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Original Article

Effect of GPE-AGT nanoparticle shRNA transfection system mediated RNAi on early atherosclerotic lesion

Ping Lu, Lifen Yuan, Yuqiang Wang, Qin Du, Jing Sheng

Department of Geriatrics, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, No 639 Zhizaoju Road, Shanghai, 200011, China

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Abstract: Objective: To investigate the effects of RNA interference targeting AGT on early atherosclerotic lesion in the hypertensive state. Methods: Hypertension and atherosclerosis rats were treated with GPE nanoparticles carrying AGT shRNA. Systolic blood pressure and heart rate were measured for 2 consecutive weeks. Three days after treatment, the mRNA and protein expressions of AGT in the liver were measured by PCR and western blot assay, respectively. The blood levels of AGT and Ang II were determined by ELISA. H&E staining and electron microscopy were performed. Results: Three days after AGT shRNA treatment, the mRNA and protein expressions of AGT in the liver were markedly reduced and the blood levels of AGT and Ang II dramatically decreased as compared to the remaining 3 groups ($P < 0.05$). Three days after AGT shRNA treatment, the blood pressure was reduced by 27 ± 4mmHg when compared with that at baseline ($P < 0.05$). About 11 days after AGT shRNA treatment, the blood pressure began to increase. The blood pressure remained unchanged in the remaining 3 groups. Microscopy showed the atherosclerotic lesions were markedly attenuated in AGT shRNA treated rats but the liver and kidney functions remained stable ($P > 0.05$) when compared with the remaining 3 groups. Conclusion: Transfection with GPE nanoparticle carrying AGT shRNA can stably lower the blood pressure and improve the atherosclerotic lesions which lead to the delayed development of early atherosclerotic lesions in hypertension rats with concomitant atherosclerosis.

Keywords: Angiotensinogen, RNA interference, hypertension, atherosclerosis, spontaneously hypertensive rat, animal model

Introduction

Epidemiology shows the incidence of hypertension is about 25% in the elderly, and half of them develop atherosclerosis (AS) concomitantly. The proportion of subjects with protuberant lesions in the aorta and coronary artery among hypertension patients is higher than that in individuals with normal blood pressure [1]. Animal studies also demonstrate that the presence of hypertension and hyperlipoidemia may increase the susceptibility to AS [2, 3]. Currently, it is imperative to identify early atherosclerotic lesions in patients with hypertension which is beneficial for the effective intervention of hypertension aiming to prevent the cardiovascular events.

Renin-angiotensin system (RAS) plays important roles in the occurrence and development of hypertension and AS [4]. Angiotensinogen (AGT) is an important component of RAS and mainly synthesized in the liver. AGT is the unique precursor of angiotensin II (AngII), and the increase in AGT may significantly elevate the generation of Ang II resulting in an increase of blood pressure [5, 6]. It has been confirmed that the blood pressure is closely related to the plasma AGT concentration ($r = + 0.39$, $P < 0.00001$) [6]. In hypertension patients, the plasma AGT level is significantly higher than that in subjects with normal blood pressure ($P < 0.01$). In animals with AGT over-expression [7], the mean arterial pressure is dramatically higher than that in the control group (159 ± 8 mmHg vs 107 ± 3 mmHg, $P < 0.05$).

Paravicini et al found reactive oxygen species (ROS) played an important role in the development of cardiovascular disease, including hypertension, atherosclerosis [8], but the production of nitric oxide (NO) was reduced and the

endothelium dependent dilation was impaired. However, inhibition of RAS (angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, selective renin inhibitor) may reduce the generation of ROS, promote the synthesis of NO in the endothelial cells, induce the vascular dilation, control the blood pressure and delay the development of AS. Thus, to inhibit the AGT, an initial substrate of RAS, may be helpful to control the blood pressure and delay the development of AS.

