suggesting that PEDF/PEDF-R interaction could generate a cellular signal. It is still not known, however, whether PEDF-R is a

FIGURE 6. Effect of mutated pigment epithelial-derived factor (PEDF) on quinolinic acid (QA)–induced excitotoxicity. (A) Schematic diagram of the predicted functional domains on the PEDF sequence and mutated PEDF used in this study. The 34-mer peptide (residues 44–77) mainly confers anti-angiogenic activity; the 44-mer peptide (residues 78–121) predominantly exhibits neurotrophic activity. (B) Representative immunoblot for full-length and mutated PEDF. The expression level of adeno- virally transduced mutated PEDF genes (PEDFΔ44–121 or PEDFΔ78–121) was similar to that of full-length PEDF (n = 4). (C) Effect of mutated PEDF on QA-induced neuronal loss. One week after injection of Ad.LacZ, Ad.PEDF, Ad.PEDFΔ44–121, or Ad.PEDFΔ78–121 into the right striatum, QA was injected in the ipsilateral striatum. There was a significant protective effect observed in Ad.PEDF- and Ad.PEDFΔ78–122–treated rats (n = 7) Values are expressed as mean ± SEM. *p < 0.0005 versus Ad.LacZ; **p < 0.0001 versus Ad.LacZ; #p < 0.01 versus Ad.PEDF. (D) Effect of PEDF on apomorphine (APO)-induced rotational behavior. Histogram shows the average changes in rotational asymmetry induced by APO stimulation 7 days after QA injection. Positive values correspond to turns ipsilateral to the lesion. There was a significant reduction of APO-induced rotational behavior in Ad.PEDF- or Ad.PEDFΔ78–121–injected rats compared with Ad.LacZ-injected rats; Ad.PEDFΔ44–121 had no effect (n = 7 for Ad.LacZ, Ad.PEDF, and Ad.PEDFΔ78–121; n = 5 for Ad.PEDFΔ44–121) Values are expressed as mean ± SEM. *p < 0.05 versus Ad.LacZ; **p < 0.05 versus Ad.LacZ, #p < 0.05 vs. Ad.PEDF.
specific receptor involved in the neuroprotective effect of PEDF.

Although the neurotrophic domains of PEDF have not yet been fully defined, a 44-amino acid fragment of PEDF corresponding to positions 78–121 has been shown to have neurotrophic activity on Y-79 retinoblastoma cells and spinal cord motor neurons (42, 43). This fragment also bound to or competed with PEDF for binding to Y79 cells, retinal cells, cerebellar granule cells, and motor neurons (42–44), whereas a 34-amino acid fragment of PEDF, corresponding to amino acid positions 44–77, has anti-angiogenic effects (45, 46). In contrast to our expectations, PEDFΔ78-121 was as effective as full-length PEDF in protecting the striatum from QA-induced excitotoxicity, whereas PEDFΔ44-121 showed reduced neuroprotective effects (Fig. 6C). Similar results were obtained when assessing the efficacy for functional improvement (Fig. 6D). Our results suggest that amino acids 44–77 of the human PEDF sequence are required for the in vivo neuroprotective effect against QA-induced excitotoxicity. Although there is currently no explanation for the discrepancy between the previously reported observations obtained with PEDF-derived peptide fragments (34-mer and 44-mer) and the adenoviral vector-expressed mutated PEDF protein used in this study, the functional role of these regions (amino acid residues 44–77 and 78–121) may vary according to the cell types or experimental paradigms. Further studies are needed to address the issue.

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is essential to physiologic events such as wound healing, whereas it plays a key role in pathologic events in diseases such as cancer and diabetes (47). Although suppression of excess angiogenesis causes a reduction of microvessel density in the eye and in cancer cells that translates into a therapeutic effect, the inhibition of new vessel formation may interfere with vascular remodeling and repair in excitotoxically injured brain. Therefore, if the pharmacologic effect of PEDF could be modulated by removing the functional region of anti-angiogenic activity, it would provide an attractive new option for the treatment of neurodegenerative disease and stroke. Additionally, identification of the functional domains with regard to neuroprotective and/or anti-angiogenic effects may contribute to the development of smaller peptides that might cross the BBB. Further studies will be required for evaluating these possibilities.

In conclusion, this study demonstrates that induced striatal expression of PEDF that is achieved by adenoviral vector-mediated gene delivery is effective in protecting striatal GABA-ergic and cholinergic neurons from QA-induced excitotoxicity; this suggests that PEDF could directly protect striatal neurons against glutamate toxicity in vivo. In addition, we also showed that PEDF attenuates the functional deficit, glial activation, and expression of inflammation-related genes in QA-treated animals. Further studies are required to clarify the precise mechanisms by which PEDF mediates beneficial effects against QA-induced excitotoxicity, but our results suggest that gene transfer of PEDF may be helpful in designing new therapeutic strategies for excitotoxicity-related diseases, including HD, brain ischemia, and AD.

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