

Local adiponectin treatment reduces atherosclerotic plaque size in rabbits

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Abstract

In this study, we investigated the *in vivo* role of adiponectin, an adipocytokine, on the development of atherosclerosis in rabbits mainly using adenovirus expressing adiponectin gene (Ad-APN) and intravascular ultrasonography. Serum adiponectin concentrations in rabbits after Ad-APN local transfer to abdominal aortas increased about nine times as much as those before transfer ($P < 0.01$), about ten times as much as the levels of endogenous adiponectin in adenovirus expressing β -galactosidase gene (Ad- β gal) treated rabbits ($P < 0.01$), and about four times as much as those in the aorta of non-injured rabbits on a normal cholesterol diet ($P < 0.01$). Ultrasonography revealed a significantly reduced atherosclerotic plaque area in abdominal aortas of rabbits infected through intima with Ad-APN, by 35.2% compared with the area before treatment ($P < 0.01$), and by 35.8% compared with that in Ad- β gal-treated rabbits

($P < 0.01$). In rabbits infected through adventitia, Ad-APN treatment reduced plaque area by 28.9% as compared with the area before treatment ($P < 0.01$) and 25.6% compared with that in Ad- β gal-treated rabbits ($P < 0.01$). Adiponectin significantly suppressed the mRNA expression of vascular cell adhesion molecule-1 (VCAM-1) by 18.5% through intima transfer ($P < 0.05$) and 26.9% through adventitia transfer ($P < 0.01$), and intercellular adhesion molecule-1 (ICAM-1) by 40.7% through intima transfer ($P < 0.01$), and 30.7% through adventitia transfer ($P < 0.01$). However, adiponectin had no effect on the expression of types I and III collagen. These results suggest that local adiponectin treatment suppresses the development of atherosclerosis *in vivo* in part by attenuating the expression of VCAM-1 and ICAM-1 in vascular walls.

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Introduction

Secreted by adipocytes, adiponectin is a collagen-like protein with significant roles in regulating the metabolism of glucose and fatty acids. Low plasma adiponectin levels are associated with insulin resistance and risk of type 2 diabetes (Spranger *et al.* 2003). Adiponectin reduces the size of atherosclerotic lesions and inhibits neointimal thickening and proliferation of vascular smooth muscle cells in injured arteries (Matsuda *et al.* 2002) and suppresses the expression of VCAMs (Ouchi *et al.* 1999). Thus, adiponectin plays significant roles in preventing atherosclerosis.

The vascular adventitia is defined as the outermost connective tissue of vessels. Recently, the adventitia was increasingly considered a highly active segment of vascular tissue that contributes to a variety of disease pathologies, including atherosclerosis and restenosis (Shi *et al.* 1996, 1997, Houtkamp *et al.* 2001, Sartore *et al.* 2001). However, no data exist concerning the role of adiponectin in atherosclerosis development through the adventitia pathway. Since blood vessels are surrounded by much adipose tissue, could

adiponectin play a protective role against atherosclerosis through paracrine pathways through adventitia? With these considerations in mind, the present study was designed to investigate the effects and mechanism of adiponectin on the development of atherosclerosis in New Zealand rabbits by local treatment using transfer of adenovirus expressing the full-length adiponectin gene to abdominal aortas and intravascular ultrasonography.

Materials and Methods

Animals and diet

Three-month-old male New Zealand rabbits weighing ≈ 1.5 – 2.0 kg and, standard and high-cholesterol diets were purchased from the Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Science (Shandong, China). The high-cholesterol diet was 1% cholesterol added to the standard diet. Each rabbit was fed 150 g/day, providing a daily intake of 1.5 g cholesterol.

The rabbits were allowed free access to water and were housed one rabbit per cage in a temperature-controlled environment. The Experimental Animal Management and Use Committee at Shandong University approved all animal use protocols.

Extraction of RNA

To clone the gene fragments, cervical back fat (brown fat) was obtained from 3-month-old male rabbits freely consuming a standard diet. The rabbits were given i.v. injections of anesthetic (3% pentobarbital sodium) at 30 mg/kg body weight. The s.c. adipose tissue was removed from the dorsal depot located over the cervical spine, rinsed with saline, then stored at -80°C until RNA extraction. Total RNA was extracted from adipocytes or blood vessels with Trizol reagent (Invitrogen) following the manufacturer's instruction. Briefly, 100 mg adipose tissue or 1 cm abdominal aorta was cut and homogenized, and then 1 ml Trizol was added. The samples were then extracted with trichloromethane and precipitated with isopropyl alcohol. After abluion with 75% alcohol, the total RNA was dissolved in 20 μl DEPC- H_2O (diethylpyrocarbonate treated water) and quantified by spectrophotometry (Eppendorf BioPhotometer, Hamburg, Germany) at 260 nm/280 nm and stored at -70°C .

Cloning of the rabbit adiponectin gene fragments

For cloning the adiponectin gene using RT-PCR, total RNA was denatured by heating at 75°C for 5 min, then chilled on ice. Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), oligo (dT), dNTP, and H_2O were added for a total volume of 20 μl . The reaction was incubated at 37°C for 60 min, and then heated to 95°C for 5 min, thus inactivating the reverse transcriptase and denaturing the template cDNA complexes.

