Effects of angelica polysaccharides on the proliferation of mouse skeletal muscle satellite cells in hematopoietic microenvironments *in vitro***

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Abstract

BACKGROUND: It is hopeful that skeletal muscle satellite cells (SMSCs) can be served as seed cells for hematopoietic reconstitution. Angelica polysaccharides (APS) can not only promote hematopoietic stem cells and hematopoietic progenitor cells proliferation and differentiation, but also change the growth characteristics of SMSCs.

OBJECTIVE: To investigate the effects of APS on the proliferation of mouse SMSCs in different culture environments.

METHODS: SMSCs were procured by a modified method from new born mouse. The α-actin protein of the SMSCs was examined by immunohistochemistry at 5 days after culture. SMSCs were cultured and synchronized for 24 hours in the 96-well plate. After that, SMSCs were assigned into the blank control group, marrow stroma cell supernatant group, APS DMEM/F12 groups (contained 50, 100, 200, 300, 400 mg/L APS) and the marrow stroma cell conditioned medium (disposed by 50, 100, 200, 300, 400 mg/L APS in DMEM/F12). The proliferation of SMSCs was determined by MTT.

RESULTS AND CONCLUSION: The α-actin was positive in the cultured SMSCs. MTT results demonstrated that, SMSCs showed a proliferative property in the marrow stroma cell conditioned medium groups. Additionally, the marrow stroma cell conditioned medium can effectively alter growth characteristics of SMSCs in a dose-dependent manner.

INTRODUCTION

Angelica sinensis is a common traditional Chinese medicine prominent for functions in nourishing blood and activating blood circulation. Plenty of pharmacological studies found that Angelica sinensis plays its role *via* Angelica polysaccharides (APS), a main effective constituent of angelica sinensis, which promote hematopoietic function[1-2]. Skeletal muscle satellite cells (SMSCs) are muscle-derived stem cells, located between the sarcolemma and basement membrane of terminally-differentiated muscle fibers, and have the potential to proliferate and differentiate[3-4]. The hematopoietic potential of SMSCs has reported in China and abroad[4-6]. The aim of this paper is to investigate the effects of APS on the proliferation of mouse SMSCs in different culture environments, and to provide an experimental basis for the research of promoting recovery of hematopoietic function using Chinese drugs.

MATERIALS AND METHODS

Design: An *in vitro* cytology observation.

Time and setting: The experiment was performed at the Tertiary Laboratory of Pathology, Tianjin University of Traditional Chinese Medicine, from April 2007 to December 2009.

Materials: Ten male, 5-day-old, Kunming suckling mice, and 15 Kunming mice, weighing 20 g, were provided by the Experimental Animal Center of Tianjin University of Traditional Chinese Medicine [Animal certification number: SCXK(Jin)2009-001]. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[7].

Main reagents used are as follows:

Reagent	Source
DMEM/F12 culture medium, fetal bovine serum	Shanghai Shangbao Biological Technology Co., Ltd, China
Type Ⅱ collagenase, trypsin	Sigma, USA
α-actin monoclonal antibody, MTT, dimethyl sulfoxide	Tianjin Yitailong Science and Technology Development Co., Ltd., China
APS	Gansu Min County Kangda Biological-Technological Co., Ltd. , China

Methods

Preparation of marrow stroma cell conditioned medium: The bilateral femurs were obtained from 20 g Kunming mice under aseptic condition, cultured in DMEM/F12 medium, pipetted, filtered to prepare single cell suspension, and suspended in DMEM/F12 supplemented with 10% fetal bovine serum after centrifugalization. The cells were seeded in a 10 cm culture flask. The suspended cells were discharged and the culture medium was exchanged at 24 hours after culture. After that, the culture medium was renewed every third day. When these cells grew adhere to the wall at 7 days, the culture medium were replaced by DMEM/F12 supplemented with APS (100, 200, 300 mg/L, respectively) for 1 day.

The supernatant was harvested, filtrated by 0.22 μm filtration membrane, and discharged cells or cell debris.

