Original Article

Tight junction proteins and gap junction proteins play important roles in high fat dietary atherosclerosis pathogenesis

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Abstract: Atherosclerosis (AS) is a leading chronic diseases with high death rate in industrialized countries, where AS is caused by many factors. Studies show that tight junction protein (TJP) and Connexin family play important roles in heart and blood vessel function and health. Changing Gap Junction Protein (Cx43 Cx45 and Cx46) and Tight Junction Protein (Zo-1, Claudin-1 and Occludin-1) may have effects on AS pathogenesis. Here, we construct a rat model on a high-fat diet to explain the relationship between atherosclerosis pathogenesis and TJP/GJP changes. Our results showed that compared with control group, the weight of treatment group increased significantly with the formation of atheromas and the artery wall infiltration with adipose tissue in histological section. Our qPCR and western blotting results of heart coronary and artery endothelial tissue showed that Cx43/45/46 and claudin-1 were significantly down-regulated in coronary artery. In FITC-Inulin transwell experiment, a paracellular permeability test, we found that primary endothelial cell of high-fat diet rat group showed higher permeability compared to control group. Immunofluorescence staining experiment showed less Cx43 and Zo-1 protein expression and more CD14 monocyte penetration in heart and aorta wall in high-fat diet rat group. Our results suggested that atheroma formation might be due to the loss of TJP and GJP, causing higher permeability in rat coronary artery and thus promoted the pathogenesis of atherosclerosis.

Keywords: Tight junction protein, gap junction protein, atherosclerosis

Introduction

Atherosclerosis, also called as arteriosclerotic vascular disease or AS, is characterized by artery chronic degeneration and gradual changing of artery wall [1] which is due to the growth of the connective tissue, deposition of the cholesterol, fatty acid and calcium carbonate in the inside and outside cells [1, 2]. Collagen and proteoglycan gather in the arteries, which become harden and thicken and finally lose flexibility [3, 4]. AS is a widespread disease, but little were carried out. Symptoms of AS are severe, such as once the disease comes, it will come out angina pectoris, myocardial infarction, stroke, and other deadly diseases, mortality rate is high, the harm is great [3-5]. Hardening of the artery is a complex biological process, which involves quite a lot tissues (like epithelial cells, smooth muscle, monocytes, macrophages and platelets), along with various hormones and cytokines. Therefore so far there is not good atherosclerosis model and diagnosis technology [3, 6]. Therefore, it is important to study participation AS.

Previous studies have shown that AS is tightly associated with TJP and mang diseases. Tight junction protein TJP (tight junction protein) is served as adhesive structures between cells. Cell adhesion is the basic conditions for the robust stability of organic structures, and also as the factor of cell movement and the adjustment of function. It is also important for cell differentiation and cell proliferation [2, 7]. Tight junction is made of a bite protein occludin,
closed protein Claudin, adhesion molecules (junction adhesion molecule JAMs) and closed small ring protein ZO-week cytoplasm 1/2/3 [4, 7-9]. Scaffold proteins include claudins which are tightly coupled, and participate in the intercellular signal transduction. Aberrant expression of Claudin protein level has strong implications for tumorgenesis, cancer invasion and metastasis [8].

Gap junction (GJ), communication links, refers to the connection between two adjacent cells. Connection channel is arranged in a special membrane structure [1, 10-12]. Cell-cell interactions are mediated through gap junction intercellular space connection communication (GJIC) [10]. It passes on ions, small molecule metabolites and secondary signal, the formation of participating in the predictor of material exchange between cells metabolism and electrical coupling. It plays an important role in metabolism regulating, internal environment stability, cell proliferation and cell differentiation. Connection channel are not form single gap junction, but form a tightly bunched polymer or spot, ranging from a few to thousands. It varies with groups of different development stages. Components of gap junction channels are junction proteins (connexin, Cx) [1, 12]. Cx comes from a membrane transport protein family, which is channel of transmembrane ion and small molecules communication [1, 3, 12].