Degenerative primers were designed from highly conserved regions of mouse and human adiponectin sequences and used to amplify adiponectin cDNA (forward primer, 5'-ACACCTCCAGGGCTCAGGATGCT-3'; reverse primer, 5'-TCAGTTGGTATCATGG TAGAGAAG-3'). The transcribed DNA was amplified by PCR for 35 cycles with DNA polymerase (Promega) as follows: denaturation at 94°C for 30 s (3 min in the first cycle), annealing at 55°C for 30 s, and extension at 72°C for 2 min (5 min in the last cycle). The PCR product was separated and purified with the EZNA Gel Extraction Kit (OMEGA Bio-tek Inc., Doraville, GA, USA) according to the manufacturer's protocol, and then sequenced (Invitrogen). The PCR product was cloned into a pGEM-T Vector System I (Promega) and named pGEM-T-APN.

Recombinant adenovirus

Primers were designed to clone the adiponectin gene fragment from pGEM-T-APN by the use of the KOD-Plus DNA Polymerase kit (TOYOBO Co. Ltd, Osaka, Japan;

forward primer, 5'-CGGAATTCACACCTCCAGGGCTCAGGATGCT-3'; reverse primer, 5'-ACGCGTCGACTCAGTTGGTATCATGGTAGAGAAG-3'). A 771-bp fragment was extracted with use of QIAquick Gel Extraction kit (OMEGA Bio-tek Inc.) and digested with restriction enzymes (EcoRI and Sall) (New England Biolabs, Beverly, MA, USA). The product was ligated with PSU-CMV: a shuttle plasmid (pSU) containing cytomegalovirus (CMV) promoter vehicle (Sangon Biological Engineering & Technology and Service Co. Ltd, Shanghai, China) and transformed into *Escherichia coli* competent cells (Sangon). Plasmid DNA extracted from positive clones was cut with restriction enzymes, co-transferred with plasmid Pbhge3, including the right arm of type 5 adenovirus, to 293 cells (Microbix Biosystems Inc., Toronto, Ontario, Canada) and then purified by CsCl gradient centrifugation. The final product, named adiponectin-producing adenovirus (Ad-APN), was a tumor-specific non-proliferation adenovirus expressing adiponectin gene.

Protein expression in *Pichia pastoris*

Expression and purification of the protein were carried out with a pGAPZ α *Pichia* Expression Kit (Invitrogen) following the manufacturer's instruction. Briefly, cDNA containing the coding region of mature rabbit adiponectin was amplified by PCR (sense primer: 5'-GAATTCATGCTGTTGCTGCAAGCTGT-3'; antisense primer: 5'-GCGGCCGCGTTGGTATCATGGTAGAG-3') (containing EcoRI and NotI sites), then inserted into the pGEM-T vector by T/A cloning strategy, leading to a subcloning vector pGEM/APN (containing adiponectin gene). By sequencing, the pGEM/APN was confirmed to contain adiponectin gene, which was then inserted between the EcoRI and NotI sites of pGAPZ α (containing an amino-terminal His-tag) leading to pGAPZ α -APN. The pGAPZ α -APN was transformed into *E. coli* DH5 α . *E. coli* DH5 α strains were obtained from our laboratory and the restrictive enzymes were purchased from New England Biolabs.

After the above transformation, plate transformation was mixed onto low salt Luria-Bertain (LB) plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with 25 $\mu\text{g}/\text{ml}$ Zeocin (Invitrogen) and Zeocin-resistant colonies were selected. Zeocin-resistant transformants were inoculated into 2 ml low salt LB medium with 25 $\mu\text{g}/\text{ml}$ Zeocin, grown overnight at 37°C with shaking. The pGAPZ α -APN, linearized by cleaving with Bln I (New England Biolabs), was transformed into the GS115 yeast (obtained from our laboratory) by electroporation (Bio-Rad Laboratories). The transformants were plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing Zeocin (100 $\mu\text{g}/\text{ml}$) for positive selection. The recombinant yeasts were cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose) and grown in flasks at 30°C with shaking (250–300 r.p.m.) overnight. The supernatant was transferred to a separate tube and stored at -80°C until ready to assay. The culture media used in this study were purchased from Oxoid Ltd, Basingstoke, Hants, UK.

Western blot analyses

Sample proteins were separated by SDS-PAGE on 14% polyacrylamide gels and then transferred to nitrocellulose membrane. Following blocking with 5% non-fat milk, the blots were incubated with anti-His tag mouse monoclonal antibody (1:1000 dilution, Appligen Technologies Inc., Beijing, China) and rabbit adiponectin polyclonal antibody (1:100 dilution, Boster, Wuhan, Hubei, China) at 4 °C overnight. After overnight incubation, the blots were incubated with secondary antibody conjugated to horseradish peroxidase (1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then visualized with enhanced chemiluminescence.

