Treatment of Atherosclerosis by Transplantation of Bone Endothelial Progenitor Cells Over-Expressed Paraoxonase-1 Gene by Recombinant Adeno-Associated Virus in Rat

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Endothelial dysfunction/loss is a key event in the development of vascular diseases, including native atherosclerosis (AS). Recent studies have shown that endothelial progenitor cells (EPCs) have the ability to repair endothelial cells that have been lost or damaged following AS. As a result, the therapy of transplanting EPCs is a promising option for the treatment of AS. However, the therapeutic effect on AS with only EPCs transplantation has not been satisfactory. The upregulation of those genes, which prevent the progress of AS in EPCs, is a novel therapeutic strategy for AS. Because it can reduce macrophage foam cell formation and protect endothelial cells from the oxidation of low-density lipoprotein (ox-LDL), paraoxonase-1 (PON1) gene is a candidate for gene therapy in AS. In this study, a recombinant adeno-associated virus (rAAV) vector carrying the human paraoxonase-1 (hPON1) gene (rAAV-PON1) was constructed, and endothelial progenitor cells (EPCs) transfected with rAAV-PON1 were transplanted into the atherosclerosis model of Sprague-Dawley rats (SD rats). The results of doppler ultrasound and histological analysis showed that the group transplanted with the hPON1 gene-transfected EPCs was superior to the group transplanted only with the EPCs and was also better than the group with hPON1 gene injection alone. The results indicated that rAAV-mediated hPON1 gene-transfected EPCs is a potentially valuable new tool in the treatment of atherosclerosis.

Key words atherosclerosis; paraoxonase-1; endothelial progenitor cell; adeno-associated virus; gene therapy

Atherosclerosis (AS), a kind of general arterial disease, is a leading disease throughout the world in terms of high morbidity and mortality. Currently, available therapies for AS patients are inadequate and the effects of drug and surgery are unsatisfactory. Therefore, the development of novel therapies is needed. The cell-based gene therapy strategy holds promise for the treatment of AS.

Previous studies have demonstrated that endothelial dysfunction is an early hallmark of atherosclerotic disease. Classical risk factors for atherosclerosis concur to determine endothelial damage. Cell-based therapy for the injured endothelium has been proposed as a novel strategy for preventing the atherosclerosis formation. Recently it has been demonstrated that progenitor cells derived from bone marrow are capable of homing to the injured endothelium. Upon arrival at the injured endothelial layer, endothelial progenitor cells (EPCs) may differentiate into endothelial cells, proliferate and replace the injured endothelial cells. Published data from animal studies have revealed that EPCs effectively contribute to restoring endothelial function and diminishing neointimal formation after arterial injury. Moreover, EPCs could be locally delivered to prevent atherosclerosis formation in the carotid artery with an injured endothelium. Unfortunately, in many clinical trials, these beneficial effects are not observed in patients with coronary artery disease, due at least in part to the risk factors for AS that impair EPC function. Although risk factors for AS hinder the application of EPCs in transplants for the treatment of cardiovascular disease, we hypothesized that genetic modification of EPCs with paraoxonase-1 (PON1) could counter the effects of these risk factors and enhance the biological function of EPCs.

The direct role of PON1 in reducing oxidative stress was demonstrated in studies using the PON1 knockout mouse model and the human PON1 transgenic mouse model. Studies showed that the oxidation of low-density lipoprotein (LDL) in the artery wall is a major cause of the initiation and progression of atherosclerosis. High-density lipoprotein (HDL) can protect against the development of atherosclerosis and attenuate the oxidation of LDL. Data from in vitro experiments suggest that PON1 is a protective factor inhibiting LDL oxidation and HDL oxidation. In addition, transgenic mice overexpressing PON1 exhibited significantly less aortic lesions relative to wild-type controls. PON1 was shown to possess the ability to reduce cholesterol and oxidized lipid influx, inhibit macrophage cholesterol synthesis, stimulate macrophage cholesterol efflux, and reduce macrophage foam cell formation. Moreover Exposure of cultured EPC to oxidized LDL induces a dose-dependent impairment of their functional activity, accelerates the rate of EPC senescence, possibly by telomerase inactivation, and can be associated with up to a 70% reduction in EPC numbers. The role of PON1 in reducing oxidative stress protected EPC from oxidation of low-density lipoprotein (ox-LDL). These studies suggest that the PON1 gene is a candidate for gene therapy in AS.