RNA interference (RNAi) refers to introduce of small interfering RNA (siRNA) of 19-25 bp with characteristic structure into mammalian cells in which the expression of homologous mRNA is specifically interfered [9]. This technique can specifically inhibit the expression of target gene. However, for chronic diseases, application of synthetic siRNA alone usually fails to achieve favorable therapeutic efficacy, and transfection of siRNA in the aid of vectors is preferred in the treatment of chronic diseases. Although the virus mediated transfection has a high transfection efficiency, this technique may induce the immune response, has virulence, potential carcinogenicity and limited DNA load and the assembling of virus expressing target gene is difficult. Thus, increasing investigators pay attention to the non-viral vector mediated transfection. Among non-viral vectors, polyethylenimine (PEI) polycation complex is widely used [10, 11]. In our previous study, the bPEI (800 Da) with low cytotoxicity was used to construct the high molecular weight PEI derivative (PEI-Et) with urethane bond. In vitro experiment showed PEI-Et had low cytotoxicity and its transfection efficiency was higher than that of PEI (25 kD) [12]. On the basis of these findings, with the aid of massive asialoglycoprotein receptors (ASGP-R) on the hepatocytes, the specific ligand galactose was linked to Et to form Gal-PEG-Et (GPE). Then, target gene was introduced to GPE which was then transfected into hepatocytes (published elsewhere).

In the present study, spontaneously hypertensive rats (SHR) were intragastrically treated with large dose VitD3 and fed with high fat food to prepare the hypertensive rats with AS. Then, GPE-AGT shRNA was injected via the tail vein aiming to down-regulate the AGT expression in the liver and subsequently reduce the generation of Ang II and lower the blood pressure followed by observation of atherosclerotic lesions. Our findings may provide evidence for the clinical prevention and treatment of AS.

Materials and methods

Ethics statement

All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of Shanghai Jiaotong University School of Medicine (SHJT 2011-0002), and conformed to the National Institute of Health guidelines on the ethical use of animals.

Animals, establishment of animal model and grouping

A total of 24 adult female SHR aged 16 weeks (270-320g) were housed separately in cages (3-5 rats per cage) at 24 ± 2℃ in an atmosphere with the humidity of 60~65% in a standard 12h : 12h light-dark cycle. Animals were given ad libitum access to food and water.

Animals were divided into two groups. In control group, animals were intragastrically treated with normal saline for 3 days and fed with general food (n = 6). In the AS group, rats were intragastrically treated with VitD3 and fed with high fat food to introduce AS to SHR (n = 18) [13]. In the control group, normal saline (500μl) was injected via the tail vein once every 10 days for nine times. In the AS group, animals were further divided into three subgroups (n = 6 per group): GPE-AGT shRNA group (rats were injected with 500μl of GPE-AGT nanoparticle siRNA [50ng/μl; GPE nanoparticle: plasmid DNA = 30 : 1], negative control group (rats were treated with 500μl of GPE-NC nanoparticle siRNA via the tail vein) and blank control group (rats were treated with 500μl of normal saline via the tail vein). Treatment was done for 9 times.

Detection of mRNA and protein expression of AGT in liver

At 3 days after injection, 3 rats were randomly collected from each group and the liver was collected for the detection of AGT expression. Total RNA was extracted with Trizol reagent and the 2μg of RNA were used to reverse transcribe into cDNA. MA3000P thermal cycler (Applied

Table 1. Primers for real-time PCR.

Genes	Sequences	Expected size
AGT	Forward 5'- CATCTTCCCTCGCTCTCTG -3'	175 bp
	Reverse 5'- GCCTCTCATCTTCCCTTGG -3'	
β-actin	Forward 5′- CTGTCCCTGTATGCCTCTG -3′	217 bp
	Reverse 5′- TGTCACGCACGATTTCC -3′	

Table 2. Blood lipid before and after experiment (n=3)

Group		TC	TG	LDL-C	HDL-C
AS	Before	2.15±0.80	0.70±0.16	0.19±0.05	0.74±0.18
	After	4.13±0.58▲	1.60±0.00▲	1.52±0.30▲	0.76±0.00
Control	Before	2.13±0.75	0.64±0.17	0.24±0.06	0.76±0.16
	After	2.14±0.70	0.62±0.10	0.23±0.00	0.75±0.20