Construction of atherosclerotic rabbit models

We created the atherosclerotic model as described (Chen *et al.* 2004). Briefly, a balloon catheter was inserted into the abdominal aorta of rabbits ($n=70$) through the right femoral artery, and the aorta was injured by engorged balloons. Surviving rabbits were randomly divided into the following four groups: 1) Ad-APN transfer through intima ($n=18$), 2) adenovirus expressing β -galactosidase gene (Ad- β gal) transfer through intima ($n=12$), 3) Ad-APN transfer through adventitia ($n=18$) and 4) Ad- β gal transfer through adventitia ($n=12$), and received high-cholesterol diet for 3 months.

Intravascular ultrasonography procedure

Rabbits were anesthetized intravenously with 3% pentobarbital sodium at 30 mg/kg body weight. The left femoral artery was dissected and punctured. A detecting head for intravascular ultrasonography (Galaxy, Boston Scientific SciMed, Natick, MA, USA) was set at the distal end of the thoracic aorta and dragged back slowly along a guide wire (0.5 mm/s). Abdominal images were recorded.

Transfer of adenovirus

After ultrasonography, transfer of Ad-APN or Ad- β gal (Sangon) (6×10^9 pfu/ml, 0.5 ml for each rabbit) to abdominal aortic intima was as follows. The abdominal cavity was opened and the abdominal aorta dissociated. A locus from the renal artery gap extending downward 3 cm was chosen to transfer the adenovirus. First, blood supply was temporarily blocked from the two ends of the transfer position, and then Ad-APN or Ad- β gal was injected into the lumen of the vessel at the transfer position. Blood flow was recovered after 20 min.

The method for adventitia transfer was as follows. The abdominal aorta was isolated from the surrounding tissue, and then a polyethylene tube (3 cm long; inner diameter, 6 mm; outer diameter, 8 mm) was cut longitudinally to open the tube which was placed around the artery. The procedures for cuff placement were as described (Hisrosimi *et al.* 1987,

Yamasaki *et al.* 2003) with some modification. Ad-APN or Ad- β gal (6×10^9 pfu/ml, 0.5 ml for each rabbit) was injected into a space between the cuff and the artery and the two ends of the cuff were stuffed with gelatin sponge. Cuff and gelatin sponge were removed after 20 min.

Collection of samples and plasma data analyses

On day 14 after gene transfer, surviving rabbits ($n=50$) were anesthetized and underwent repeat ultrasonography, then abdominal aortas were harvested. Blood samples were collected and the level of total cholesterol, high-density lipoprotein cholesterol, triglyceride, and low-density lipoprotein cholesterol were determined by use of an automatic biochemistry analyzer (Hitachi).

ELISA assay

Adiponectin levels in rabbit serum and in the supernatant of cultured *Pichia pastoris* were quantitatively determined through immunoassays. A kit consisting of rat anti-human adiponectin enzyme (BPB Biomedicals, Inc., Franklin, CA, USA) was applied and the measurements were performed following the manufacturer's instructions.

Histochemical analyses

Frozen cross-sections (8 μ m thick) of abdominal aortas embedded in optimal cutting temperature compound after overnight fixation in 10% formalin were mounted on slides. For the analyses of plaque size, the three sections (100 μ m apart) from each rabbit on the 14th day after adenovirus transfer were stained with Oil Red O (ORO). The lesion size was quantified with the use of Image-Pro Plus 5.0 software (MediaCybernetics, Silver Spring, MD, USA) and the mean values were determined. For immunohistochemical analyses, paraffin-embedded cross-sections (5 μ m thick) were incubated with either rabbit adiponectin polyclonal antibody (Boster), mouse vascular adhesion molecule-1 (VCAM-1) monoclonal antibody (Santa Cruz), mouse intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody (Santa Cruz), mouse collagen I (Abcam, Cambridge, MA, USA), or mouse collagen III (Calbiochem, Darmstadt, Germany) monoclonal antibody.

Real-time PCR analyses

VCAM-1, ICAM-1, and type I and III collagen cDNA were cloned with total RNA of abdominal aortas by RT-PCR. The relative quantitation of target gene expression was determined by use of LightCycler (Roche) following the manufacturer's protocol. To quantify the expression of VCAM-1 and ICAM-1, real-time PCR involving the use of the SYBR Green kit (TaKaRa, Dalian, Liaoning, China) was carried out according to the manufacturer's instructions. For quantification of type I and III collagen level, TaqMan

hydrolysis probes were utilized (sense and antisense primers are shown in Table 1). The conditions for real-time PCR were as follows: denaturation at 95 °C for 30 s, then the 40 cycles at 95 °C 0 s, annealing for 10 s, and extension at 72 °C for 10 s. The annealing temperatures for the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), VCAM-1, ICAM-1, type I and III collagen were 60, 60, 58, 58, and 58 °C respectively. The expression ratio of target gene to GAPDH represents the mRNA level. Data were analyzed with the use of LightCycler 4.0 (Roche).

