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Research Paper

Inhibitory effect and mechanism of chuanxiongzine on multiplication of VSMC

Qingxian Li[a], Yuhua Liao[a], Huiling Zhang[b], Yanying Jiang[c], Yanfu Wang[a,*]

[a]*Department of Cardiology, Union Hospital, Tong Ji Medical College, Hua Zhong University of Science & Technology, Wuhan 430022, China*

[b]*Department of Cardiology, Affiliated Hospital, Jining Medical College, Jining, 272029, China*

[c]*Department of Ophthalmology, Affiliated Hospital, Jining Medical College, Jining 272029, China*

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Abstract

Objective: To study the inhibitory effect of chuanxiongzine on vascular smooth muscle cell (VSMC) proliferation and explore its molecular biology basis. **Methods**: we selected the VSMC cultured 4~8 generation from rat aorta thoracalis as research object. The objects were divided into four groups(Ⅰ)control group, (Ⅱ)chuanxiongzine(50 μg/ml)group, (Ⅲ)chuanxiongzine (100 μg/ml) group and(Ⅳ) chuanxiongzine(200 μg/ml) group. The inhibitory effect of chuanxiongzine on VSMC proliferation was investigated by cell counting, MTT and ^{3}H-TdR incorporation assay. In order to illuminate the molecular biology mechanism of chuanxiongzine inhibiting VSMCs proliferation, the expression of proliferating cell nuclear antigen (PCNA) and C-myc were detected. **Results**: Chuanxiongzine could inhibit the proliferation of VSMC significantly in a dose- and time-dependent manner, compared with control group ($P < 0.05$). The expression of PCNA and c-myc were inhibited obviously and correlated with the concentration of chuanxiongzine ($P < 0.05$). **Conclusion**: Chuanxiongzine may play a considerable role in VSMC proliferation process. The inhibitory effect of chuanxiongzine in a dose- and time-dependent manner can be realized via down regulating the expression of PCNA and c-myc. In this study, The great theoretical fundament about Chinese medicine, which is used to treat atherosclerosis (AS), has been obtained.

Keywords: chuanxiongzine; vascular smooth muscle cell; proliferating cell nuclear antigen; c-myc

INTRODUCTION

Atherosclerosis (AS)is a common and serious disease involving many tissues and organs[1]. The pathogenesis of AS has been focused on in recent years. The fundamental pathological changes of AS are cell proliferation and degeneration, of which, VSMC proliferation plays a critical role [2]. Berditt et al proposed that the mechanism of AS might be similar to that of tumors in 1975. Recent studies have shown that AS might belong to benign tumor of VSMC[3]. The process of AS formation is similar to that of some tumors, and its occurrence and development depend on the result of cell proliferation and apoptosis.

Traditional Chinese medicine is the essence of conventional iatrology in China. Obvious effectiveness of Chuan xiong zine can be found by promoting blood flow and dissolving congestion of general complex prescription of traditional Chinese herbs, the main component is chuanxiong and its major active ingredient is chuanxiongzine [4]. Previous studies have shown that not only could chuanxiongzine dilate small artery-vein, resist platelet collection, but also might inhibit VSMC proliferation.

Studies on PCNA have shown that there existed critical link of signal conduction inthe process of DNA duplication, cell cycle and fission [5,6]. C-myc proto-oncogene might play an important role during the process of VSMC proliferation, too. The VSMC gene expression would increase quickly, when the cells proliferate vigorously[7].

*Corresponding author.

E-mail address: wyf1369@yahoo.com.cn.

In this study, we investigated the inhibitory effect of chuanxiongzine of different concentrations on VSMC proliferation. In order to illuminate the mechanism of chuanxiongzine at molecular biology level, we also detected the expression of PCNA and c-myc, which have been closely related with VSMC proliferation.

MATERIALS AND METHODS

Reagents

Chuanxiongzine injection(Beijing Four-ring Pharmaceutical Co., Ltd.); ^{3}H-TdR(Department of Isotope Labeling, AERE, China); MEM culture solution and HEPES (GIBCO); calf serum (Sijiqing Biotechnology Company, Zhejiang); 5%TCA; scintillation fluid; c-myc antibody(mouse anti rat, Santa Cruz); PCNA antibody (mouse anti rat, Zymed); SP routine dyeing kit (Zymed).

Laboratory apparatus

CO_2 cell incubation (Queue), superclean bench, microscope (Olympus), auto double purified water distilling apparatus, electrothermal homeothermial drying oven, desk-top disinfector, 96-well enzyme labeling apparatus, liquid scintillation counter (Beckman-LS3801), multichannel cell harvestor, scintillating dise, etc.