Note: ▲P<0.05 vs AS before experiment group

Biosystems) was employed for real time quantitative PCR. The primers (Invitrogen) are shown in **Table 1**. The conditions for PCR were as follows: pre-denaturation at 95℃ for 10 min, 40 cycles of denaturation at 95℃ for 30s, annealing at 60℃ for 30s and extension at 72℃ for 45s. The house keeping gene β-actin served as an internal reference. The expression of target gene was normalized to that of β-actin as the relative expression. At the same time, total protein was extract from the liver and the protein expression of AGT was determined by western blot assay.

Detection of serum AGT and Ang II by ELISA

At 3 days after shRNA treatment, 3 rats were randomly selected from each group, and 1ml of blood was collected from the femoral vein. After centrifugation at 2000rpm/min for 20min, the supernatant was collected and stored at -80°C. The serum AGT and Ang II were measured by ELISA with kits according to the manufacturer's instructions.

Measurement of blood pressure and heart rate

Before and after shRNA treatment, the systolic blood pressure (SBP) [14] and heart rate were measured via the tail artery at rest with PowerLab ML125/R system. Measurement was done for consecutive 3 times followed by averaging.

Pathological examination of blood vessels

After anesthesia, an incision was made in the neck and the left common carotid artery was exposed and 1 cm of carotid artery was collected, fixed in 10% formalin for 12 ~ 24h and embedded in paraffin followed by HE staining for pathological examination under a light microscope (Nikon, Japan). Another segment of carotid artery was fixed in 2% glutaric dialdehyde in PBS at 4C° for 12~24h for the examination of ultrastructure by electron microscopy (CMI20, Philip, Netherlands).

Blood lipid and safety

Before experiment, 8 weeks after treatment and at the end of experiment, 3 rats were randomly selected from each group and venous blood (1ml) was collected from the femoral vein. The total cholesterol (TC), total triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea nitrogen and creatinine were determined by using an automatic biochemical analyzer in the Department of Laboratory of our hospital.

Statistical analysis

Data were expressed as means ±standard deviation (SD) and statistical analysis was performed with the SPSS version 17.0 statistical