Statistical analyses

Data were analyzed using an ANOVA procedure. A value of $P < 0.05$ was considered statistically significant. Data are presented as means \pm S.E.M. SPSS for Windows Version 13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Cloning of rabbit adiponectin

To investigate whether rabbit adiponectin gene might have the same function as that in humans and mice, we used RT-PCR and adipocytes RNA to clone rabbit adiponectin with translation start code (atg) and stop code (tga) (GenBank DQ334867). The open reading frame for rabbit adiponectin is 735 bp long, and when translated, yields a protein of 244 amino acids. Rabbit adiponectin is highly homologous to that of humans (86.4%; GenBank NM004797) and mice (81.5%; GenBank NM009605), with 85.7 and 85.3% identity at the amino acid level respectively.

Serum adiponectin and expression of adiponectin in vitro

Serum samples were collected from rabbits before aorta injury, before and after adenovirus transfer, and subjected to

ELEISA assay. Serum adiponectin concentrations in rabbits after Ad-APN intima and adventitia transfer increased about nine times as much as those before transfer (intima: 9.17 ± 0.61 vs 1.02 ± 0.06 $\mu\text{g/ml}$, $P < 0.01$; adventitia: 9.27 ± 0.23 vs 1.02 ± 0.06 $\mu\text{g/ml}$, $P < 0.01$). After transfer, the serum adiponectin concentrations elevated to a level about ten times higher than the level of endogenous adiponectin in Ad- βgal -treated rabbits (intima: 9.17 ± 0.61 vs 0.92 ± 0.05 $\mu\text{g/ml}$, $P < 0.01$; adventitia: 9.27 ± 0.23 vs 0.90 ± 0.05 $\mu\text{g/ml}$, $P < 0.01$), and about four times higher than those in the aorta of non-injured rabbits on a normal cholesterol diet (2.45 ± 0.17 $\mu\text{g/ml}$, $P < 0.01$) (Fig. 1).

As shown in Fig. 2, western blotting with the anti-His tag monoclonal antibody or adiponectin polyclonal antibody indicated a single predominant band at approximately 42 kDa (containing 9.3 kDa α -factor signal peptide and 2.5 kDa C-terminal His-tag). Adiponectin level in the supernatant of cultured *Pichia pastoris* was 0.29 ± 0.02 $\mu\text{g/ml}$.

Lipid characteristics of adenovirus-infected rabbits

No significant difference was observed between cholesterol and triglyceride levels in Ad-APN- and Ad- βgal -infected rabbits (Table 2).

Atherosclerosis was reduced by regional transfer of Ad-APN

Ultrasonography revealed a significantly reduced atherosclerotic plaque area, in abdominal aortas of rabbits infected through intima with Ad-APN, by 35.2% compared with the area before treatment (3.98 ± 0.32 vs 2.58 ± 0.12 mm^2 , $P < 0.01$), and by 35.8% compared with that in Ad- βgal -treated rabbits (4.02 ± 0.31 vs 2.58 ± 0.12 mm^2 , $P < 0.01$) (Fig. 3). The lumen area stenosis (LAS) was also reduced, by 32.7% (35.07 ± 2.43 vs $23.60 \pm 1.97\%$, $P < 0.01$) and 24.1% (31.09 ± 2.03 vs $23.60 \pm 1.97\%$, $P < 0.01$) respectively. In rabbits infected through adventitia, Ad-APN treatment

Table 1 Primers for real-time PCR used in this study

	GenBank locus	Primer sequence	PCR product (bp)
GAPDH	AB231852	S 5'-GAACGGGAAACTCACTGGCAT-3'	110
		A 5'-CCTTCTTGATGTCGCATACCTTAGC -3'	
		P 5'-CTCCAGGCGGCAGGTCAGGTCCAC-3'	
VCAM-1	AY212510	S 5'-AGTCCCTCGTCCATCGTG-3'	122
		A 5'-GAAAGAGGCTGTAGGTCC-3'	
ICAM-1	AB128157	S 5'-TGCTCCGCCCTTCCACCAG -3'	136
		A 5'-TGGCACCACGCAGTCCTC -3'	
Collagen I	D49399	S 5'-AGACCCTCCTCACTCCTGAAG-3'	163
		A 5'-TCGCCAGTAGAGAAATCACAGTAA-3'	
		P 5'-CGGAAGAACCAGCTCGCACCTGC-3'	
Collagen III	S83371	S 5'-CATTGGCCCTGTTTGCTTT-3'	116
		A 5'-GTTGGTCACTTGACTGGT-3'	
		P 5'-CCAACCTTCTCTGAAGCCCCA-3'	

GAPDH, glyceraldehyde phosphate dehydrogenase; VCAM-1, vascular adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; S, sense primer; A, antisense primer; and P, probe.