Adeno-associated virus (AAV) has been one of the most promising viral vectors for gene therapy. Aside from the potential to persistently express the transgene following integration, AAV as a nonpathogenic virus is able to efficiently transduce slowly dividing or nondividing cells without severe immune responses. The human clinical trials using AAV have demonstrated safety. AAV was emerged as a classical vector for gene replacement therapies and has been used for treating lipid disorder in the past decade. Some studies demonstrated that long-term, nearly complete correction
of a lipid disorder is feasible using AAV gene therapy. In the present study, we investigated whether combination therapy with AAV2 encoding human PON1 and bone marrow derived EPCs could have better therapeutic effect than administration either of them alone.

MATERIALS AND METHODS

Plasmid Construction and Preparation of Recombinant Adeno-Associated Virus (rAAV) The total RNA from the human fetal liver was kept stored in −80 °C. Single-stranded cDNA was synthesized from a 10-μg human fetal RNA. Human PON1 was amplified from the cDNA by polymerase chain reaction (PCR) with a sense primer 5′-CCG AAT TC CAATGGCGAAGCTGATTGCGCT-3′ (containing the EcoRI site) and an antisense primer 5′-CGC TCG AG GAG-CTCACTA GT AAAG AGCTTTG-3′ (containing the XhoI site). The PCR was carried out with 2 units of LA Taq polymerase (TaKaRa Biotechnology Co., Ltd., China) in a final volume of 50 μl using the following conditions: 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 45 s at 52 °C and 1 min at 72 °C; and a final extension of 10 min at 72 °C. The PCR product was isolated by gel extraction. The gel extraction product was then digested with EcoRI and XhoI (TaKaRa Biotechnology) and then recloned into the EcoRI and XhoI sites of pAAV-IREs-green fluorescent protein (GFP) (Stratagene). The PON1 gene was cloned into the multiple cloning sites (MCS) of the expression plasmid pAAV-IREs-GFP containing the internal ribosome entry site (IRES) sequence followed by GFP. All of the regions were under the control of a CMV promoter. pAAV-PON1-IREs-GFP (pAAV-PON1) was analyzed by restriction enzyme EcoRI and XhoI digestion and sequenced at the Shanghai Invitrogen Biological Technology Co., Ltd. for testing the correctness of its sequence and transgene direction.

Large quantities of AAV-2 vectors were prepared by using the AAV Helper-Free System (Stratagene). One hundred 100-cm² plates of HEK293 cells grown at 80% confluence and transgene direction.

EPCs Culture and Characterization Normal male healthy Sprague-Dawley (SD) rats (200—300 g) were served as a marrow source. The rats were sacrificed by cervical dislocation. Bone marrow mononuclear cells (BMMNCs) were isolated by flushing rat femur bones and via density gradient centrifugation with Histopaque-1083. The isolated cells by density-gradient centrifugation and plated on fibronectin-coated (Sigma) culture dishes and maintained in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with 10% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF, R&D) and basic fibroblast growth factor (bFGF, R&D). After culture at 37 °C with 5% CO₂ for 4 d, non-adherent cells were removed by washing with phosphate buffered saline (PBS). The medium was replaced, and the culture was maintained through day seven. Cellular morphology study and fluorescein isothiocyanate-labeled vWF, Flk-1 (eBioscience) and Dil-acLDL (Molecular Probes) staining were used to observe the endothelial phenotype of EPCs.