Cell culture

All procedures were carried out as described by Zhao Sanmei [9], taking out of the aorta thoracalis, culture the VSMC of the blood vessel, undertake passage after 6~7 days when the "peak-valley" interlaced pykno-cell layer formed. The cultured cells in the study were demonstrated by contrast phase microscope and transmission electron microscope. The passage cells were digested by using mixed liquor of 0.125% parenzyme and 0.02% EDTA. The 4th~8th generation cultured cells were employed as res earch object.

Methods

The cells were plated at 2×10^5 cells/ml in 6-well cell culture plates and incubated in 2% fetal bovine serum/MEM, 5% CO_2 in air at 37°C for 16 h. After washing away nonadherent cells, according to the difference of experimental group, the cells were fed with different final concentration chuanxiongzine: Ⅰ (0 μg/ml); Ⅱ (50 μg/ml); Ⅲ (100 μg/ml); Ⅳ (150 μg/ml). And then, cell counting was carried out at 24, 48 and 72 h, after cultured continuous for 24, 48 and 72 h, re spectively, and every well was counted for 3 times.

MTT assay[8]

The digested VSMCs were diluted to 2×10^4 cells/ml and plated at 96-well cell culture plates (200 μl/well). Simultaneously, different concentrations of chuanxiongzine were added. MTT (20 ml, 10 μg/ml) was added into each well after cultured for 48 h, and then the cells were incubated for 48h in the same condition. After that, DMSO 200 μl was added. After thoroughly mixed, we detected the absorption value (570 nm) of every well at 96-well enzyme labeling apparatus.

^{3}H-TdR incorporation assay[9]

The cells were cultured with chuanxiongzine for 48 h, and then every well cells were fed with ^{3}H-TdR 18.5 Kbq. In order to make 3H-TdR incorporate into de novo synthetic DNA, the cells were cultured for another 6 h. After having discarded nutrient solution and washed the cells with D-Hanks for 2 times, and then NaOH(1 ml, 0.1 M) was added to dissolve the cells. The radioactivities (counts per minute, CPM) of samples were detected by liquid scintillation counter.

Immunohistochemical staining

The cells of 6-well culture plates were fixed by 2% paraformaldehyde for 20min. Then the polyclonal antibody of anti-c-myc and anti-PCNA (1:50 dilution) were added in the culture plates, incubated at 37℃ for 1h. After having been washed away unconjugated antibody, the signal was magnified by actin. Routine-SP dyeing was carried out according to the protocol provided by the kit. Ⅰ Group with PBS was taken as negative control group. Outcome assessment was peaformed as follows: the number of positive cells was counted with light microscope (×400), and the percentage of positive cells was calculated.

Statistical analysis

Statistical analysis was performed with SPSS (Version 13.0). Data were presented as mean±SD. The differences between the groups were evaluated by Student's test. $P < 0.05$ was considered statistically significant.

RESULTS

VSMC proliferation was inhibited by chuanxiongzine

To examine the inhibitory effect of chuanxiongzine on VSMC proliferation, we calculated the percentage of positive cells. As shown in ***Fig. 1***, the inhibitory effect of chuanxiongzine on VSMC prolif-

eration could be found in a dose- and time-dependent manner (compared with control group, $P < 0.05$).

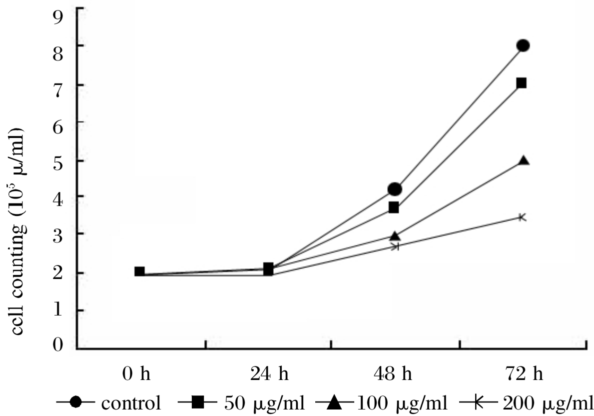

Fig 1 **Effect of chuanxiongzine on VSMC proliferation.**

Chuanxiongzine reduced the OD_{570} value

As shown in ***Fig 2***, the OD_{570} value was inhibited obviously by chuanxiongzine, and the inhibitory effect took on a dose-dependent manner (compared with control group, $P < 0.05$).