Previous studies suggest that one of major causes of atherosclerosis occurs is endothelial cell injury and adhering of monocyte to endothelial cells. Tight junction proteins and clearance proteins play an important role in the process. Somebody thinks that the human heart is given priority to with Cx40, 43, 45 [6, 11, 13]. Cx43 was mainly observed in the intercellular space of main connection from previous studies, which is highly expressed in myocardial tissue, macrophages, connective tissue cells, endothelial cells, endothelial cell, EC) and smooth muscle cells (smooth musclecell, SMc), fibroblasts, lens, corneal epithelium cells and also expressed in other tissues [13, 14]. Cx43 is encoded by a 2768 bp segment, which consists of 2768 base pairs (base pair, bp) of the three complementary cDNA. The composition of cDNA code contains a 1146-bp open reading frame, 43 kd for coding the molecular weight is single peptide, it contains 378 amino acids, so it is named Cx43 [11, 13]. Cx43 is synthesized in the endoplasmic reticulum ribosomes, and then gathered with six connection protein in golgi complex to form a half channel and finally transported to the plasma membrane, Cx43 was phosphorylated during the process of transportation, relating to the most structural protein. Cx43 have a short half-life, about a few hours [14]. Gap junction spot (also called gap junction plaque) consists of several Cx43 proteins, which is a transmembrane protein, and the quantity of Cx43 directly affects the GJIC function. Plexiform gap junction channels (GJ spot), which is composed of Cx43, can be influenced by the cAMP and microfilament. Phosphorylation of Cx43 is closely related to the function of the GJ. Studies have shown that gap junction protein 43 (Cx43) is the most important link protein in mammalian heart; studies have shown that it is essential for the normal differentiation of the heart and development. Abnormal expression of Cx43 leads to a variety of cardiovascular diseases and congenital malformation of the heart [6, 11-14].

In human, Cx45 is observed in two different places, one is myocardial cell surface, and the other is both sides of the dish. Cx45 distribution is different, which influences the regulation of the cardiac systolic function. The variation of gap junction protein 46 (Cx46) is associated with many diseases, such as breast cancer, cataract, etc [12]. The G143R missense mutation on connexin (Cx) 46 was recently reported to be associated with congenital Coppock cataracts [10, 16].

Common symptoms of atherosclerosis are high blood cholesterol, triglycerides, high-density lipoprotein cholesterol (hdl-c) and abnormal lipoprotein electrophoresis pattern. Additionally, most atherosclerosis patients suffer from the III or the IV type high lipoprotein hematic disease [5]. Furthermore, people bare symptoms of obesity, high cholesterol or diabetes are liable to suffer atherosclerosis as well [2, 5]. The research has confirmed that atherosclerosis is tightly associated with individual diet. However, whether high-fat diet leads to AS, and if so, to what extent does high-fat diet contribute to AS remains in the air. In order to address this problem, we established a high dietary fat rats’ model. By analyzing the expression level of Cx43/45/46, Occludin-1 and Claudine-1, along with histochemistry features of hyperglycaemia and blood vessels, we found that high-
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Table 1. The list of primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5‘ to 3’)</th>
<th>amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6-F</td>
<td>TGCGTGCTGGAGTC</td>
<td>96 bp</td>
</tr>
<tr>
<td>U6-R</td>
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<tr>
<td>R-zo-1-F</td>
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<tr>
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</tr>
<tr>
<td>R-cx46-F</td>
<td>TGGCTGACCTTGGTGCTTCA</td>
<td>160 bp</td>
</tr>
<tr>
<td>R-cx46-R</td>
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<tr>
<td>R-cx43-F</td>
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<td>243 bp</td>
</tr>
<tr>
<td>R-cx43-R</td>
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<tr>
<td>R-cx45-R</td>
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<tr>
<td>R-claudin-1-F</td>
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<td>237 bp</td>
</tr>
<tr>
<td>R-claudin-1-R</td>
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<td></td>
</tr>
<tr>
<td>R-occludin-1-F</td>
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<td>183 bp</td>
</tr>
<tr>
<td>R-occludin-1-R</td>
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Materials and methods

