Inhibitory effect of the paraoxonase gene on the formation of rabbit coronary atherosclerosis

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1. Introduction

In recent years, studies have shown that because of antioxidant, anti-inflammatory, anti-atherosclerosis (AS) activity of high-density lipoprotein cholesterol (HDL-C), it is closely related to the coronary atherosclerotic heart disease. With the further research, the anti-AS effect of HDL-C is closely related to its binding protein, especially the paraoxonase (PON-1)1, PON-1 is an arylesterase which is related to HDL, it is believed that the PON-1 and its gene family play a role in the cardiovascular disease, type 2 diabetes and other diseases related with AS. There are at least three members of this family, the latest research think that PON-1 is mainly related to lipid metabolism2.

Based on this, this study used the rabbit with coronary atherosclerotic as research object, to explore the effect of extracardiac fat local transfection PON-1 on coronary atherosclerosis formation.

2. Materials and methods

2.1. Materials

Twenty general New Zealand male white rabbits, weight 2.0–2.2 kg, aged three months, were provided by the Animal Center of the Medicine Laboratory of XX University. pEGFP–PON–1 plasmid were constructed and preserved by the molecular biology laboratory of the medical college of XX University.
2.2. Main reagents

Liposome (lipofectamine 2000) was purchased from Invitrogen Corp., Mouse anti-rabbit PON-1 antibody was purchased from Thermo. Fluorescently labeled goat anti-mouse secondary antibody was purchased from Gene Company. The paraoxonase gene ELISA kit was purchased from Sigma, USA.

2.3. Grouping and preparation of rabbit coronary atherosclerosis model (AS model)

All rabbits were randomly divided into four groups: Group A (control group), Group B (AS model group), Group C (AS model + empty plasmid group), Group D (AS model + PON-1 group), with 5 in each group. The AS models of rabbits were established based on the Ishikawa method\[3\]. The animals were raised in separate cage, and were given the diet of fat feed formulation (80.5% basic diet + 5% egg yolk powder + 0.5% cholesterol + 4% lard) respectively\[4\]. They were allowed to drink freely, with continuous feeding for 4 weeks. And anesthesia the rabbits after 8 weeks by injecting of 3% pentobarbital sodium through the marginal ear vein, and the pleural was opened rapidly. A total of 50 μL of saline was injected via pericardial cavity in the control group and the AS model group, 50 μL empty plasmid or PON-1 gene in the model + empty plasmid group and AS model + PON-1 group via pericardial cavity. Chest was opened and the adipose tissue of epicardium was extracted, and the rapid frozen sections were prepared. The fluorescence microscope was used to observe fluorescence intensity, and then to make sure that transiently transfection was successfully implemented after the injection of pericardial cavity. Then all animals were fed with high fat diet for 4 weeks after chest closed, the peripheral blood and epicardial fat were collected for detection.

2.4. Biochemical analysis

After feeding for 4 weeks, blood samples (2 mL) were collected from the ear vein in each experimental group. Serum was separated to determine TC, TG, HDL-C and low density lipoprotein cholesterol (LDL-C).

2.5. Western blot detection

Rabbits were anesthetized with pentobarbital sodium again, then the chest was opened, tissue of anterior descending of the left coronary artery vascular was extracted. They were rinsed by normal saline, then dried by the filter paper, put into liquid nitrogen and transferred to refrigerator (−80 °C) for Western blot. Protein homogenates extract was added into epicardial adipose tissue which was stored at −80 °C, at the ratio of 1:9. Total protein was extracted to make the protein quantification, then was denatured at 95 °C–100 °C for 5 min, and 30 μg total protein was taken for electrophoresis and transmembrane respectively. Mouse anti-rabbit PON-1 antibody was added, diluted at 1:1 000, swayed and incubated overnight at 4 °C. After washing, second antibody after was added, and diluted at 1:5 000. It was shaked and incubated for 1 h avoid light at room temperature. The membranes were washed, and were observed under fluorescence system scan. With β-actin as an internal reference, the gray integral value of each strip was recorded. The statistical analysis was performed by sample integral value / internal reference integral value ratio.

