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Traditional Chinese medication Tongxinluo attenuates apoptosis in ox-LDL-stimulated macrophages by enhancing Beclin-1-induced autophagy

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ABSTRACT

In advanced atherosclerosis, a large number of necrotic core increases plaque vulnerability, which leads to the occurrence of acute atherothrombotic cardiovascular events. Macrophage apoptosis plays an important role in secondary necrosis. The present study aimed to examine and describe the effect of the traditional Chinese medication Tongxinluo (TXL) on macrophage apoptosis in advanced atherosclerotic plaques and to explore its mechanism. By observing the effect of TXL on ox-LDL-stimulated macrophage apoptosis, it was shown that TXL significantly inhibited ox-LDL-induced apoptosis of macrophages by enhancing autophagy. Therapeutic mechanism of TXL included increasing the expression of Beclin-1 and improving the dissociation of Bcl-2-Beclin-1 Complex. Apolipoprotein E knockout (apoE−/−) mice with a high fat diet were divided into four groups: saline group (Saline gavage), low dose TXL group (0.38 g/kg/d, gavage), medium dose TXL group (0.75 g/kg/day, gavage), and high dose TXL group (1.5 g/kg/day, gavage). 4 weeks after carotid-artery surgery, lentiviral of Beclin-1 silencing was injected through the tail vein. TXL treatment significantly reduced macrophage apoptosis dose-dependently and the result was blocked by Beclin-1 silencing. In addition, the increased Lc3b dots by TXL almost localized to macrophages in advanced atherosclerotic plaque. Compared with the same dose of TXL shBeclin-1 group, plaque area and vulnerability index of TXL groups decreased. The anti-apoptosis effects of TXL on atherosclerosis was related to the improvement of autophagy via Beclin-1.

1. Introduction

Cardiovascular disease is one of the three leading causes of chronic noncommunicable diseases that cause the most deaths, and goes far beyond the cancer's death rate. From 2006 to 2016, the number of deaths caused by global cardiovascular and cerebrovascular diseases increased by 14.5% [1].

Atherosclerosis is a type of circulatory system diseases which was closely linked to cardiovascular and cerebrovascular events (ischemic heart disease and brain disease). Its mechanism is complex with many factors involved. Macrophages play a crucial role in the progression and development of atherosclerosis [2]. Due to the injury of the vascular endothelium, a large amount of monocyte chemotactic factor is released, monocytes in the blood are recruited, migrated under the endothelium, and induced to macrophages. Macrophages entering the vessel wall constantly phagocytose lipoproteins until they become foamy cells [3]. In advanced plaques, macrophage apoptosis can lead to plaque necrosis and aggravation of inflammation, resulting in the production of acute intraluminal thrombus caused by vulnerable plaque, leading to unstable angina, myocardial infarction and even heart sudden cardiac death or stroke [4]. Therefore, inhibiting such apoptosis may be a very important approach to delay the progression of atherosclerosis, improve plaque stability and thus reduce the incidence of acute cardiovascular events in the future

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treatment.

Autophagy is a lysosomal-dependent cell self-protection mechanism which is evolved in the response to external environmental stimuli during eukaryotic cell evolution and is also an important cell death-related mechanism as well as apoptosis. The relationship between apoptosis and autophagy is very complicated, which changes with the external environment [5]. Experiments by knocking out Atg7 or Beclin-1, or utilizing the autophagy inhibitor 3-MA have shown that inhibition of autophagy inhibits caspase activation and reduces apoptosis [6–8]. And in contrast, by inhibiting apoptosis, the induction of autophagy markers is accelerated and the death of some cells is reduced, which means autophagy is an upstream functions of apoptosis.

Tongxinluo is a traditional Chinese medicine made of 12 kinds of ingredients. Since its approval by the State Drug Administration of China in 1996, it has been widely used in the treatment of atherosclerosis, coronary heart disease, angina pectoris, myocardial infarction and stroke etc. [9,10]. Previous experimental study found that Tongxinluo has a variety of roles in the aspect of cardiovascular system protection, such as anti-oxidation, anti-inflammatory, anti-angiogenesis and protection of vascular endothelial function. Tongxinluo could stabilize atherosclerotic rabbit vulnerable plaque; inhibit plaque inflammatory angiogenesis; promote myocardial ischemia in mice after myocardial angiogenesis and so on [11–14]. However, it remains unclear whether Tongxinluo affects the stability of the plaque by regulates macrophage apoptosis in advanced atherosclerotic plaques.