Subjects and samples

SD rats (14 weeks) was purchased from Sino-British Ltd. Rats were randomly mixed, and divided into two groups, one group of the experimental group and another group as the control group. The experimental group of rats fed the way to feed the oil 6 ml per day per rat and mixed with oil as the sole feed. Rat food of control group received no treatment. The two groups of rats were drinking deionized water. Rats were fed three months. Three-month period, every two weeks for a body weight of rats were weighed. Monthly anatomically one pair of rats gets back dorsal aorta blood vessels and heart. They were divided into three parts, one was for HE staining pathology, one was for qPCR, and one was for western blotting. Rats were weighed periodically data tables and statistics SD rat animal level, the initial weight of rat weight changed growing. And the overall-like recorded rats.

Primary cell culture

Vascular endothelial cell line was obtained from the Shanghai Cinoaisa Institute, and they respectively come from a test group SD rat and a control group SD rat. Vascular en-do-thelial cells were grown in Dul-becco’s Modified Eagle Medium/F12 (DMEM/F12) (Gibco) with 20% Fetal Bovine Serum (FBS) (Gibco), streptomycin (10 mg/mL) (Sangon)/penicillin (10 KU/mL) (Sangon)/am-photericinB (2-50 ug/ml) (Sangon) at 37°C with 5% CO2 supplement.

Transwell paracellular permeability assay

Make the vascular endothelial cells which have been cultured become cell suspension, pave cell suspension to the transwell. Both experimental group and control group pave the three holes, the cell number is 4*10^4 every hole. After adding cell, respectively the 0 d, 3 d, 6 d, 9 d, make Cell permeability test by transwell. Add 50 ul Inulin-FITC (Gibco) into every under test hole, finish this process in one minute. After adding Inulin-FITC (gibco), take out every hole down solution 50 ul at 1 min, 5 min, 15 min, 30 min, 45 min, 1 h, 2 h. Separately add them into 96-well-plate, and then dropwise add complete medium 50 ul in the 96-well-plate. At last the number of OD is determined by enzyme-labeled instrument in 500 nm wavelength.

Real time quantitative PCR

Using Trizol regent to isolate the heart and blood vessel's total RNA from tissue samples of Rattusnorvegicus, the quantity and quality of RNA were confirmed with a NanoDrop 1000 (NanoDrop, Thermo Scientific-Waltham, MA, USA), the primers were designed using primer primer6.0 software and synthesized from Generay Biotech (Table 1). For gene specific reverse transcription, based on the specification of ReverTta Ace qPCR RT Kit (Toyobo) synthesized the first strand. Quantitative real time PCR were conducted on FTC-3000 (Funglyn, Canada) with SYBR Green Fast qPCR kit (KAPA), thermal cycling parameters were as follow. Under the following conditions: 95°C for 3 min (enzyme activation), the next stage were repeated 40 times : 95°C for 5 s (denaturation), and 60°C for 30 s (annealing/extension/data acquisition). Data were analyzed by the 2-△△Ct algorithm.
Western bolt

Cell lysates were prepared using RIPA buffer (Sigma) containing protease inhibitors (Roche), subsequently agitated on ice for 30 minutes. Pierce™ BCA Protein Assay Kit (Pierce) was used to determine the protein concentration. Protein electrophoresis was performed with Mini-PROTEA III (Bio-Rad). In 10% polyacrylamide gels (Tris/glycine), proteins were separated and transferred onto polyvinylidene fluoride membrane (Bio-Rad). Primary and secondary antibodies were labeled subsequently. Antibodies included Cx43 (rabbit polyclonal anti-Cx43, 1:1000, Sigma-Aldrich), Occludin-1 (rabbit polyclonal anti-Occludin-1, 1:1000, Abcam), GAPDH (rabbit polyclonal anti-GAPDH; 1:2500, Abcam). Goat anti-rabbit IgG-HRP secondary antibody was purchased from Santa Cruz. Experiments were performed in triplicate.

