Angelica Sinensis Polysaccharide Mediates Sirt1 to Inhibit P53/P21 Pathway and Delay Hematopoietic Stem Cell Aging

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To study the effect of Angelica sinensis polysaccharide (ASP) on the expression of Sirt1/p53/p21 in radiation-induced aging hematopoietic stem cells (HSCs) of mice, and to explore the possible mechanism of ASP regulating the aging of HSCs. Methods: C57BL/6J mice were randomly divided into control group, model group and ASP group. The model group was irradiated with X-ray 3.0Gy/8F to establish the aging model of HSCs in mice. ASP group was given ASP intragastric administration during irradiation; The control group and the model group were given equal volume of normal saline. HSCs were sorted by immunomagnetic beads, and cell cycle was detected by flow cytometry The aging cells were observed by β-galactosidase staining. The directional differentiation ability of HSCs was observed by mixed colony culture (CFU-Mix). The expression of Sirt1, p53 and p21 protein was detected by Western blot. Results: Compared with the control group, X-ray can significantly increase the proportion of HSCs G_1 cells, the rate of SA-β-Gal positive cells and the expression of p53 and p21 protein in aging group (P<0.05). The expression of Sirt1 and mixed colony forming ability were decreased (P<0.05). Compared with the model group, ASP inhibited the increase of aging HSCs G_1cell ratio, SA-β-Gal positive cell rate and p53 and p21 protein expression (P<0.05). At the same time, Sirt1 expression and mixed colony forming ability were increased (P<0.05). conclusion: ASP may inhibit p53/p21 pathway and delay the aging of mouse HSCs by regulating Sirt1.

Key words: Angelica sinensis polysaccharide; Sirt1; Hematopoietic stem cells; Cell senescence; p53/p21
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At present, people are deeply studying the mechanism of aging, and the research and development of anti-aging drugs are gradually deepening. Traditional Chinese medicine has a long history of anti-aging in China, and it plays a unique role in delaying aging with its advantages of overall balanced regulation and less

side effects. Angelica sinensis polysaccharide (ASP) is the main active component of Angelica sinensis, which has pharmacological effectssuch as promoting hematopoiesis, regulating immunity, resisting oxidation, resisting tumor and resisting radiation[1, 2]. Silencing information regulator factor 2-related enzyme 1 (Sirt1) is a type III histone/protein deacetylase dependent on nicotinamide adenine dinucleotide$^+$, which plays a key role in prolonging cell life and aging, inflammation and anti-oxidative stress[3]. In this study, based on the aging HSCs mode[3], the effects of angelica polysaccharide (ASP) on Sirt1/p53/p21 expression of aging HSCs in mice induced by radiation were observed, and the possible mechanism of ASP regulating the aging of HSCs was discussed, which provided experimental basis for preventing and developing anti-aging drugs.

MATERIALS AND METHODS

Main drugs and reagents

ASP was purchased from Shaanxi Ciyuan Biotechnology Co., Ltd., with purity ≥95%; The Anti-Sca-1 MicroBead Kit was purchased from Miltenyi company. Sa-β-gal training kit was purchased from Cell Signaling company. Lymphocyte separation solution was purchased from Axis-Shield company. MethoCult GF M3434 was purchased from Stem Cell Technologies. Sirt1 rabbit anti-sheep antibody was purchased from Abcam Company, and p53 and p21 rabbit anti-mouse polyclonal antibodies were purchased from Bioworld Technology Company. BCA protein quantitative kit and chemiluminescence enhancer were purchased from Pierce company; The internal reference GAPDH antibody and horseradish peroxidase (HRP) labeled secondary antibody kit were purchased from Jackson Company.

Animal grouping and treatment

Seventy-two healthy and clean C57BL/6J mice, aged 6-8 weeks, weighing 16-20 g, were provided by Animal Center of Chongqing Medical University [CertificateNo.: SCXK (Chongqing) 2007-0001]. All animal experiments were approved by the Ethics Committee of Chongqing Medical University.They were randomly divided into control group, model group and ASP group, with 24 rats in each group. The ASP group and the model group were treated with x-ray irradiation of 3.0 Gy (irradiation energy 6MeV, time 1 min, height 100cm, area 25 cm×25 cm) once every 10 days for 8 times, with a total of 24 Gy. on the same day of irradiation, 200mg/kg ASP and equal volume normal saline were given to the stomach, once every other day for 40 times[3]. The control group was given equal volume of normal saline. The animals were killed on the 10th day after the last irradiation.

