Original Article

Testin on Atherosclerosis in Rabbits

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Abstract

Background: The expression of *TES*, a novel tumor suppressor gene, is found to be down-regulated in the left anterior descending aorta of patients with coronary artery disease (CAD) compared with non-CAD subjects. This study aimed to investigate the expression of *TES* during the development of atherosclerosis in rabbits.
Methods: Thirty-two New Zealand rabbits were randomly divided into a normal diet (ND) and high-fat diet (HFD) groups. Body weight and serum lipid levels were measured at 0, 4, and 12 weeks after diet treatment. The degree of atherosclerosis in thoracic aortas was analyzed by histological examinations. The expression of Testin in the tissue samples was inspected via immunohistochemical and immunofluorescence confocal microscopy. Real time-polymerase chain reaction and Western blot analysis were performed to evaluate the expression of *TES*/Testin at mRNA and protein levels in the aortic tissues.
Results: After 12 weeks postenrollment, rabbits in HFD group had a higher level of serum lipids and atherosclerotic plaque compared to ND group ($P < 0.05$). Testin expression was detected at high levels in the endothelium and a weak expression on the subendothelium area. The expression of *TES* mRNA was markedly reduced by 10-fold in the aortic tissues in the HFD group compared with the ND group ($P = 0.015$), and the protein level was also significantly decreased in the HFD group ($P < 0.05$).
Conclusions: Reduced *TES*/Testin expression is associated with the development of atherosclerosis, implicating a potentially important role in the pathogenesis of atherosclerosis.

Key words: Atherosclerosis; Rabbit; Testin

Introduction

Atherosclerosis is currently the preeminent health problem worldwide.[1] Pathogenesis of atherosclerosis is a complicated process involving many pathogenic factors. Previous studies have implicated that endothelial dysfunction plays an important role in the onset and progression of atherosclerosis.[2,3] However, the etiology of the disease is still not fully understood.

TES, a novel tumor suppressor gene, is located in a common fragile site on human chromosome 7q31.2, designated as FRA7G. It is predicted to encode a highly conserved protein of 421 amino acids named Testin.[4] Testin has been found to play an important role in focal adhesion[5] and is present in the cytoplasm. Testin is ubiquitously expressed in normal tissues as a negative regulator of cell growth.[6] Recently, it has been reported that the expression of *TES* was decreased in the left anterior descending aortic tissue from patients with coronary artery disease (CAD) compared with non-CAD subjects.[7] In this report, we aimed to determine the cellular origin of *TES* expression in aorta and examine whether its expression is affected in the aortic tissue during the pathogenesis of atherosclerosis.

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Methods

Animal studies

Thirty-two healthy purebred New Zealand rabbits were used at the age of 3–4 months with body weights from 1.5 to 2.0 kg. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Tianjin Medical University. The rabbits were randomly divided into two groups as the following: (1) The normal diet (ND) group ($n = 12$) were fed on 100 g/d of ND; (2) High fat diet (HFD) group ($n = 20$) were fed on the mixture of 100 g/d of 1% cholesterol, 5% lard, 10% egg yolk powder and 84% ND. Body weight and serum lipid levels

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were measured at 0, 4 and 12 weeks after enrollment. The animals were killed at the end of 12-week treatment.

Atherosclerotic model studies

Thoracic aortas were rapidly removed, cleaned of adventitia, cut into 5-mm ring segments. The specimens were fixed in 4% formaldehyde and sectioned for routine Hematoxylin and Eosin staining. Tissue structure and pathological features of atherosclerosis were examined under a microscope (Olympus, Japan).

Testin detection

The detection of Testin on tissue samples was inspected via immunohistochemical and immunofluorescence staining. After fixation, the tissue sections were rehydrated twice for 5 min in phosphate buffer saline (PBS), blocked for 1 h in PBS with 10% normal goat serum followed by incubation in a humid chamber with 50 μl of anti-Testin primary antibody (1:100 dilution; ABcam, ab78499, USA) and anti-CD31 primary antibody (1:50 dilution; ABcam, ab9498, USA) in 10% serum overnight at 4°C. The slides were rinsed twice for 5 min in PBS before being incubated for 2 h in a humid chamber with 50 μl of anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (1:100 dilution; Sigma, USA) solution to visualize Testin. Stained sections were rinsed twice for 5 min in PBS, stained for 10 min with 50 μl of 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) in 200 ml PBS, then rinsed again twice for 5 min in PBS, and mounted with an aqueous mounting medium before being examined under confocal microscope (Olympus, Japan).