Matrigel basement membrane matrix (BD Biosciences) was used for assessment of the potential of EPCs forming tube-like structures. 1×10⁴ cells/well (96-well) were seeded were reseeded on solidified Matrigel in EBM-2 containing 10% FBS. After additional 12 h of incubation at 37 °C, capillary morphogenesis was examined under an inverted microscope.

EPCs Gene Transfer and PON1 Expression After reaching 80% confluence, EPCs were transduced with either rAAV-GFP or the rAAV-PON1 at 1.0×10⁸—5.0×10⁸ v.g./cell in EGM-2 without FBS for 2 h at 37 °C. Following infection, the medium was replaced with fresh EGM-2 with 10% FBS, and the cells were placed in a six-well culture plate. After 72 h, the expression of the transgene was detected under an inverted fluorescent microscope.

The expression of the human PON1 gene was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). At 48 h after gene transfer, the total RNA was extracted from the transduced EPCs using Trizol reagent (Invitrogen) according to the manufacturer’s directions. The cDNA was reverse transcribed using AMV reverse transcriptase (RT) (TaKaRa Biotechnology ). The PCR amplifications were performed using specific primers for human PON1. The primer sequences were as follows: forward primer 5′-CCGAAATTC-CAATGGCGAAGCTGATTGCGCT-3′, reverse primer 5′-CGTCGAGGAGTTCACAGTAAAGAGCCTG-3′, β-actin primer: forward 5′-CGTTGACATCTCGGAGGA-3′, reverse, 5′-AGCCACAGATCCACACAG-3′.

The Establishment of an AS Rat Model Forty male healthy SD rats (200—300 g) were randomly divided into five groups (Table 1), with 8 rats per group. For three months, the control group (A) was fed with basic food, while the other groups were loaded with a high-fat diet. The levels of plasma total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) were determined with Biochemical Analyser (Unicel Dxc800, Beckman). The pathological changes of rat aorta were evaluated via ultrasonography after three month high fat diet. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhongshan Medical University and conformed to the Guide for the Care and Use of Laboratory Animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tr>
<td>A Control</td>
<td>PBS</td>
</tr>
<tr>
<td>B Untreated</td>
<td>PBS</td>
</tr>
<tr>
<td>C PON1/EPC</td>
<td>EPCs transduced with AAV-PON1</td>
</tr>
<tr>
<td>D AAV-PON1</td>
<td>AAV-PON1</td>
</tr>
<tr>
<td>E GFP/EPC</td>
<td>EPCs transduced with AAV-GFP</td>
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Animals published by the National Institutes of Health, China.

After three month of dietary treatment, the rats were injected via the tail vein with either vehicle, rAAV-PON1 vectors (1 × 10^5 v.g./1 ml PBS), EPCs transduced with rAAV-PON1 (transduction after 72 h, 2 × 10^6 cells/ml PBS), or EPCs transduced with rAAV-GFP (2 × 10^6 cells/ml PBS) (as shown in Table 1). After transplantation, all animals were fed with basic food and survived the experiments.

**Detection of Doppler Ultrasound**
Two months after the transplantation, the condition of atherosclerosis in the aorta was evaluated with Doppler ultrasound. The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate. Three separate measurements were done for each rat, and the results were averaged.

**Tissue Collection and Preparation**
Animals were sacrificed by the intravenous injection of potassium chloride at two months after cell transplantation. The abdominal aorta and liver were harvested and divided into two parts. One part of these tissues was fixed overnight in 4% paraformaldehyde and embedded in paraffin for hematoxylin and eosin (H&E) staining and immunohistochemistry staining. The other part was snap-frozen in liquid nitrogen and stored at −80°C for RT-PCR, cryosectioning and western blot analysis.