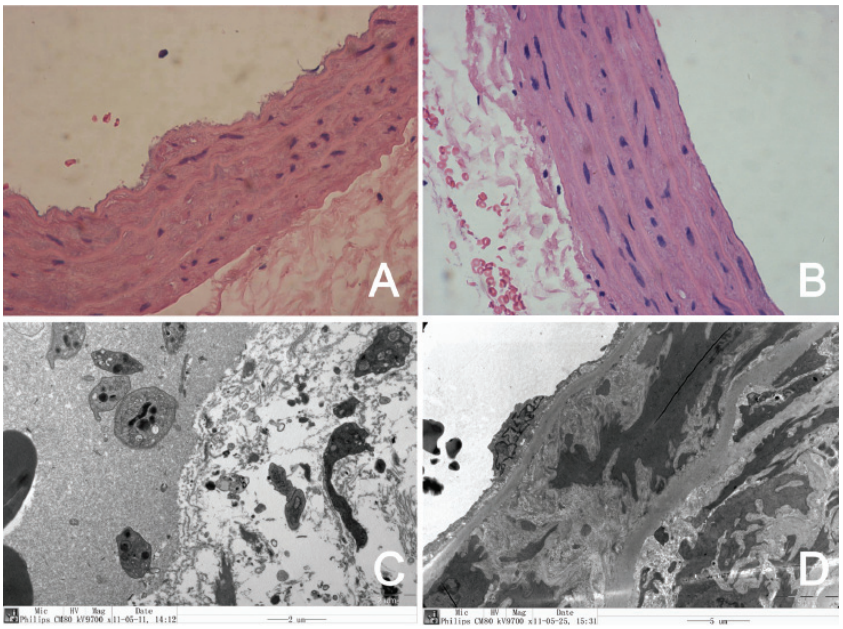

Figure 1. Pathological examination (×400) and electron microscopy (×9700) of carotid artery from SHR. The SHRs were intragastrically treated with VitD3 and fed with high fat food. Eight weeks later, the pathological examination by H&E staining and electron microscopy of carotid artery were done. A-B: light microscopy; C-D: electron microscopy. Figure 1A shows evident calcification in the media of carotid atery of SHR with AS which had disordered structure. Fiure 1B showed the carotid artery had complete structure, each layer was arranged regularly; no foam cells or calcification were found. Figure 1C shows elastic membrane was fractured, platelet aggregated, and degenerated organelles were found in the smooth muscle cells, macrophages and smooth muscle cells phagoctoing lipid were identified in the vascular wall in the AS group. Figure 1D indicates that the endothelial cells and smooth muscle cells were normal in morphoogy, subendothelial structures were orderly arranged, the elastic layer was clear, but no lipid drop or calcium deposition was noted in the control group.

software. Comparisons were done by t test and a value of $P < 0.05$ was considered statistically significant.

Results

Establishment of AS animal model with SHR

The SHRs were intragastrically treated with VitD3 and fed with high fat food. Eight weeks later, the blood lipid was measured (**Table 2**). Results revealed the blood lipid level was markedly increased in the AS group as compared to the remaining groups ($P < 0.05$). Eight weeks later, the pathological examination by H&E staining and electron microscopy of carotid artery were done (**Figure 1**). Pathological examination revealed the evident calcification and disordered structure in the media of carotid artery (**Figure 1A**). As shown in **Figure 1B**, the carotid artery in the control group had ordered structure, and foam cells and calcification were not noted. Electron microscopy (**Figure 1C**) revealed the elastic layer was fractured, platelet aggregated, degenerated organelles were found in the smooth muscle cells, and macrophages and smooth muscle cells ph-agocytosing lipid were identified in the vascular wall in the AS group. As shown in the **Figure 1D**, the endothelial cells and smooth muscle cells were normal in morphology, subendothelial structures were orderly arranged, elastic layer was clear and lipid drop and calcium deposition were not noted in the control group. These findings demonstrate that AS is successfully introduced to SHR after intragastrical treatment with VitD3 and feeding with high fat food.

AGT expression following treatment with shRNA

The mRNA expression of AGT in the liver was measured by real-time PCR (**Figure 2A**). The mRNA expression of AGT in the shRNA treated animals was markedly reduced by 61.72% and 61.94% when compared with the blank control group and negative control group, respectively ($P < 0.05$). This suggested that GPE-AGT shRNA significantly reduced the mRNA expression of AGT in the liver.

Western blot assay was employed to detect the protein expression of AGT in the liver (**Figure 2B-C**). Results showed the protein expression of AGT in the shRNA treated animals was markedly reduced as compared to the blank control group and negative control group ($P < 0.05$), which was consistent with findings in real time PCR.