Isolation and culture of SMSCs: SMSCs were isolated from the skeletal muscle of 5-day-old mice's hind limbs. After fat removal, skeletal muscles were washed with DMEM/F12 culture medium and cut into pieces, washing, and discharging the upper liquid and floating tissues. The cells were digested in type Ⅱ collagenase (0.1%) at 37 ℃ for 25 minutes,

centrifuged at 1 000 r/min for 10 minutes.
The supernatant was discarded, and the cells were digested in trypsin (0.125%) for 5 minutes at 37 ℃, centrifugation, followed by discarding supernatant. Culture medium was added into the cells, collected filtrate, centrifugated, and differential velocity adherent for 1 hour in DMEM/F12 supplemented with 20% fetal bovine serum. The suspension at a density of $1\times10^5/cm^2$ was incubated in an incubator. The culture medium was firstly renewed after 5 days, and then replaced every second day. An inverted microscope was used to observe SMSCs.
Experimental grouping: The cells were assigned into the blank control group, marrow stroma cell supernatant group, APS DMEM/F12 groups (contained 50, 100, 200, 300, 400 mg/L APS) and the marrow stroma cell conditioned medium (disposed by 50, 100, 200, 300, 400 mg/mL APS in DMEM/F12).
SMSCs identified by α-actin immunohistochemistry: Cell slides were fixed by 40 g/L paraform, incubated with α-actin first antibody (1:300) overnight at 4 ℃, followed by incubation with biotin labeled second antibody for 30 minutes at room temperature, colored, dehydrated, cleared, and mounted. A light microscope was used to observe SMSCs.
Cell proliferative activity determined by MTT: SMSCs at a density of 1×10^7/L were seeded into 96-well plate, and incubated using culture medium supplemented with 20% fetal bovine serum in 5% CO_2 at 37 ℃ for 5 days. After that, the cells were intervened by grouping methods, cultured for 24 hours, further incubated for 4 hours with added 20 μL MTT (5 g/L), the culture medium was suck out, and 150 μL dimethyl sulfoxide was added, shuck for 10 minutes, finally, the absorbance value was measured at the 570 nm of an enzyme-linked analyzer.

Main outcome measures
Immunohistochemistrical results and cell proliferative activities.

Statistical analysis
The data were expressed as Mean ± SD. Statistical analysis was performed using one-way analysis of variance with SPSS 16.0 software.

RESULTS

Morphological changes of SMSCs under an inverted microscope
SMSCs presented with spherical shape, with strong optical activity following double-enzyme digestion and differential adhesion. After 12 hours in culture, some adherent SMSCs were observed. At 48 hours in culture, a great number of mononuclearcells appeared, presented with fusiform or atractoid. After 72 hours, SMSCs were turned to be slender, with mutually connections, indicating SMSCs turned to phase of symplasts. At 4 days after culture, well-differentiated cells exhibited spontaneous contractility under an inverted microscope (Figure 1). Myotubes and spindle-shape myocytes showed parallel arrangement and interval distribution, once in a while, satellite cell cytoplasm inserted into myotube cytoplasm through the myotube surface or one end of satellite cells, demonstrating the mature process of satellite cell fusion.

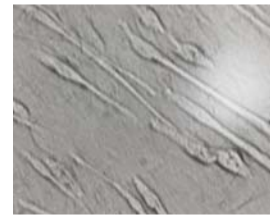

Figure 1 Morphological changes of skeletal muscle satellite cells at 7 d after culture (Inverted microscope, ×200)

Identification of SMSCs using α-actin immunohistochemistry
Immunohistochemistrical results showed that SMSCs, rather than fibroblasts, were positive to α-actin (Figure 2).

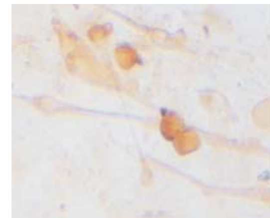

Figure 2 Positive expression of α-actin in skeletal muscle satellite cells

Proliferation of SMSCs in different culture conditions were determined by MTT (Table 1).

Table 1 Effects of culture conditions on skeletal muscle satellite cells survival (*n*=8)

Group	A ($\bar{x}\pm s$)	Cell survival ratio (%)
Blank control	0.083±0.006	0
Marrow stroma cell supernatant	0.090±0.007	108
Angelica polysaccharides		
50 mg/L	0.085±0.003	102
100 mg/L	0.090±0.006	107
200 mg/L	0.096±0.007	115
300 mg/L	0.114 ± 0.011^{ab}	137
400 mg/L	0.093±0.003	112
Conditioned medium		
50 mg/L	0.126 ± 0.006^{ab}	150
100 mg/L	0.135 ± 0.011^{ab}	161
200 mg/L	0.158 ± 0.002^{ab}	189
300 mg/L	0.154 ± 0.002^{ab}	184
400 mg/L	0.149 ± 0.004^{ab}	179

$^aP < 0.05$, $^bP < 0.01$, *vs.* blank control group

APS could promote SMSCs proliferation in a dose-dependent manner at low concentrations (50–200 mg/L), but the function would be weakened at high concentration. Compared with the blank control group, the promotion was more evident in the marrow stroma cell conditioned medium groups ($P < 0.05$). Particularly, marrow stroma cell conditioned medium can effectively alter growth characteristics of SMSCs in a dose-dependent manner in concentrations of 50–200 mg/L, and the optimal dose was 200 mg/L.