**Histological Analysis**
Cross sections of the artery were cut to a thickness of 5 μm and stained according to the standard protocols for H&E staining. The specimens were examined using an AXIOVET-40 inverted microscope (Zeiss, Germany). The degree of AS was evaluated by immunohistochemical staining of the aortas according to the manufacturer’s protocol. The primary antibodies used were as follows: rabbit anti-rat endothelial nitric oxide synthase (eNOS) antibody, rabbit anti-rat ICAM-1 antibody, rabbit anti-rat apolipoprotein E (apoE) antibody, and rabbit anti-rat extracellular signal-regulated kinase (ERK) antibody (Boster, cat: BA0364, cat: BA0541, cat: BA0647, cat: BA1657, respectively). An ABC kit and a DAB kit were used for immunohistochemical staining (Boster, cat: SA1022, cat: AR1022, respectively). Digital images of 20 fields (×100) from 5 sections were randomly selected from each animal for image analyses (Image-Pro Plus Version 4.0). The values of the positive areas were analyzed. To examine the expression of the green fluorescent protein (GFP), the tissues stored at −80°C were embedded in optimal cutting temperature (OCT) compound at −20°C. Sections (10 μm) were cut on a cryostat and placed on glass slides. GFP expression was observed using an inverted fluorescent microscope.

**mRNA Expression by RT-PCR**
To detect the mRNA expression levels changes of these genes after transplation, eNOS, ICAM-1 and apoE expression was primarily assessed by means of RT-PCR. The methods of extracting total RNA and reverse transcription were same as the above. PCR amplification of these sequences from harvested cDNAs used these primers: eNOS primer 1 (forward) 5′-CACCGATA-CAACATACTTGGAG-3′ and primer 2 (reverse) 5′-CAAGCCATACAGGATAGTCG-3′. ICAM-1 primer 3 (forward) 5′-TATCGGGATGGAAGTCTC-3′ and primer 4 (reverse) 5′-GGCCGTAATAGGTGTTAAGT-3′. apoE primer 5 (forward) 5′-GAGGGGATATAAAGCCTACACAA-3′ and primer 6 (reverse) 5′-GCTCAACGGATAGCACTCA-3′. Rat β-actin primer 7 (forward) 5′-CGTTGACATCCG-TAAAGA-3′ and primer 8 (reverse) 5′-AGCCACCAATC-CAACAGCAG-3′.

**Western Blotting**
A standard Western blotting protocol was used to detect PON1 protein expression in aortas. The same amount of protein extracted from aortas was separated on SDS-PAGE under reducing and denaturing conditions and transferred to Hybond nitrocellulose membranes. The membranes were incubated in a 1:400 dilution of polyclonal rabbit anti-PON1 antibody (Santa-Cruz), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2000, Santa-Cruz). β-Actin (1:400, Santa-Cruz) was used as the loading control. The signal was detected using the ECL Plus kit (GE Healthcare Life Science, Piscataway). Western blotting was performed on a sample of all animals in each group and at least twice for each animal.

**Statistical Analysis**
Results are expressed as mean ± S.D. Comparisons between groups were analyzed by a one-way ANOVA. p values <0.05 were considered statistically significant. All analyses were performed with SPSS 13.0 software.

**RESULTS**

**Identification of the Constructed pAAV-PON1 (pAAV-PON1-IRES-GFP)**
The rAAV encoding hPON1 was constructed. The human PON1 cDNA fragment was cloned into the multiple cloning site of pAAV-PON1-GFP. The PON1 PCR products were 1068 bp on a 1.5% agarose gel. The 1068 bp fragment encoding human PON1 (1—355 amino acids) was collected by gel extraction. The gel extraction product was digested with EcoRI and Xhol and was cloned into the sites between EcoRI and Xhol in pAAV-IRES-GFP. The successfully constructed plasmid pAAV-PON1 could be cut into two fragments, 6.1 kb and 1068 bp, by EcoRI and Xhol. Moreover, the result of the sequence was confirmed to be correct (data not shown).