Real-time polymerase chain reaction analysis

Total RNA was isolated from aortic tissue using the TRIzol solution (Invitrogen, USA). Total of 1 μg RNA was used for first-strand complementary DNA synthesis using Random Primer (Invitrogen, USA) and SuperScript II Transcriptase (Invitrogen, USA) according to manufacturer's instruction.[8] Real-time polymerase chain reaction (PCR) was performed using IQ SYBR Green Supermix (Bio-Rad, USA) with CFX-96 Real-time PCR Detection System (Bio-Rad, USA). The primers used for *TES* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control in the reaction were: 5'-CCTGTCCAGAACCAGGCATT - 3'/5' - TTCTTTCGGTACTGTGCCCC-3' (*TES* gene, 98 bps), and 5'-TGAGAATCTGCCCCTCTTCAC - 3'/5'-CGTTGCTGTCGAGACTTTATTGA-3' (GAPDH, 110 bps).

Western blot analysis

Total protein extract was prepared from aortic tissue by homogenize tissue in Radio Immunoprecipitation Assay Lysis Buffer (Sigma, R0278, USA). An equal amount of 20 μg protein was used for immunoblot from each sample. Proteins were separated on NuPAGE SDS-PAGE Gel (Invitrogen, USA) and transferred to a nitrocellulose transfer membrane (Whatman , UK) according to the manuscript's instruction.[9] The primary antibodies used for protein detection included anti-Testin (1:500 dilution; ABcam, ab78499, USA) and GAPDH (1:1000 dilution; Santa Cruz, USA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (ABcam, USA). Proteins were visualized by enhanced chemiluminescence system (Western Blot Detection Kit, GE Healthcare). Developed signals were digitally recorded and quantified with Melanie two-dimensional gel software analysis (SIB, Switzerland).

Statistical analysis

All data was analyzed using SPSS 13.0 (SPSS Inc., USA). Continuous variables were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance among groups. For comparison of data before and after dietary intervention, a paired Student's *t*-test was used. $P < 0.05$ was considered to be statistically significant.

Results

Atherosclerosis developed in rabbit following high-fat diet

We examined the extent of atherosclerosis in rabbits fed with HFD and ND for 12 weeks. As expected, all of the rabbits in HFD group had a significantly higher level of serum lipids [Table 1] and presence of atherosclerotic plaque in the thoracic aorta compared with ND group [Figure 1]. These results confirmed the development of atherosclerosis in rabbits on HFD.

Predominant expression of the Testin protein in the endothelium layer of aorta and plaque

By immunostaining in aortic tissues with anti-Testin antibody and confocal microscopy, we observed Testin signal was predominantly detected in the endothelium as identified with CD31 staining. A relatively weaker signal was also detected in the subendothelial areas [Figure 2a–2c]. Moreover, the majority of atherosclerotic plaque displayed Testin immunoreactivity on segments bearing the plaque but also on those distal from the plaque [Figure 2d–2g]. Significant

Table 1: Levels of serum lipids in two groups at different periods (mmol/L, mean ± SD)

Characteristics	ND group	HFD group
At 4 weeks		
TG	0.77 ± 0.22	1.34 ± 0.82*
TC	1.18 ± 0.87	26.31 ± 11.05*
LDL-C	0.49 ± 0.57	9.87 ± 3.64*
HDL-C	0.52 ± 0.28	5.03 ± 1.12*
At 12 weeks		
TG	0.87 ± 0.37	2.13 ± 1.97*
TC	1.15 ± 0.46	45.88 ± 14.55*
LDL-C	0.44 ± 0.19	14.05 ± 4.08*
HDL-C	0.44 ± 0.25	5.33 ± 0.72*

*Compared with ND group, $P < 0.05$. TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High- density lipoprotein cholesterol; SD: Standard deviation; ND: Normal diet; HFD: High-fat diet.