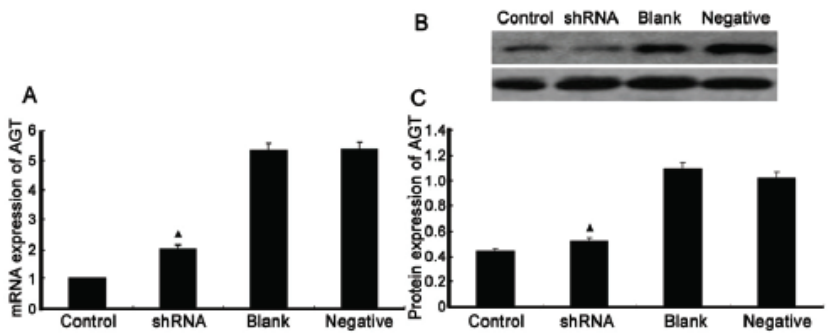

Figure 2. mRNA and protein expression of AGT in the liver following treatment. SHR were divided into two groups. In control group, animals were intragastrically treated with normal saline for 3 days and fed with general food. In the AS group, rats were intragastrically treated with VitD3 and fed with high fat food to introduce AS to SHR. In the control group, normal saline (500 μl) was injected via the tail vein once every 10 days for nine times. In the AS group, animals were further divided into three subgroups (n=6 per group): GPE-AGT shRNA group (rats were injected with 500 μl of GPE-AGT shRNA [50 ng/μl; GPE nanoparticle : plasmid DNA = 30 : 1], negative control group (rats were treated with 500 μl of GPE-NCshRNA via the tail vein) and blank control group (rats were treated with 500 μl of normal saline via the tail vein). At 3 days after injection, 3 rats were randomly collected from each group. The livers were collected and total RNA and protein were extracted for PCR and western blot assay. A. The mRNA expressions of AGT in the four groups. (B-C) the protein expression of AGT in the four groups. The mRNA and protein expressions of AGT were determined by densitometric analysis and normalized by that of β-actin. Data are presented as the means ± SE vs. negative control group, ▲P<0.05.

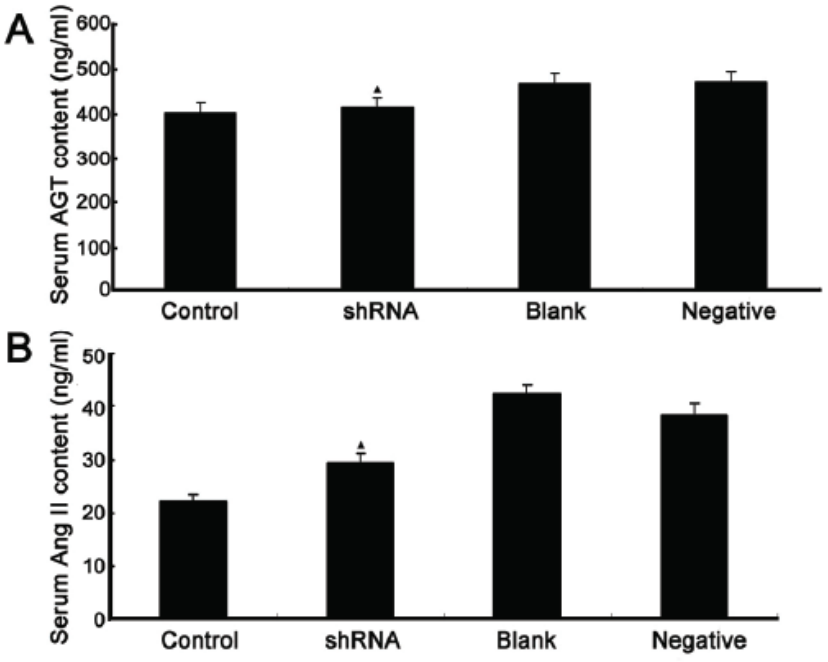

Figure 3. The serum contents of AGT and Ang II. 3 rats were randomly selected from each group, and 1 ml of blood was collected from the femoral vein. After centrifugation at 2000 rpm/min for 20 min, the supernatant was collected and stored at -80°C. The serum contents of AGT and Ang II were measured by ELISA. A. The serum contents of AGT in the four groups. B. The serum contents of Ang II in the four groups. Data are presented as the means ± SE vs. negative control group, ▲P<0.05.

ELISA was performed to measure the blood contents of AGT and Ang II (**Figure 3**). In the shRNA treated animals, the serum AGT content was markedly reduced by 15% as compared to the blank control group and negative control group ($P < 0.05$). In addition, in the shRNA treated animals, the serum Ang II content was dramatically reduced by more than 30% when compared with the blank control group and negative control group ($P < 0.05$). These findings demonstrated that treatment with GPE-AGT shRNA could significantly reduce the mRNA and protein expression of AGT accompanied by reduction in serum cotents of AGT and Ang II.