2.6. HE staining

Rabbit anterior descending branch of the left coronary artery was extracted in all groups, fixed in formalin for 1d. Paraffin-embedded sections were 5 μm in slice thickness. They underwent HE staining, dehydration, transparent, and cementing. Intima-media thickness of the left coronary artery was measured, intima–media thickness ratio was calculated.

2.7. Statistical analysis

The result was expressed by mean±SD. Date were analyzed by ANOVA. Single factor analysis of variance was used to compare among groups, \( P<0.05 \) was regarded as statistical significance.

3. Results

3.1. TC, TG, HDL-C, LDL-C level of the rabbit peripheral serum

Compared with the blank group, TC, TG and LDL-C levels of the rabbit peripheral blood were significantly increased in the high-fat feeding group, while HDL-C was significantly reduced \( P<0.05 \); There was no significantly difference in TC, TG, HDL-C and LDL-C between the AS model group and the AS model + empty plasmid group \( P>0.05 \), which confirmed that the empty plasmid transfection did not affect serum TC, TG, HDL-C, LDL-C levels; In AS model + PON-1
group and the AS model + empty plasmid group, TC and LDL-C levels were significantly decreased ($P < 0.05$) (Table 1).

**Table 1**
TC, TG, HDL-C, and LDL-C levels of rabbit peripheral serum (mmol/L).

| Groups  | TC     | TG     | HDL-C  | LDL-C  |
|---------|--------|--------|--------|--------|
| Group A | 2.85±0.47 | 0.39±0.08 | 1.59±0.18 | 1.15±0.25 |
| Group B | 22.38±2.82 | 0.65±0.13 | 1.05±0.12 | 24.28±2.35 |
| Group C | 21.89±3.10 | 0.59±0.14 | 1.08±0.15 | 23.96±2.06 |
| Group D | 19.28±0.68 | 0.58±0.13 | 1.07±0.14 | 21.83±2.36 |

*Compared with group A, $P < 0.05$; *Compared with group B, $P < 0.05$; *Compared with group C, $P < 0.05$.

3.2. **PON-1 levels of the tissue of anterior descending of the left coronary artery vascular in each group**

The PON-1 levels of the tissue of anterior descending of the left coronary artery vascular were significantly decreased in the AS model group (0.97±0.16) and the AS model + empty plasmid group (0.92±0.15). There was no difference between the AS model group and the AS model + empty plasmid group ($P > 0.05$). The PON-1 levels in AS model + PON-1 group (1.89±0.24) were significantly higher than the control group (1.53±0.19) and the AS model group and the AS model + empty plasmid group ($P < 0.05$).

3.3. **HE staining of anterior descending of the left coronary artery vascular in each group**

HE staining showed in the normal group, the endothelium of pulmonary vessels were smooth, the endothelial cells distributed symmetrically, there was no dysplasia or obvious abnormalities. The AS model group and the AS model + empty plasmid group showed arrangement disorder in the vascular endothelial cells (Figure 1). The intimal thickening was significant, intimal/medial thickness ratio were (17.1±2.5)% and (17.4±2.4)%, significantly higher than the ratio of normal control group which was 0 ($P < 0.05$). There was no statistically significant between the AS model group and the AS model + empty plasmid group ($P > 0.05$). The intimal and medial thickening were much significant of the AS model + PON-1 group than the normal group, intimal/medial thickness ratio were (10.5±1.6)%, significantly higher than the ratio of normal control group which was 0 ($P < 0.05$), but it was reduced much significantly than the AS model group and the AS model + empty plasmid group ($P < 0.05$) (Table 2).