**Characterization of EPCs**
BMMNCs were cultured for 7 d resulted in a cobble-stone, EC-like morphology (Figs. 1A, B). After 2 weeks in culture, almost all of the cells had the ability to take up Dil-ac-LDL, and more than 90% of the cells expressed Flk-1 and vWF (Figs. 1D—F), which are generally accepted as the important features of EPCs. The cells were passaged several times without discernible alterations in morphology and growth characteristics. In addition, endothelial cell colonies were formed when the cells were plated at a low density (Fig. 1C). The cultured EPCs formed capillary-like structures on Matrigel (Fig. 1G).

**Transduction of EPCs Using rAAV-PON1 in Vitro**
We performed the transduction of EPCs using AAV-PON1 in vitro. EPCs were transduced with either rAAV-GFP or the rAAV-PON1 at 1.0 × 10⁴—5.0 × 10⁵ v.g./cell. After 72 h, the expression of the transgene was detected under inverted fluorescence microscope. The expression of GFP was correlated with viral doses (Figs. 2A—C). The transduction efficiency reached plateau at 1.0 × 10⁵ v.g./cell, which was used for the rest experiments of transducing EPCs. To confirm the expression of the human PON1 gene, RT-PCR using human PON1-specific primers was performed 48 h after the gene transfer. The rat β-actin was detected by RT-PCR as an internal control. As shown in Fig. 2E, the expression of the human PON1 gene was observed in AAV-PON1-transduced EPCs but not
in non-transduced EPCs.

**Establishment of the AS Rat Model** After 3 months of high fat diet feeding, the rats developed atherosclerotic plaques on aorta. The accumulation of a small amount of foam cells and smooth muscle cells under the injured endothelium could be observed under microscope. The plasma levels of TC, TG and LDL-C levels of high-fat diet rats were significantly higher than these of normal-diet rats at three months after giving the high-fat diet (Table 2).

**Detection by Doppler Ultrasound** Two months after the transplantation, the condition of atherosclerosis in the aorta was observed with Doppler ultrasound. In the control group, the aortic tunica intima of rats was slicking, and there was no thickening or plaque on the vessel wall (Fig. 3A). The ultrasound changes of atherosclerosis were found in the aorta of rats in the untreated group (Fig. 3B), including increased intima-media thickness and a rough and discontinuous intima. The small hyperechoic spot showed the existence of plaque (Fig. 3B). There was no obvious plaque in the aortic tunica intima of rats in every treated group, but the intima was thickened in the aorta of rats in the GFP/EPC group (Fig. 3E).

**Histological Analysis** We evaluated the possible positive effectiveness of the gene transfer on the development of the atherosclerotic plaques and assessed the entire length of the aorta, taking representative sections at intervals. The morphometric analysis showed a reduction of atherosclerosis at the artery in the PON1/EPC, rAAV-PON1 and GFP/EPC groups compared to the untreated group. In particular, in the PON1/EPC group, we found that the aortic arch was free of plaques (Fig. 4A-3). All untreated rats (untreated group) showed aortic atherosclerotic lesions (Fig. 4A-2). Although the PON1-treated rats (rAAV-PON1) displayed a number of plaques in their aortas similar to those found in untreated rats, an obvious reduction of pathological changes in the rAAV-PON1 group (Fig. 4A-4) was seen. Compared with the pathological changes in the treated group, those in the GFP/EPC group were more obvious than those in the treated group (Fig. 4A-5).

To determine whether the transplanted EPCs were localized to the atherosclerotic lesion, EGFP-expressing endothelial cells were observed in cryosections of the arteries using direct fluorescence microscopy. Two months after the transplantation, EGFP-expressing endothelial cells were detectable on the endothelium of the aorta in the PON1/EPC and GFP/EPC groups but undetectable in other groups (Figs. 4A-8, 10).

To investigate the effects of the treatments on the gene expression in the artery of experimental atherosclerotic rats, histological sections were performed with immunohistochemical staining to detect the expression of eNOS, ICAM-1, apoE, and ERK. As shown in Fig. 4B, the cells staining brown were positive for rat eNOS, ICAM-1, apoE and ERK. The values were expressed as the percentage of cell-positive areas in the lesions by immunohistochemical staining (Figs. 4A-8, 10).