Blood pressure and heart rate

As shown in **Table 3**, there was no significant difference in the blood pressure among groups before experiment ($P > 0.05$). Three days after shRNA treatment, the blood pressure was reduced by about 27 ± 3mmHg as compared to that before experiment ($P < 0.05$). Thereafter, the blood pressure reduced slowly, and the reduction of blood pressure last for about 8 days. The maximal amplitude of blood pressure reduc-

Table 3. Blood pressure before and after treatment in each group (Means±SD, mmHg)

Time point	Control group	shRNA group	Blank control group	Negative control group
0 d	193±5	200±6	195±6	196±6
1 d	194±7	190±4	196±8	197±5
2 d	196±6	184±6	197±7	191±7
3 d	197±4	173±7▲	196±8	193±6
5 d	195±4	171±4▲	194±6	192±5
7 d	192±5	169±5▲	196±7	191±6
11 d	191±6	175±5	193±5	193±4

Note: ▲P<0.05 vs before treatment

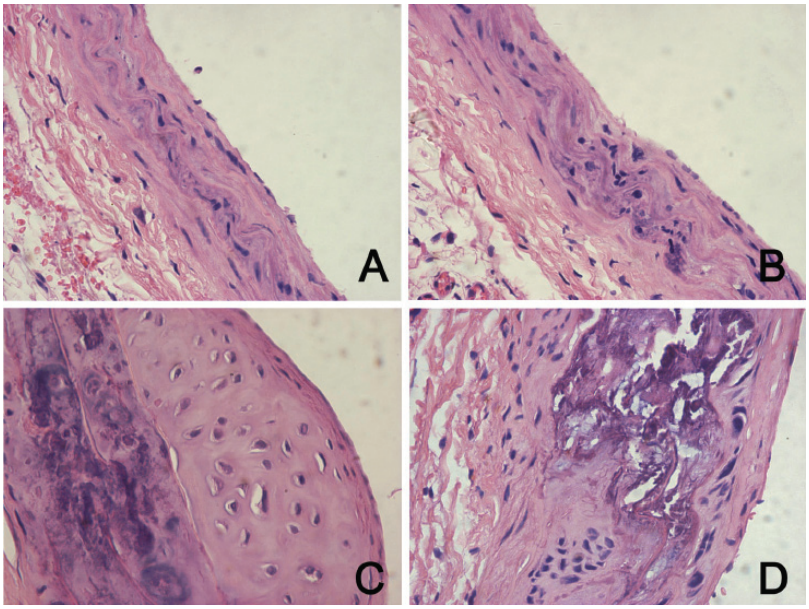

Figure 4. H&E staining of carotid artery in the four groups (×400). A: control group, B: shRNA group, C: blank control group, D: negative control group. Figure 4A shows that each layer of vascular wall was clear and ordered arranged, no foam cells or calcification were found, and a few shedding endothelial cells were found . Figure 4B shows that each layer of vascular wall was clear, the media was slightly disordered, a few foam cells and calcification were noted. Figure 4C shows that the hyperplasia was found in the intima of carotid artery and plaque-like protrusion was found, calcification was obvious and foam cells were found under the intima. Figure 4D (Figure 4D) shows that calcification was evident in the media of carotid artery disordered structure was also found.

tion was about 31mmHg and the blood pressure began to increase about 11 days after treatment. In addition, there heart rate remained unchanged after treatment in each group (P< 0.05).

H&E staining of carotid artery

H&E staining showed that each layer of vascular wall was clear and ordered arranged, foam cells and calcification were not found and a few shedding endothelial cells were found in the control group(**Figure 4A**). In the shRNA treated animals (**Figure 4B**), each layer of vascular wall was clear, the media was slightly disordered, a few foam cells and calcification were noted. In the blank control group (**Figure 4C**), the hyperplasia was found in the intima of carotid artery and plaque-like protrusion was found, calcification was obvious and foam cells were found under the intima. In the negative control group (**Figure 4D**), calcification was evident in the media of carotid artery disordered structure was also found.