Immunofluorescence stain

Sections were fixed in 10% formalin for 12 hours, and then embedded in paraffin. 3-μm sections were stained with primary antibody and fluorescent labeled secondary antibody. 100 times magnification field microscopy is applied to capture typical images through Olympus IX71 microscope and Q-IMAGE camera. Antibodies included Cx43 (rabbit polyclonal anti-Cx43, 1:1000, Sigma), Zo-1 (rabbit polyclonal anti-Zo-1, 1:1000, Abcam) and CD14 antibody (rabbit polyclonal anti-CD14, 1:1000, Bioss).

Results

Compared with the control group, the trend of rat weight of which group is treated by high fat diet show higher body weight than that of normal diet. (Figure 1) However no significant difference was found between the two groups probably due to a small number of samples. In histological section, sub-endothelial fat infiltration and atheromas were shown which indicated success of establishment of model. (Figure 2B) FITC is one of the polymers which can pass through the TJPs formed cell-cell layer. We used Spectrophotometer to determine the concentration of Inulin - FITC passing through the cellular layer from upper well to lower one. Once the TJPs between endothelial cells are destroyed, Inulin-FITC can easily through the cell layer as (Figure 3). Dyeing cell culture in different time point, the value of relative fluorescence intensity was measured. The RFI value in the control group and treatment group increased slowly with the passage of time in 0 d and 3 d two time points and showed no significant difference between control group and high fat diet group. However, in 6 d and 9d tests, several time points were revealed to have a higher permeability in high fat diet group. Thus it can draw the conclusion: two groups of cells in 3 d and 6 d were to form the tight junction protein which show higher permeability while cells of high fat diet treatment group in 6 d and 9 d showed dysfunctional paracellular barrier.

By qRT-PCR assay, the expression of Claudin-1, Cx43, Cx45 and Cx46 amount is lower in the treatment group than the control group in heart coronary artery. Occludin-1 and ZO-1 showed no difference in this tissue between two groups. While it is interesting that only expression quantity of Occludin-1 showed significant lower in aorta endothelia in the high fat diet group. These results suggested that coronary artery is more sensitive to endothelial lesion and atherosclerosis formation than aorta (Figure 4). We select Cx43 and Claudin-1 as TJP and GJP representatives for western blotting validation on coronary artery tissues (Figure 5).

By immunofluorescence staining, while the expression of Zo-1, Cx43 is lower and discontinuous in the treatment group than the control group in heart coronary artery, CD14 positive monocyte is shown prone to infiltrate aorta wall in experimental group (Figure 6).

Discussion

In this study we found that a high-fat diet lead to quite a lot histological and functional changes in both coronary and aorta artery, which
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We successfully establish a rat high fat diet model, which helped us conduct further researches on the biological mechanism of atherosclerosis pathogenesis. Adipose infiltration and atheromas were served as markers of the model. qRT-PCR and WB experiments were performed to evaluated three major proteins of GJP, Cx43, Cx45, Cx46, and three TJP for ZO-1, Claudin-1 and Occludin-1. Remarkably, we found that all GJPs and Claudin-1 were down-regulated in high-fat diet rat group, compared with control.

Figure 2. Sub-endothelial fat infiltration and atheromas were shown which indicated success of establishment of model. High fat diet model was successfully established as shown in (B) compared to normal control shown in (A).

Figure 3. The RFI value in the control group and treatment group showed no significant difference between control group and high fat diet group in 0 d and 3 d. However, in 6 d and 9 d tests, several time points were revealed to have a higher permeability in high fat diet group.
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**Figure 4.** The expression of Claudin-1, Cx43, Cx45 and Cx46 amount is lower than the control group in heart coronary artery in high fat diet group. However, only Occludin-1 showed significantly lower in aorta endothelia in the high fat diet group compared to normal control.

**Figure 5.** The protein expression level of Claudin-1 and Cx43 from control group and high fat diet group were validated by western blotting assay in heart coronary artery tissues. Results showed the concordance with qRT-PCR quantitation results.
Figure 6. Immunofluorescence staining shows that the expression of Cx43 (A), Zo-1 (B) is lower and discontinuous in the treatment group than the control group in heart coronary artery, CD14 positive monocyte (C) is shown prone to infiltrate aorta wall in experimental group.
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Disclosure of conflict of interest

None.

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References