**Figure 1. Characterization of EPCs Derived from Rat Bone Marrow in Vitro**
(A) Monolayer of EPCs with a cobblestone morphology after 7–10 d in culture; (B) Wright’s Giemsa staining of EPCs; (C) the formation of EPCs colonies; (D) positive staining for vWF; (E) Flk-1 immunostaining; and (F) Dil-acLDL uptake. These cells formed capillary-like on Matrigel (G). Scale bar: 200 µm (for A, C, G); 100 µm (for D, E); and 50 µm (for B, F).

**Figure 2. EPCs Transfected with Various Titers of rAAV after 72 h**
(A, B, C) rAAV-GFP; (D) rAAV-PON1. The titers: (A) 1.0×10⁴; (B) 1.0×10⁵; (C) 5.0×10⁵; and (D) 1.0×10⁶. Scale bar: 100 µm (for A—D). (E) The expression of human PON1 mRNA by RT-PCR in AAV-PON1-transduced EPCs. The size of the PCR products for human PON1 and β-actin were 1068 and 173 bp, respectively. Lane 1: 1 kb DNA marker; lane 2: EPCs transduced with AAV-PON1; lane 3: non-transduced EPCs; and lane 4: 100 bp marker.

**Table 2. The Level of Serum Liquid of High-Fat Diet Rat in the Third Month**

<table>
<thead>
<tr>
<th>Group</th>
<th>TC</th>
<th>TG</th>
<th>LDL-C</th>
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<tbody>
<tr>
<td>A</td>
<td>3.59±0.90</td>
<td>1.23±0.40</td>
<td>0.75±0.15</td>
</tr>
<tr>
<td>B</td>
<td>22.55±4.16*</td>
<td>2.16±0.65*</td>
<td>29.67±6.74**</td>
</tr>
<tr>
<td>C</td>
<td>23.05±2.85*</td>
<td>2.06±0.77*</td>
<td>27.91±5.63**</td>
</tr>
<tr>
<td>D</td>
<td>23.88±0.27*</td>
<td>1.9±0.61*</td>
<td>32.23±2.81**</td>
</tr>
<tr>
<td>E</td>
<td>22.45±2.50*</td>
<td>1.8±0.48*</td>
<td>27.7±5.08**</td>
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</table>

*p<0.05, **p<0.01 vs. control group. A: normal diet. B, C, D and E: high-fat diet.
4C—F). To detect the function of endothelial cells, tissue sections were stained with an antibody against eNOS, and the mean values of the positive percentage in the PON1/EPC group were significantly higher than those of other treated groups (8.69±1.53%, 15.08±1.31%, 11.43±1.64% and 12.00±0.97% for untreated groups, PON1/EPC, AAV-PON1, and GFP/EPC, respectively; p<0.05), and there was no obvious difference between the control group and the untreated group (Fig. 5C). The expression of ICAM-1 was observed. A great deal of brown positive stained material was found in the intimas and the medias in the untreated group, and the positive percentage was 17.17±2.09%, which was the highest value of all the groups (p<0.05). In all treated groups, the positive percentage of ICAM-1 was lowest in the PON1/EPC group (p<0.05, Fig. 4D). The expression of apoE and ERK was the same as the expression of ICAM-1 (Figs. 4E, F).

**Analysis of mRNA Expression in Aorta** The expression of eNOS, ICAM-1 and apoE gene at the mRNA level was evaluated by RT-PCR two months after transplantation (Figs. 5A—C). The values were expressed with correspondence optical density (OD) value. The results showed that the expression of mRNA level was different in each group (Fig. 6). The correspondence OD value of eNOS mRNA in untreated group was significantly lower than that of other groups (9.60±1.71, 0.65±0.27, 6.19±0.84, 3.37±1.45, and 4.35±0.69 for groups control, untreated, PON1/EPC, AAV-PON1,GFP/EPC; p<0.01), indicating eNOS low expression in AS. In treated groups, the expression of eNOS mRNA was increased, especially in PON1/EPC group (Fig. 6A). Correspondence OD value of ICAM-1 and apoE mRNA were highest in all groups (p<0.05), indicating ICAM-1 and apoE excess expression in AS, but they were significantly lower than that of treated groups, as shown in Figs. 6B and C.