The purpose of this study is to examine and describe the therapeutic effects of TXL on macrophage apoptosis and plaque stability both in vitro and in vivo, and to explore the underlying mechanisms.

2. Materials and methods

2.1. Ethics statement

The present research program was approved by Ethics Committee of Shandong University Qilu Hospital. All in vivo programs comply with the Guidelines for the Care and Use of Laboratory Animals published in the National Institutes of Health (8th edition, 2011) and the Chinese Ministry of Health Animal Management Code (Item No. 55,2001).

2.2. TXL preparation

TXL ultrafine powder was obtained from Yiling Pharmaceutical Co. Ltd (Shijiazhuang, China). For in vitro experiments, TXL ultrafine powder was resolved ultrasonically in serum-free RPMI 1640 (Gibco, USA). The solution was centrifuged at 3500 rpm for 20 min. The supernatant was filtered through Sterile Syringe Filter (Merck KGaA, Germany) [15]. For in vivo experiments, TXL ultrafine powder was dissolved in saline, and administrated to mice daily.

2.3. Cell culture

Human acute monocytic leukemia cell line (THP-1) was obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin. To differentiate cells into macrophages, 160 nM phorbol myristate acetate (PMA) was used overnight. THP-1 macrophages were treated with or without TXL for 24 h and then stimulated with 50 mg/L recombinant human ox-LDL for 12 h.

2.4. TUNEL assay

Apoptosis was measured using terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL). TUNEL was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Merck KGaA, Germany).

2.5. Flow cytometry

Apoptosis was measured using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, USA). Apoptotic cells were examined within 1 h using flow cytometer 2.6(Becton-Dickinson, USA).

2.6. siRNA and RNA interference

Beclin-1 siRNA and negative control siRNA (GenePharma, China) was used in Opti-MEM (Gibco, USA) with Lipofectamine 3000 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The DNA target sequence for Beclin-1 siRNA is 5′-CAGTTTGGCACAATCAATA-3′.

2.7. Immune coprecipitation

Cell lysates were pre-cleared and incubated with cognate antibodies and 100 μL Protein A magnetic beads (Pierce, USA) overnight at 4 °C with gentle rotation. Magnetic beads-bound immunoprecipitates were rinsed and subjected to SDS–PAGE.

2.8. Atherosclerosis animal model protocol

Male ApoE−/− mice (8 weeks old, 18–23 g) were purchased from Peking University Animal Research Center (Beijing, China) and given an atherogenic diet (1.25% cholesterol and 40% cocoa butter). The atherosclerotic models were created as previously described [16]. The mice were randomly divided into four groups: a saline group (Gavage saline), a low-dose TXL group (TXL-L, Gavage dose of 0.38 g/kg/day), a medium-dose TXL group (TXL-M, Gavage dose of 0.75 g/kg/day), and a high-dose TXL group (TXL-H, Gavage dose of 1.5 g/kg/day). 4 weeks after the carotid-artery surgery [17], 200 μL lentivirus was injected through the tail vein. 4 weeks after transfection, all mice were euthanized.

2.9. Lentiviral silencing

Lentiviral vector pGLV3/H1/GFP + Puro (pGLV3) was purchased from GeneChem (Shanghai, China). Short-hairpin RNA sequence targeting Beclin-1 and scrambled control RNA were cloned into the vector.

2.10. Immunofluorescence staining

Aorta roots fixed in 4% formaldehyde were placed in OCT compound to make 5 μm thick sections. Aorta roots sections were blocked with 5% BSA and incubated with primary antibodies overnight at 4 °C. After rinsed with PBS, sections were incubated with Alexa Fluor 488 or Alexa Fluor 594 conjugated secondary antibodies. Nuclei were stained with DAPI (Roche, Germany).

2.11. Histopathology and immunohistochemistry

Aorta roots sections were stained with haematoxylin and eosin (H&E) for plaque morphology, with oil red O for lipids and with sirius red for collagen. Sections were incubated with alpha-SMA (1:100) for smooth muscle cells (SMCs) and Moma-2 (1:100) for macrophages overnight at 4 °C. The sections were then incubated with secondary

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