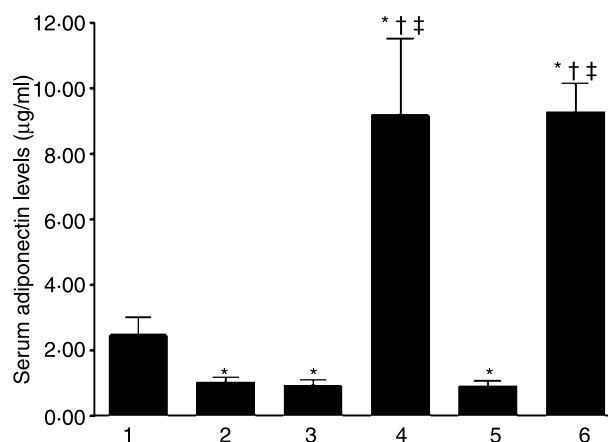


Figure 1 Serum adiponectin concentrations in rabbits. 1, Aorta non-injured rabbits on normal cholesterol diet; 2, aorta injured rabbits on high-cholesterol diet before adenovirus transfer; 3, after transfer Ad- β gal through intima; 4, after transfer Ad-APN through intima; 5, after transfer Ad- β gal through adventitia; 6, After transfer Ad-APN through adventitia. The symbol * indicates significant difference ($P < 0.01$) vs serum adiponectin concentrations in aorta non-injured rabbits on normal cholesterol diet; † compared with rabbits before adenovirus transfer; and ‡ compared with rabbits after Ad- β gal transfer. The values are means \pm S.E.M.

reduced plaque area by 28.9% as compared with the area before treatment (4.08 ± 0.41 vs 2.90 ± 0.23 mm², $P < 0.01$) and 25.6% compared with that in Ad- β gal-treated rabbits (3.90 ± 0.16 vs 2.90 ± 0.23 mm², $P < 0.01$) (Fig. 3). Likewise, the LAS was reduced, by 23.6% (34.19 ± 2.12 vs 26.13 ± 1.88 %, $P < 0.01$) and 19.4% (32.43 ± 1.54 vs 26.13 ± 1.88 %, $P < 0.05$) respectively.

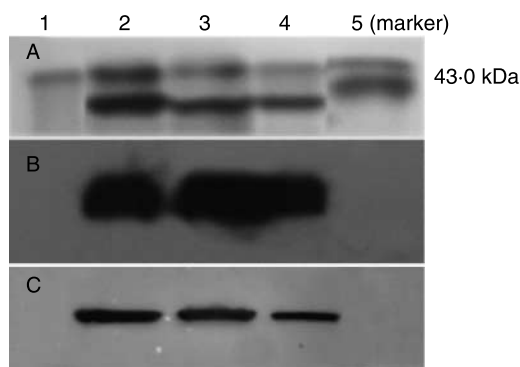


Figure 2 SDS-PAGE (A) and western blot analysis of the supernatant of cultured *Pichia pastoris* (B, using anti-His tag monoclonal antibody; C, using adiponectin polyclonal antibody). Line 1 is a blank pGAPZ α *Pichia* vector (not containing adiponectin gene) sample control and lines 2–4 are samples from the supernatant of cultured *Pichia pastoris* containing the recombinant rabbit adiponectin gene. Line 5 contained the molecular weight markers (not visible in image of blot). Both anti-His antibody and adiponectin polyclonal antibody detected a single predominant protein at ≈ 42 kDa (containing 9.3 kDa α -factor signal peptide and 2.5 kDa C-terminal His-tag).

In rabbits with Ad-APN infection through intima, the atherosclerotic plaque area as seen on ORO staining (Fig. 4) was reduced significantly by 35.8% (2.57 ± 0.30 vs 1.65 ± 0.15 mm², $P < 0.01$) and the maximal thickness of plaque was reduced by 26.7% (0.75 ± 0.04 vs 0.55 ± 0.03 mm, $P < 0.01$) as compared with those in Ad- β gal-treated rabbits. In rabbits infected through adventitia, the plaque area and maximal thickness of plaque were reduced by a similar significant level.

Adiponectin inhibits mRNA expression of VCAM-1 and ICAM-1

Immunohistochemical analyses revealed that adenovirus-derived rabbit adiponectin abundantly adhered to cells in the atherosclerotic plaque of rabbits through intima transfer. In rabbits treated through adventitia, adiponectin adhered to cells among adventitia (Fig. 5A).

When compared with Ad- β gal treatment, infection with Ad-APN through intima showed significantly suppressed mRNA expression of VCAM-1 (by 18.5%, 20.70 ± 1.29 vs 16.87 ± 0.88 %, $P < 0.05$) and expression of ICAM-1 (by 40.7%, 2.70 ± 0.68 vs 1.60 ± 0.19 %, $P < 0.01$). Rabbits infected through adventitia showed significantly suppressed mRNA expression of VCAM-1 (by 26.9%, 21.70 ± 0.79 vs 15.87 ± 0.58 %, $P < 0.01$) and expression of ICAM-1 (by 30.7%, 2.70 ± 0.21 vs 1.87 ± 0.19 %, $P < 0.01$) compared with Ad- β gal-treated rabbits. However, rabbits infected through intima or adventitia showed no significant difference in atherosclerotic plaque area, LAS, plaque maximal thickness or mRNA level of VCAM-1 and ICAM-1 in the abdominal aorta (Fig. 5B).

As well, Ad-APN- and Ad- β gal-treated rabbits did not differ in expression of type I and III collagen, regardless of infection through intima or adventitia.