**Transgene Expression in the Aorta** Western blot analysis of homogenates from aortic tissues of animals sacrificed two months after transplantation demonstrated that the hPON1 protein was expressed in the PON1/EPC and rAAV-PON1 groups but not in the other groups (Fig. 7), confirming the success of targeted gene expression.

**DISCUSSION**

Atherosclerosis is the primary cause of coronary artery disease and is rapidly becoming the leading cause of death and disability in the entire world. Endothelial dysfunction is an early hallmark of atherosclerotic disease. Several studies have shown that EPCs contribute to reendothelialization of the injured vessels and have potentially protective roles in endothelial dysfunction and in the early stages of atherosclerosis formation.27,28) A study reported by Werner et al. showed that repair of endothelial cell damage is modulated not only by adjacent vessel structures but also by bone marrow-derived EPCs, which might help to prevent advanced vascular lesion formation.29) EPCs can be localized in the bone marrow and peripheral blood or can reside in the arterial wall. These cells show a cobblestone morphology and exhibit a high capacity for proliferation. In addition, they can be passaged several times without discernible alterations in cell morphology or growth characteristics. In our study, EPCs were cultured from rat bone marrow. The cultured cells showed a cobblestone morphology and displayed a high proliferative potential when we observed the growth curve and formation of the colonies. Moreover, the cultured EPCs formed capillary-like structures on Matrigel. EPC phenotype was further confirmed by the ability of these cells to express endothelial cell markers (vWF, Flk-1) and to take up ac-LDL.

EPCs can repair the injured endothelium, but they cannot decrease the plaque, which limits their therapeutic usefulness in AS. Cell-based gene therapy is a rapidly growing area of research. This approach offers several advantages over viral gene delivery systems and cells alone, which are currently being used to treat human diseases. Cells can be cultured in vitro and genetically modified to secrete high levels of a therapeutic protein, such as hPON1, that can inhibit atherosclerotic plaque formation. Studies have shown that rPON1 retards the oxidative modification of LDL.30) Moreover, hPON1 prevents atherosclerosis development largely by reducing oxidative stress in the circulation and by preventing macrophage foam cell formation.31) In our experiment, the hPON1 gene was cloned into the multiple cloning sites (MCS) of the expression plasmid pAAV-IRE-GFP. The recombinant AAV vector rAAV-PON1 was constructed, and the expression of green fluorescence was observed in HEK293 cells. Moreover, we successfully transduced the PON1 gene into EPCs derived from the bone marrow of rat and demonstrated that hPON1 expression from transduced EPCs was assured with RT-PCR.

Two months after transplantation, the results of ultrasound and histological assessment showed that the PON1/EPC group had a higher therapeutic effect than the administration of either of them alone in the AAV-PON1, GFP/EPC or non-treatment groups. To investigate the effects of the treatments on the gene expression in the artery of experimental atherosclerotic rats, immunohistochemical staining to detect the expression of eNOS, ICAM-1, apoE, ERK. In addition, the expression of eNOS, ICAM-1 and apoE gene at the mRNA level was evaluated by RT-PCR. The first stage of atherosclerosis is considered to be endothelial dysfunction accompanied with changes in some genes, including eNOS, ICAM-1, and PON1, and GFP/EPC, respectively; AA V -PON1,GFP/EPC;
apoE and ERK gene. Endothelial cells are able to produce both vasoconstrictive and vasodilating substances. One of the most important regulatory and vasoactive substances produced by endothelial cells is nitric oxide (NO). NO is produced in the endothelium by endothelial NO synthase (eNOS). The development of atherosclerosis is, however, accompanied by an early deficit of NO and a decrease in the expression of endothelial NO synthase. Adhesion mole-