**Table 2**
Intimal and medial thickness of rabbits left coronary artery in each groups.

| Groups  | intimal thickness (μm) | medial thickness (μm) | intimal/medial thickness ratio (%) |
|---------|------------------------|-----------------------|-----------------------------------|
| Group A | 0                      | 193.5±11.4            | 0                                 |
| Group B | 55.3±4.2               | 323.6±23.5            | 17.1±2.5*                         |
| Group C | 58.6±4.8               | 335.8±24.2            | 17.4±2.4*                         |
| Group D | 25.7±3.0               | 244.3±18.8            | 10.5±1.6*                         |

*Compared with group A, $P < 0.05$; *Compared with group B, $P < 0.05$; *Compared with group C, $P < 0.05$.

**Figure 1.** Histological changes of rabbit left coronary in each group (HE stain, ×10).
Group A: control group; Group B: AS model group; Group C: Empty plasmid group; Group D: PON-1 group.

4. Discussion

With the development of the study of cardiovascular disease, a large number of risk factors have been discovered. Although the pathogenesis is yet not clear, the abnormal lipid metabolism plays an important role in the process of coronary atherosclerosis. Currently more and more research concerned about the gene cluster (PON-1) which is located at human chromosome 7q21.3-q22.1, among which the PON-1 was more closely related to the lipid metabolism. PON-1, also known as aryl dialkyl phosphate enzyme, is a glycoprotein synthesized in the liver, which combined with HDL-C and hydrolyze organic phosphates, aromatic esters, lactones, and LDL-C and other lipid peroxides. Therefore PON-1 can inhibit the accumulation of a variety of lipid peroxides, and it is linked to cardiovascular disease[5]. In this study, we intended to improve the local environment of coronary PON-1 expression levels by the transfection of PON-1 gene in the local epicardial adipose, hoping to achieve the purpose of anti-coronary atherosclerosis.
Many studies think that the PON-1 gene was closely related to atherosclerosis, coronary heart disease and the diabetes, and PON-1 is the main component which can determine the lipid metabolism capacity and the antioxidant activity. The animal studies of mice showed that atherosclerosis is not only associated with PON-1 expression, but also related to the degree of its activity and the genetic sensitivity of mouse. In this study, we allowed plasmid which contain the objective PON-1 confined in a closed pericardial, thus it can continue to maintain contact with the surrounding tissue due to environmental constraints. PON-1 gene transfection was successfully observed by the re-examination, Western blot analysis discovered that PON-1 gene transfection can significantly improve PON-1 levels of the rabbit coronary, and can also reduce TC and LDL-C levels effectively, alleviate the pathological changes of intimal and medial. These results indicated that the increased expression of the local PON-1 gene of epicardial fat can suppress coronary atherosclerosis. Consider the mechanism of its occurrence is mainly based on the fat-vascular regulatory axis theory, we believe that the endocrine regulation and feedback loop exists between the cardiac fat pad tissue and surrounding blood vessels (such as coronary artery), thus can interact and regulate the formation of atherosclerosis.

After PON-1 gene successfully transfected the extracardiac fat, the PON-1 expression of local fat pad was significantly increased. Through the regulation of fat-vascular regulation axes, the expression of coronary vessel wall cells and intercellular PON-1 were significantly increased. Because PON-1 protein can decomposed lipid peroxides of LDL-C, it can reduce phagocytosis of the arterial wall macrophages to oxidized low-density lipoprotein particle, slow down the accumulation of arterial wall lipid. Studies suggest that in addition to inhibiting the oxidation of LDL-C, PON-1 protein can also combined with the serum HDL, protect the integrity of HDL and prevent HDL oxidation. The HDL itself has the function of anti-coronary atherosclerosis, so effectively maintain of HDL function can clear excess cholesterol of the tissue and inhibit the inflammatory reaction so as to against coronary atherosclerosis.

In summary, increased PON-1 expression levels of the epicardial adipose tissue is expected to become a new therapeutic approach to the prevention and treatment of coronary atherosclerosis. If we can solve the problem of local sustained release function of the PON-1 gene, it will provide new treatments for the prevention and treatment of the coronary atherosclerosis.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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