Discussion

Traditionally, adipose has been considered a simple energy storage tissue, but mounting evidence suggests that it can produce and secrete many bioactive substances, collectively referred to as adipocytokines. One of these, adiponectin, has significant roles in regulating the metabolism of glucose and fatty acids, and in protecting against atherosclerosis. Adiponectin is a collagen-like protein, whose gene is located on human chromosome 3q27 and is named as *apM 1* gene. The full-length of *apM 1* gene is 17 kbp, which includes 3 exons and 2 introns. The full-length of *apM 1* mRNA is 4517 bp (Scherer *et al.* 1995, Hu *et al.* 1996, Maeda *et al.* 1996).

In the present study, we report for the first time the sequence of rabbit adiponectin and provide the amino acid sequence of the deduced protein. Sequence analyses showed that both the coding region and the amino acid sequence share high homology with adiponectin of humans and mice. The anti-His tag monoclonal antibody and adiponectin polyclonal antibody detected a single predominant protein

Table 2 Triglyceride and cholesterol levels (mmol/l) of rabbits infected with adenovirus in abdominal aorta

	TG	TC	LDL-C	HDL-C
Infection through intima				
Ad-βgal (n=10)	1.11 ± 0.22	19.54 ± 1.14	16.86 ± 1.21	2.18 ± 0.27
Ad-APN (n=15)	1.12 ± 0.14	23.72 ± 2.47	20.86 ± 2.40	2.35 ± 0.22
Infection through adventitia				
Ad-βgal (n=10)	1.43 ± 0.22	24.68 ± 2.54	21.49 ± 2.30	2.54 ± 0.45
Ad-APN (n=15)	1.12 ± 0.16	23.92 ± 1.67	21.07 ± 1.57	2.32 ± 0.28

Fifty rabbits survived the experiment. Values are mean ± s.e.m. TG, triglyceride level; TC, total cholesterol level; LDL-C, low density lipoprotein cholesterol level; HDL-C, high density lipoprotein cholesterol level; Ad-βgal, adenovirus expressing β-galactosidase gene; Ad-APN, adenovirus expressing the rabbit adiponectin gene.

of ≈ 30 kDa (subtracting 9.3 kDa α-factor signal peptide and 2.5 kDa C-terminal His-tag) in the supernatant of cultured *Pichia pastoris*. The adiponectin protein is similar in size to the single protein detected in human serum by the monoclonal antibody (Yoda-Murakami *et al.* 2001). The results suggest a similar function of adiponectin protein in rabbits as in other species in improving insulin resistance (Kubota *et al.* 2002, Maeda *et al.* 2002), stimulating fatty-acid oxidation (Yamauchi *et al.* 2002), and preventing vascular stenosis (Matsuda *et al.* 2002).

Okamoto *et al.* (2002) reported that injection of Ad-APN into the tail vein of apolipoprotein E-deficient mice inhibited lesion formation by 30%. In another study, low plasma

adiponectin levels were found to be associated with progression of subclinical coronary atherosclerosis in people with type 1 diabetes and in non-diabetes subjects independently of other cardiovascular risk factors (Maahs *et al.* 2005).

For further investigation of the protective role of adiponectin in atherosclerosis, we constructed a replication-defective adenovirus, expressing the rabbit adiponectin gene (Ad-APN) and transferred the adenovirus to abdominal aortas of an atherosclerotic rabbit model. The serum adiponectin concentrations in rabbits after 3 months high-cholesterol diet significantly decreased about two times compared with those in aorta non-injured rabbits on a normal cholesterol diet. After adenovirus transfer, the serum adiponectin concentrations of