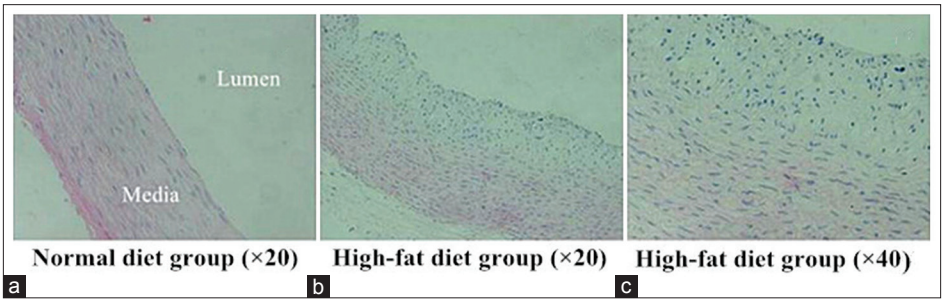

Figure 1: High incidence of atherosclerotic plaque in high-fat diet (HFD) group when compared with natural diet (ND) group (Hematoxylin and Eosin staining). Representative sections from aortic tissues in HFD group (b and c) showed atherosclerotic plaque, whereas ND group (a) was normal.

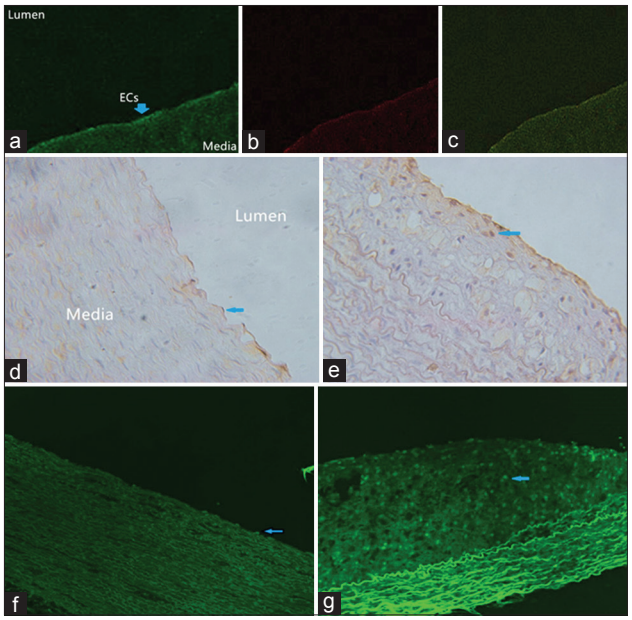

Figure 2: Expression of Testin protein in the normal and atherosclerotic aortic tissues. (a–c, × 10) The signal for Testin co-localized with the CD31 signal, indicating that Testin was expressed in endothelial cells (arrow); (d and e, × 20; f and g, × 10) Immunohistochemical and Immunofluorescence staining analysis of the normal aorta and plaque with an anti-Testin antibody. It showed that Testin was expressed predominantly in the endothelium of normal aorta and plaque (arrows).

differences in Testin signal intensities were observed among different segments, suggesting that Testin expression may correlate and contribute to vascular phenotypic heterogeneity and its possible role in atherosclerosis

TES expression in aortic tissues associated with atherosclerotic status

We assessed the expression levels of the *TES* gene in the aortic tissues obtained from HFD and ND groups. Real-time PCR analysis demonstrated that *TES* mRNA was markedly reduced by approximately 10-fold in the HFD group compared with the ND group ($P = 0.015$) [Figure 3a]. Using Western blot analysis, on the same experimental subjects showed Testin expression was decreased at protein level in the HFD group in comparison with atherosclerotic-free ND group ($P = 0.01$) [Figure 3b and 3c]. These results suggested that reduced *TES*/Testin expression was associated with HFD induced atherosclerosis.

Discussion

In this study, we characterized the expression change of *TES*/Testin in aortic tissues during HFD induced atherosclerosis using a well-established rabbit model. Our data showed that *TES*/Testin levels were significantly decreased in the HFD treated aortic tissues at both mRNA and protein levels. Using confocal analysis, we detected that Testin was mainly expressed in $CD31^+$ luminal endothelium while a weaker expression was also detected in the subendothelium area.

In this study, *TES*/Testin was expressed in endothelium, and was down-regulated in atherosclerotic tissues, which suggested a possible role for Testin in the pathogenesis of atherosclerosis; however, the underlying mechanism is not clear and needs to be further investigated. There are several possibilities that we can speculate based on our observations. First, the cellular composition of the aortic tissues in atherosclerotic subjects may be altered from basal status. Our data showed that Testin was expressed predominantly in the endothelium. In fact, the loss of endothelial cells (ECs) is a hallmark pathological in atherosclerotic tissue, which can lead to reduced expression of Testin. Second, endothelium dysfunction due to pro-atherosclerotic and pro-inflammatory stimulation in the aortic tissue following HFD can lead to down-regulation of Testin expression by genetic and epigenetic regulatory machinery. Indeed, it has been demonstrated that 5' CpG island methylation of the *TES* gene can silence its expression in gastric cancer,[10] and a similar mechanism may have occurred in atherosclerotic endothelium. Third, *TES* gene expression can also be regulated by miRNAs, which may be induced in the process of atherosclerosis. Finally, single nucleotide polymorphisms (SNPs) may contribute to regulating the expression of *TES* gene.[11,12] These possible mechanisms should be the subject of future investigations.