Fig. 4. Histological Assessment Two Months after the Transplantation

A (1—5): H&E staining from the rat artery of each group. Atherosclerotic lesions are shown in the untreated group (A-2). A (6—10): Fluorescence photomicrograph of rat carotid artery frozen sections. EGFP-expressing endothelial cells are shown on the endothelium of the aorta in the PON1/EPC and GFP/EPC groups (8, 10). Scale bar: 50 μm (for A1—5). (B) Representative images of rat aortic histological sections stained for eNOS, ICAM-1, apoE, and ERK. The endothelium stained with brown was positive. Scale bar: 50 μm (for B). (C) eNOS positive percentage. (D) ICAM-1 positive percentage. (E) ApoE positive percentage. (F) ERK positive percentage. **p<0.01 vs. group B, *p<0.05 vs. group B, #p<0.05 vs. group A, $p<0.05 group vs. C.
cules, such as ICAM-1, play a significant role in the process of atherosclerosis because they cooperate with chemokines and mediate the increased adhesion of mononuclear and neutrophil leukocytes. While ICAM-1 is constitutively expressed on resting endothelial cells, it is up-regulated by proatherogenic factors. Human apolipoprotein E (apoE) is a 34-kDa polymorphic plasma protein that plays a key role in clearing remnant lipoproteins and in delivering the intracellular cholesterol from peripheral tissues to the liver. ApoE is not only synthesized by the liver but also in the brain and by resident macrophages in the atherosclerotic wall, where it exerts atheroprotective actions independent of its role in lipid metabolism. Therefore, the expression of apoE can be detected in aortic atherosclerotic lesions. Ox-LDLs, proinflammatory cytokines and shear stress can all induce the expression of lectin-like oxidized LDL receptor-1 (LOX-1). LOX-1 also contributes to cell proliferation and migration through extracellular signal-regulated kinase (ERK) 1/2 activation.

Fig. 5. The Effects on the Aortic Gene Expression Two Months after the Transplantation
(A) The expression of eNOS mRNA by RT-PCR. The size of the PCR products for eNOS and β-actin was 496, and 173 bp, respectively. (B) The expression of ICAM-1 mRNA by RT-PCR. The size of the PCR products for ICAM-1 and β-actin was 325, and 173 bp, respectively. (C) The expression of apoE mRNA by RT-PCR. The size of the PCR products for apoE and β-actin was 335, and 173 bp, respectively. Lane 1: 100 bp marker; Lane 2: control group; Lane 3: untreated group; Lane 4: PON1/EPC group; Lane 5: rAAV-PON1 group; Lane 6: GFP/EPC group.

Fig. 6. The mRNA Expression Level
(A) The correspondence OD ratio of eNOS/β-actin. (B) The correspondence OD ratio of ICAM-1/β-actin. (C) The correspondence OD ratio of apoE/β-actin. * p<0.05 vs. group B, # p<0.05 vs. group A, $ p<0.05$ group C.

Fig. 7. Western Blot Analysis of PON1 Expression Two Months after the Transplantation
The artery samples were homogenized and processed for Western blotting using an anti-PON1 antibody. This was performed using β-actin as an internal standard, suggesting that exogenous PON1 was strongly expressed in the PON1/EPC group. A: control group; B: untreated group; C: PON1/EPC group; D: rAAV-PON1 group; and E: GFP/EPC group.

In conclusion, this study demonstrated that AAV-2 vectors could efficiently and successfully transfer therapeutic gene into EPCs for gene therapy. In addition, the transplantation group with the hPON1 gene-transfected EPCs was superior to the single transplantation group with EPCs on vascular endothelial repair but was also much better than hPON1 gene injection alone. The results indicated that the AAV-mediated
hPON1 gene-transfected EPCs are a potentially valuable new tool to be used for the treatment of AS.

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