Electron microscopy showed, in the control group, the structure was regular (**Figure 5A**), the elastic membrane was clear, lipid drop and calcium deposition were absent. In the shRNA treatment group, a few lipid drops were noted. In the blank control group (**Figure 5C**), the subendothelial structure was disordered, a lot of granule-like or fiber like substances were found, and lipid drops were noted in the smooth muscle cells. In the negative control group (**Figure 5D**), the subendothelial structure was disordered, elastic membrane was fractured, a lot of fiber-like substances were found, and degenerated organelles and lipid drops were found in the smooth muscle cells. These findings demonstrated that the AS

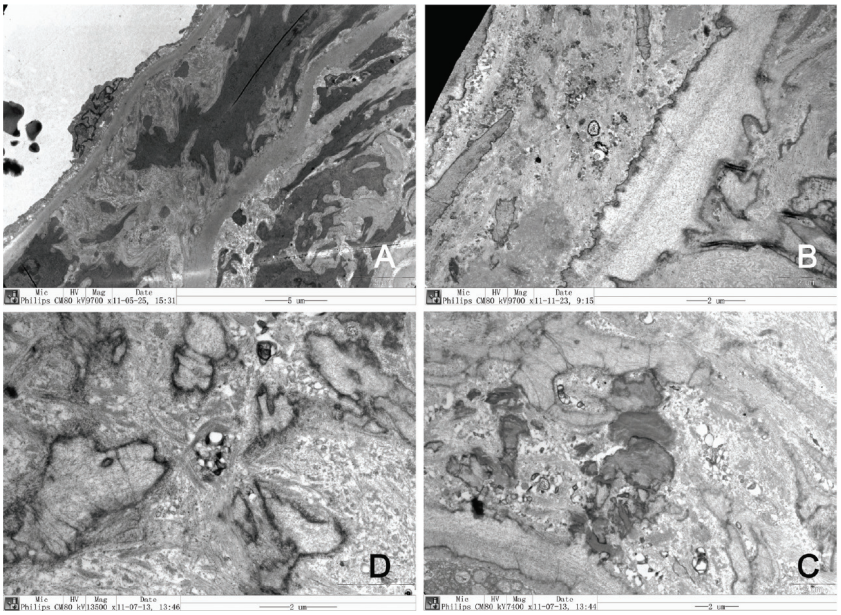

Figure 5. Electron microscopy of carotid artery in the four groups (×9700). A: control group,B: shRNA group, C: blank control group, D: negative control group. Figure 5A shows the structure was regular, the elastic membrane was clear, lipid drop and calcium deposition were absent. Figure 5B shows a few lipid drops were noted. Figure 5C shows the subendothelial structure was disordered, a lot of granule-like or fiber like substances were found, and lipid drops were noted in the smooth muscle cells. Figure 5D shows the subendothelial structure was disordered, elastic membrane was fractured, a lot of fiber-like substances were found, and degenerated organelles and lipid drops were found in the smooth muscle cells.

was attenuated after intravenous injection of GPE-AGT shRNA in SHR with AS.

Safety

In the shRNA treatment group, all animals survived. In all groups, the AST, ALT, urea nitrogen and creatinine remained unchanged ($P > 0.05$). These demonstrated that the GPE vectors had favorable safety.

Discussion

Hypertension is an important factor facilitating the occurrence and development of AS, and AS induced vascular stenosis is a cause of secondary hypertension. Thus, hypertension and AS can interact with each other [15]. In the present study, AS was introduced to SHRs, in which RNAi was employed to investigate the role of AGT in the early AS. Our findings may provide evidence for the prevention and treatment of AS.

At 8 weeks after intragastrical treatment with high dose VitD3 and feeding with high fat food, the blood lipid level was markedly increased ($P < 0.05$). Pathological examination of carotid artery by H&E staining and electron microscopy identified macrophages and smooth muscle cells phagocytosing lipid. This suggests that high dose VitD3 in combination with high fat food can induce AS in SHR, which may mimic the hypertension and AS simultaneously in humans.