DISCUSSION

Satellite cells, the myogenic progenitor cells of postnatal muscle, occupied 1%-6% total nucleated cells of skeletal muscle and lied under the basal lamina of muscle fibers in a quiescent state until they become activated, proliferate, and form new skeletal muscle, which present with growth in response to damage[8]. Recently, studies demonstrated that, adult stem cells have trans-differentiation potential. Gussoni *et al*[3,9] found that muscle-derived stem cells can differentiate into haematoblasts. The self-renewal and differentiation of SMSCs mainly depend on external signals, and determined by intracellular or external signals. In the experiment, it found that SMSCs can accelerate proliferation of hematopoietic stem cells.

Bone marrow hemopoietic microenvironment comprises stroma cells and non-cellular components. The bone marrow stroma consists of a variety of cell types, including fibroblasts, endothelial cells, osteoblasts, osteoclasts, macrophages, *etc*. Stroma cells can regulate the proliferation, differentiation and development of hematopoietic stem/progenitor cells *via* secreting various cytokines, such as stem cell factor, GM-CSF, G-CSF, M-CSF, acid or basic fibroblast growth factor, interleukin, insulin-like growth factors, transforming growth factor β and other active substances.

Angelica sinensis is a common traditional Chinese medicine famous for functions in nourishing blood and activating blood circulation. APS is a main effective constituent of angelica sinensis, which be confirmed has promotion on proliferation and differentiation of hemopoietic progenitor cells[1-2]; simultaneously, APS can promote proliferation and differentiation of human progenitor cells (CFU-Mix, CFU-GM, and CFU-MK) *in vitro*[2]. APS may increases hematopoiesis by accelerating stroma cells or endothelial cells express or secrete hemopoietic growth factor, such as GM-CSF, interleukin 3[10]. Here, marrow stroma cell conditioned medium facilitates SMSCs proliferation, maybe it associates with promotion effects of cell growth factor secreted by stroma cells, but the role needs to be confirmed by further experiment.

Bone marrow transplantation is a common method for the treatment of refractory hematological diseases. However, sources of bone marrow are restrained by many factors. Muscle-derived stem cells have widely prospect due to its hematopoietic cell plasticity. In future, stem cells can be obtained from patients, proliferated *in vitro* for directional differentiation, and reinfused into patients' bodies. This procedure can prevent immunologic rejection and avoid ethical conflict. Thus, muscle can be an alternative for hematopoietic stem cells.

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当归多糖对体外造血微环境中肌卫星细胞增殖的影响**

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摘要
背景：肌卫星细胞是造血功能重建最有希望的种子细胞来源。当归多糖对造血干细胞和造血祖细胞的增殖分化存在显著的促进作用，也可以有效改变肌卫星细胞的生长特性。
目的：观察当归多糖对不同培养条件下乳鼠骨骼肌卫星细胞增殖的影响。
方法：分离小鼠肌卫星细胞，培养5 d后采用α-肌动蛋白免疫细胞化学鉴定。将细胞接种于96孔板培养24 h，使细胞同步化；将细胞分为空白对照组，骨髓基质细胞培养上清组，加入含50，100，200，300，400 mg/L当归多糖的DMEM/F12培养基实验组及经50，100，200，300，400 mg/L当归多糖干预后骨髓基质细胞条件培养基组。经实验处理后采用MTT法检测各组细胞的增殖活性。
结果与结论：分离培养的骨骼肌卫星细胞呈α-肌动蛋白染色阳性，通过MTT法检测发现，经不同浓度当归多糖干预后的骨髓基质细胞条件培养基培养的各组肌卫星细胞增殖显著。且经当归多糖干预的骨髓基质细胞条件培养基可以有效改变肌卫星细胞的生长特性，并呈剂量依赖性。
关键词：肌卫星细胞；当归多糖；造血微环境；增殖
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