As a component of focal adhesions, Testin interacts with the cytoskeletal protein such as zyxin, talin, vasodilator-stimulated phosphoprotein, Mena, extractable nucler antigen (EVL), alphall-spectrin, actin and actin-related proteins 7A.[13-16] However, the functional role of Testin in the cardiovascular system is not fully understood. Archacki *et al*.[7] reported that overexpression of *TES* decreased monocyte adhesion to ECs or the migration of monocytes across the EC layer, while knockdown increased these activities. This result

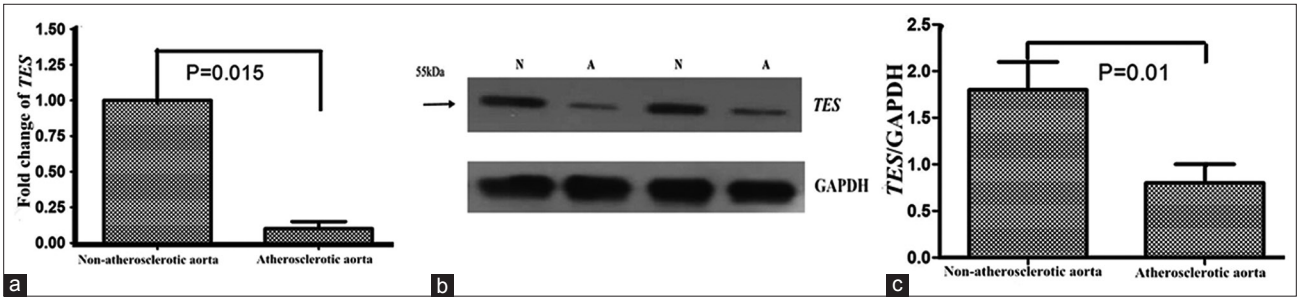

Figure 3: Decreased *TES*/Testin expression in atherosclerotic aorta compared with nonatherosclerotic samples. (a) *TES* mRNA expression from 12 nonatherosclerotic aorta compared with 20 atherosclerotic aorta arteries by real-time reverse transcription-polymerase chain reaction. The mRNA expression level of *TES* in atherosclerotic aorta was significantly lower than in nonatherosclerotic aorta arteries; (b) Testin protein expression by Western blot analysis with 12 nonatherosclerotic (N) aorta and 20 atherosclerotic aorta arteries (a) showed that Testin expression was lower in atherosclerotic aorta arteries. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control; (c) Western blot images in (b) was scanned, quantified, and plotted, which showed Testin expression was significantly decreased in atherosclerotic aorta.

indicated that *TES* may play a potentially important role in the homing and local infiltration of pro-inflammatory cells at atherosclerotic plaques, thus contributes to the initiation and progression of the pathology. In addition, Zhu *et al*.[17] have demonstrated that miR-29b decreased *TES* mRNA expression, whereas matrix metalloproteinases-2 (MMP-2) increased *TES* expression, respectively, and it is well-established that MMP-2 is associated with atherosclerosis by regulating vascularization and inflammatory response. Moreover, Magno *et al*.[18] identified Testin can also interact with calcium-sensing receptor (CaR), and influence cytoskeletal function via enhancing the CaR-mediated Rho signaling pathway. All these results suggested that Testin may play a protective role in endothelium, and its loss of expression contributes to the initiation and progression of atherosclerosis through multiple mechanisms.

In short, we characterized the expression of *TES* gene in the aortic tissue of atherosclerotic rabbits. Our results demonstrated that *TES* expression was associated with atherosclerosis. We showed that *TES*/Testin expression was significantly decreased in aortic tissues with atherosclerotic lesions compared with normal vessels. We further demonstrated that Testin was strongly expressed in $CD31^+$ luminal lining with a weaker expression in the subendothelium area. All these results indicated a potential role for Testin in atherosclerosis. Future studies are needed to fully uncover the underlying mechanisms of *TES* in the pathogenesis of cardiovascular diseases and to explore the possibility of targeting *TES* as a potential therapy.

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