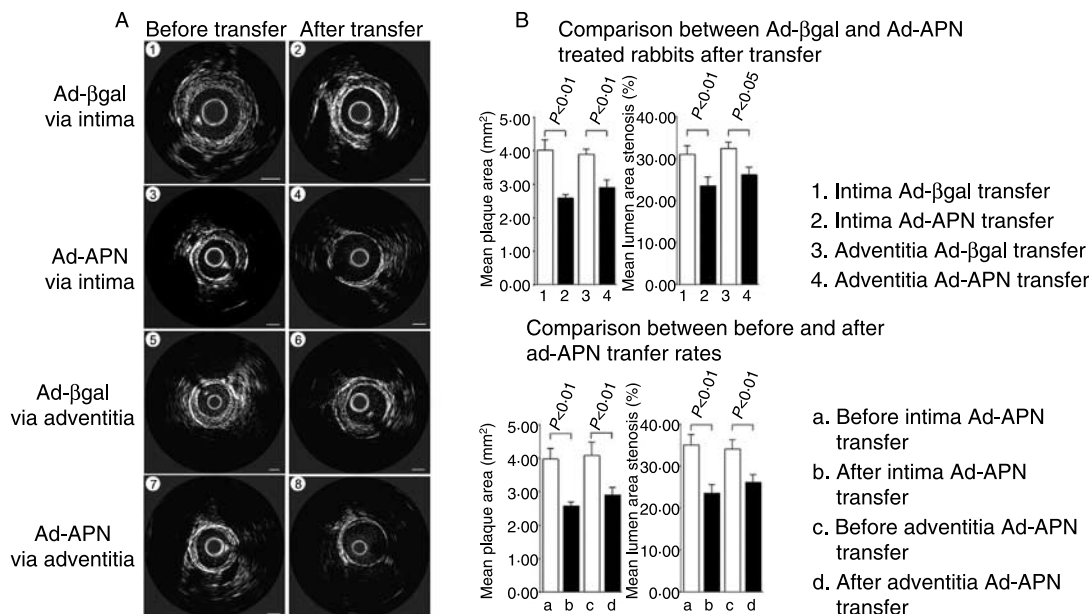


Figure 3 Intravascular ultrasonography results of Ad-APN treatment on atherosclerotic plaque in rabbits 14 days after adenovirus transfer. (A), Representative images before and after transfer of Ad-βgal or Ad-APN. 1, Before transfer of Ad-βgal through intima. 2, After transfer of Ad-βgal through intima. 3, Before transfer of Ad-APN through intima. 4, After transfer of Ad-APN through intima. 5, Before transfer of Ad-βgal through adventitia. 6, After transfer of Ad-βgal through adventitia. 7, Before transfer of Ad-APN through adventitia. 8, After transfer of Ad-APN through adventitia. Scale bars = 1 mm. (B), Quantification of atherosclerotic plaque areas in rabbits treated with Ad-βgal or Ad-APN. The average plaque area of each rabbit was determined from 10 cross-sections. The values are means ± s.e.m.

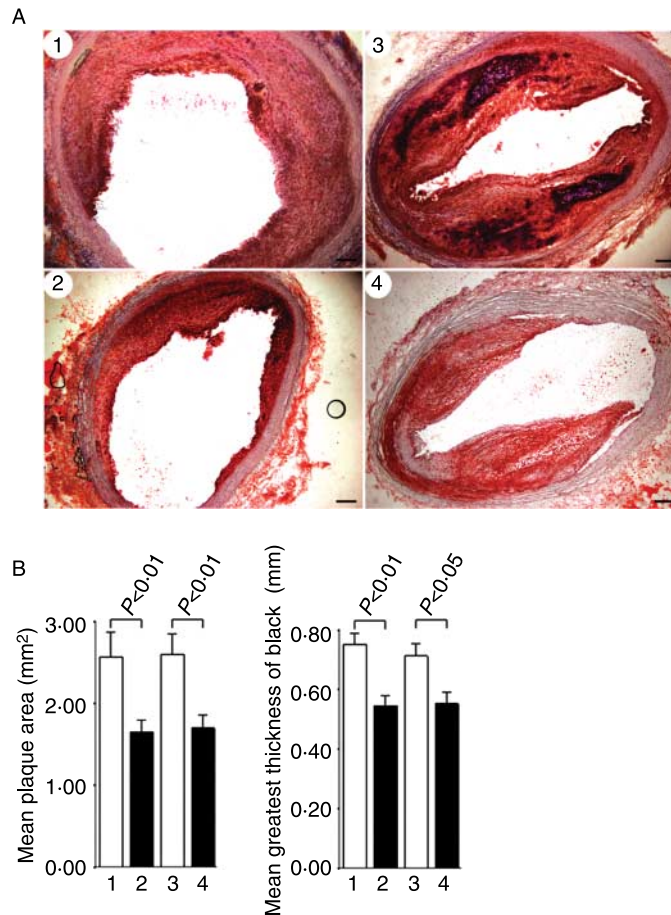


Figure 4 Oil Red O (ORO) staining results of Ad-APN treatment on atherosclerotic plaque in rabbits 14 days after adenovirus transfer. A, Representative ORO images. 1, Ad-βgal transfer through intima. 2, Ad-APN transfer through intima. 3, Ad-βgal transfer through adventitia. 4, Ad-APN transfer through adventitia. Scale bars = 200 μm. B, Quantification of atherosclerotic plaque area in rabbits treated with Ad-βgal or Ad-APN. The average plaque area of each rabbit was determined from 3 cross-sections. The values are mean ± S.E.M.

Ad-APN-treated rabbits notably increased and the atherosclerotic area was significantly reduced as seen on ORO staining and on ultrasonography. Thus, adiponectin plays a significantly protective role against atherosclerosis in rabbits.

The connective tissue adventitia in the aorta protects, supports, and nourishes the blood vessels. However, it has been found to secrete many vasoactive substances in a paracrine way to regulate the relaxation and construction of blood vessels and to influence the structure of blood vessels. Nitrogen oxide (NO) is an important vasoactive substance that could be synthesized by adventitial myofibroblast cells (Kleschyov *et al.* 2000). Zhang *et al.* (1999) reported that adventitia was a source of inducible nitric oxide synthase (NOS) in the rat aorta. The mRNA expression and protein production of iNOS of adventitia increased following

cytokine stimulation, thus increasing NO production. As reported in other studies, NO inhibits the proliferation of vascular smooth muscle cells to improve vascular stenosis (Scott-Burden & Vanhoutte 1994). In the present study, we demonstrated that the atherosclerotic plaque area of rabbits treated with adenovirus expressing adiponectin through adventitia was significantly reduced compared with that before treatment and with rabbits treated with Ad-βgal. But no significant difference was observed between two groups treated with Ad-APN through intima or adventitia transfer.