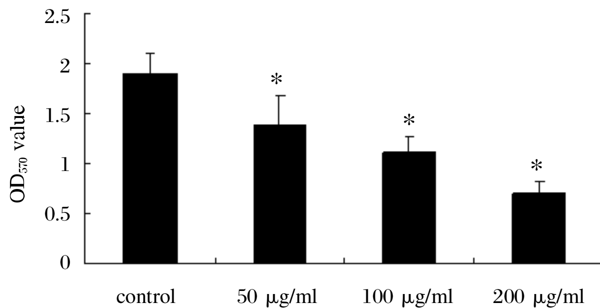

Fig 2 **Alteration of cellular OD_{570} value after being fed with different doses of chuanxiongzine**

^{3}H-TdR incorporation was depressed

To confirm that the intake of ^{3}H-TdR in VSMC could be depressed significantly by different concentrations of chuanxiongzine, we detected the radioactivities by liquid scintillation counter. The result was shown in ***Fig 3***(Compared with control group, $P < 0.05$).

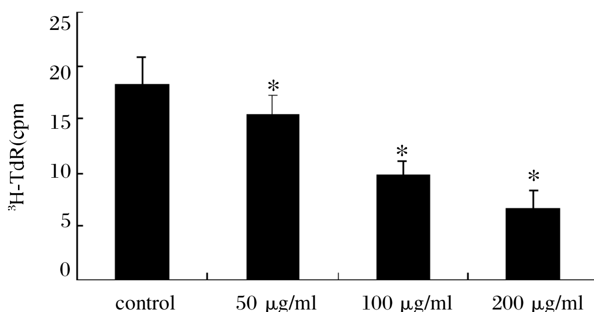

Fig 3 **Effect of chuanxiongzine on the intake of ^{3}H-TdR in VSMC.**

Chuanxiongzine could down-regulate the expression of PCNA and C-myc

As shown in ***Tab 1***, the expression of PCNA and c-myc in VSMC could be depressed obviously by chuanxiongzine with different concentrations in a dose-dependent manner (compared with control group, $P < 0.05$).

Tab 1 **The inhibitory effect of chuanxiongzine on expressions of PCNA and c-myc**

Experiment groups	positive rate (%)	
	PCNA	C-myc
Ⅰ(0 μg/ml)	91.7	95
Ⅱ(50 μg/ml)	78.5	86.3
Ⅲ(100 μg/ml)	42.7	45.7
Ⅳ(200 μg/ml)	20.3	17

DISCUSSION

VSMC is the supreme cellular composition within atherosclerotic plaque, which can capture and deposit a great quantity of cholesterol and lipid to form foam cells. VSMC also can synthesize and secrete lots of extracellular matrix, such as collagen, which can bind with ecto-fat to form fatty streak[10]. However, the appearance and development of these pathological changes mainly resulted from the abnormal proliferation of VSMC [11, 12]. The proliferation of VSMC was regulated by many growth factors [13], such as TGF-β1 and epidermal growth factor. These factors stimulate the receptors on cell surface, and then activate the signal conduction pathway, which introduced cell proliferation [14]. During this process, PCNA is the critical link of signal conduction pathway, which is imperative for DNA duplication、cell cycle and fission process[15,16]. C-myc proto-oncogene is a kind of DNA binding proteins, which plays a major role during VSMC proliferation [17]. Proliferative VSMC and other cell categories can synthesize c-myc protein and take on growth dependence. The expression of c-myc would increase quickly during the prosperity of cell proliferation; otherwise, the expression would take on low level [18, 19]. Looking for an effective medicine to resist the proliferation of VSMC and investigating its mechanism have become a focal point in the treatment of AS.

Chuanxiongzine has an inhibitory effect on the proliferation of VSMC [20]. The mechanism might be correlated with the expression of c-myc [21]. In this study, we investigated the influence of chuanxiongzine on the proliferation of VSMC via cell counting, ^{3}H-TdR incorporation and MTT assay. We demonstrated that chuanxiongzine was the inhibitor

of VSMC proliferation. We detected the expression change of regulating gene correlated with the process of VSMC proliferation via immunohistochemical staining when the cells had been incubated with chuanxiongzine. We found that there was internal relationship between PCNA, c-myc proto-oncogene and VSMC proliferation. The inhibitory effect of chuanxiongzine on VSMC proliferation might be correlated with PCNA and c-myc, whose expression might be inhibited obviously by chuanxiongzine.

In our study, we demonstrated that chuanxiongzine could obviously inhibit the proliferation of VSMC. This effect was made by regulating the expression of PCNA and C-myc. The present study may illustrate the mechanism of the occurence and development of AS and present a theore tical basis for further clinical application of chuanxiongzine.

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