The RAS involves in the liver, heart and blood vessels and includes rennin, AGT, Angiotensin-convertingenzyme, etc [15, 16]. AGT serves as an initial substrate of TAS and directly controls the activity of TAS. AGT is mainly synthesized in the liver and circulatin g AGT is derived from the liver. The structure of AGT has been elucidated. The human AGT gene locates in 1q42-43. The human AGT protein is a globular glycoprotein and its molecular weight ranges from 55 kD to 65 kDa depending on the extent of glycosylation. The precursor of AGT is first expressed and the mature AGT is a hormone precursor substance consisting of 452 amino acids. The 10 amino acids at the N terminal are corresponding to the Ang I and the remaining part represents the AGT without Ang I. In the circulation, AGT is hydrolyzed into inactive decapeptide Ang I in the presence of rennin secreted by the juxtaglomerular cells. The Ang I in the other organs including the lung is converted into active octapeptide Ang II following the hydrolysis by angiotensin converting enzyme (ACE) which is synthesized in the endothelial cells, in which two amino acids at the C terminal of Ang I are removed. Ang II is an effector in the RAS and can act on the angiotensin II receptor type 1 (AT1) leading to the contraction of arterioles, the secretion of aldosterone in the zona glomerulosa of adrenal cortex and

secretion of norepinephrine by the presynaptic membrane of sympathetic nerve ending in a positive feedback mechanism. These finally increase the blood pressure and involve in the maintenance of high blood pressure [17]. AGT is the unique precursor of Ang II, the most potent vasoactive substance. The slight change in AGT content in the circulation and focal tissues may result in significant alteration of Ang II content. Thus, the blood AGT content directly influences the generation of Ang II.

Ferrario established an AS animal model and results demonstrated that Ang II was involved in all the processes of AS including the impairment of endothelial function, lipid deposition, vascular remodeling, proliferation and migration of vascular smooth muscle cells, formation of foam cells, etc [18]. The blood flow induced mechanical pressure and percussion in the hypertensive state may cause damage to the endothelial cells inducing endothelial dysfunction [19], increase the permeability of intima to the lipid, induce the permeation of lipoprotein into the intima, lead to the migration of monocyte into the intima [20], and elicit the adhesion of platelets to the intima and migration of smooth muscle cells into the intima. These finally promote the occurrence of AS. Sustained increase of blood pressure may increase the percussion to the arteries inducing the thickening of intima [21] and the calcification of arterial intima [22], promoting the occurrence AS like calcification.

In the present study, RNAi was employed to down-regulate the mRNA and protein expression of AGT in SHR with AS (**Figure 2**). At the same time, the serum contents of AGT and Ang II (**Figure 3**) and blood pressure (**Table 3**) were significantly reduced. Thus, the percussion to the blood vessels is reduced and then the development of AS is delayed (**Figure 4-5**). These findings demonstrate that inhibition of AGT expression and reduction of Ang II production can not only lower the blood pressure but delay the development of AS.

During the atherosclerotic process, the activation of RAS may induce the over-expression of AGT which subsequently increases the production of Ang II. Ang II binds to AT1R, activates NADH/NADPH system and induces oxidative stress [23] during which a larger amount of superoxides and ROS are produced resulting in damage to the endothelial cells. Zhou et al [24] found that the endothelial cells in the microvessels and cerebral blood vessels presented with over-expression of AGT in SHR, and Ang II could bind to AT1R to induce the production of ROS resulting in vascular inflammation and damage to the endothelial cells. In a study in which patients were treated with ACE inhibitor and followed for 6 months [25], the activity of RAS was markedly inhibited, and the Ang II and markers for oxidative stress (such as malondialdehyde, lipid peroxide, etc) in the plasma were markedly reduced. These findings indirectly indicate that Ang II mediates the oxidative stress.

Taken together, our results indicate that AGT involves in the pathology of AS in an Ang II dependent manner. AGT is the unique precursor of Ang II, the most potent vasoactive substance, and thus blockage of AGT expression may significantly compromise the role of Ang II in the AS. Blockage of AGT expression may become an effective strategy for the prevention and treatment of AS. However, the sample size was small in the present study, and large scale studies are required to confirm our findings.

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Address correspondence to: Dr. Jing Sheng, Department of Geriatrics, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, No 639 Zhizaoju Road, Shanghai, 200011, China; Email: shengjing60@163.com

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