Although the mechanism for the protective contribution against atherosclerosis of adiponectin is unclear at present, the cytokine could inhibit TNF-α-induced expression of adhesive molecules by blocking nuclear factor-κB, and it could inhibit proliferation of smooth muscle cells induced by

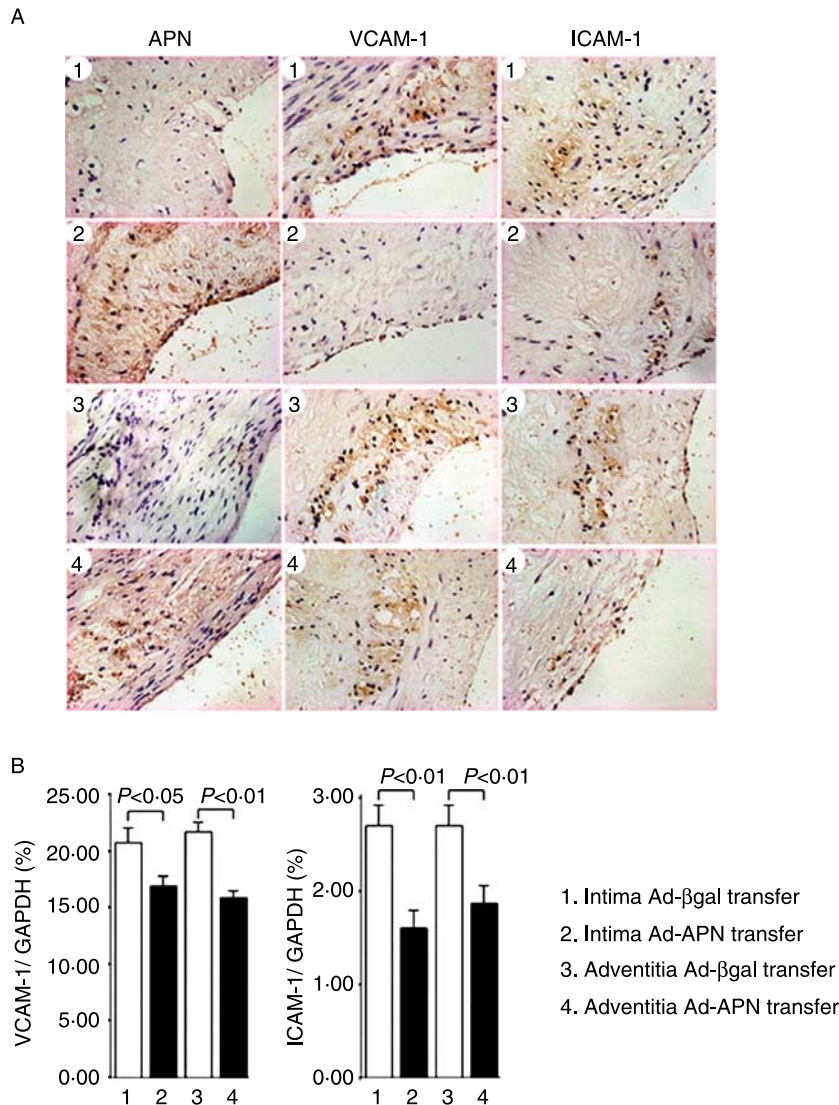


Figure 5 Effect of Ad-APN treatment on adhesion molecules in atherosclerotic rabbits 14 days after recombinant adenovirus transfer. A, Immunohistochemical detection of adiponectin, VCAM-1 and ICAM-1 in atherosclerotic lesions. Representative images are shown. 1, Ad-βgal transfer through intima. 2, Ad-APN transfer through intima. 3, Ad-βgal transfer through adventitia. 4, Ad-APN transfer through adventitia. The magnification is 400. B, mRNA expression of VCAM-1 and ICAM-1 in abdominal aortic tissue in Ad-βgal or Ad-APN-treated rabbits. The values are means \pm S.E.M.

growth factors by inhibiting mitogen-activated protein pathways (Lappas *et al.* 2005). Adiponectin has been shown to reduce atherosclerosis through attenuating endothelial inflammatory response and macrophage-to-foam cell transformation (Okamoto *et al.* 2002). Meanwhile, adiponectin suppresses the expression of NF-κB-inducible genes, including VCAM-1, in endothelial cells and class A scavenger receptor expression in monocyte-derived macrophages (Ouchi *et al.* 2001, Lappas *et al.* 2005). In the present study, we investigated the expression of VCAM-1 and ICAM-1 in

abdominal aortas of atherosclerotic rabbits treated with Ad-APN through intima and adventitia. Ad-APN treatment inhibited the expression of VCAM-1 and ICAM-1 *in vivo* but did not significantly affect the synthesis of type I and III collagen. The findings suggest that adiponectin improves atherosclerosis in part through inhibition of the expression of VCAM-1 and ICAM-1 in the vascular wall but not through regulating type I and III collagen synthesis.

In summary, we show that local Ad-APN treatment through intima or adventitia reduces atherosclerotic plaque

area, although the exact mechanism needs further investigation. Our studies have helped to elucidate the protective mechanism of adiponectin in atherosclerosis.

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