Separation and purification of HSCs

C57BL/6J mice were killed by neck removal, femur and tibia were taken out under aseptic condition, bone marrow was washed out with 1640 culture medium, single cell suspension was prepared, and bone marrow mononuclear cells were separated by adding lymphocyte separation solution. The most common marker, stem cell antigen (Sca-1), was used to identify and sort HSCs, and the cell ratio of Sca-1$^+$ detected by flow cytometry was the purity of HSCs[3].

1.4 SA-β-Gal staining was used to detect aging cells

Sca-1$^+$ HSCs in each group were collected, washed, centrifuged and discarded, added with stationary liquid, shaken and mixed uniformly, fixed at room temperature for 10min, washed again, centrifuged and discarded, added with stationary liquid and mixed uniformly, and incubated and dyed at 37℃ for 12-16 h without CO2. Smear makes the number of cells on each slide about 1×10^4, and glycerol seal is used for microscopic examination. Randomly count 400 cells, observe the positive cell morphology, count the positive cells and calculate the positive cell rate. Percentage of positive cells (%) = (number of positive cells/total number of cells) ×100%

Flow cytometry analysis of cell cycle

Sca-1$^+$ HSCs in each group were collected,

washed with PBS and fixed with ice ethanol overnight. after centrifugation, the supernatant was discarded, washed again, added with bovine pancreatic ribonuclease, incubated at 37℃ for half an hour, and reacted with propidium iodide in the dark for half an hour. The cell cycle distribution was analyzed by flow cytometry and Multicycle software.

HSCs mixed colony culture

HSCs in each group were collected respectively, and the supernatant was centrifuged after washing the culture medium. after adding 1mL methylcellulose semisolid culture medium, they were well mixed, inoculated into 96-well plates, and incubated at 37℃ for 11~12 days. the HSCs were evaluated according to the number of inoculated HSCs and the number of colonies forming unity-mixture (CFu-mix)

Western blot was used to detect the expression of Sirt1, p53 and p21 protein

Sca-1$^+$ HSCs in each group were collected, and protein samples were extracted respectively. the concentration was determined according to BCA protein quantitative kit, and then transferred to PVDF membrane after polyacrylamide gel electrophoresis separation, and sealed with 5% skimmed milk powder.The first antibody of Sirt1 (dilution ratio 1:400), the first antibody of p53 and p21 (dilution ratio 1:500) and the first antibody of GAPDH (dilution ratio 1:500) were incubated at 4℃ overnight, and the second antibody was incubated for 1h. ECL was luminescent and developed, which was analyzed by Quantity-One software. The expression level of target protein is the ratio of absorbance of target band to GAPDH band.

Statistical analysis

SPSS 18.0 statistical software was used to analyze the experimental data with mean standard deviation ($\bar{x}$ ± S). Single factor variance analysis was performed. LSD or Tamhane test was used for comparison between groups. And the difference was statistically significant with P<0.05.

RESULTS

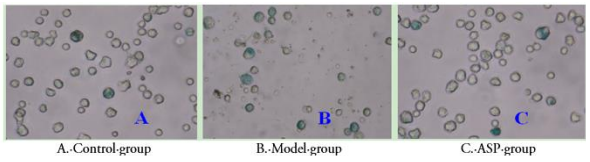
Separation and purification of HSCs

Flow cytometry showed that the percentage of Sca-1+cells in bone marrow mononuclear cells before sorting was (11.45±1.87) %, and the purity of Sca-1+cells after separation and purification reached (86.2±6.17) %, suggesting that the sorted Sca-1+ HSCs had higher purity.

HSCs SA-β-Gal staining

SA-β-Gal staining showed that the positive cells (aging cells) were large in size and blue in cytoplasm, while the negative cells were not stained.

Figure.1
Aging related SA-β-Gal staing in HSCs (×400)



The positive rate of HSCs SA-β-Gal staining in model group was (54.14±5.58) %, which was significantly higher than that in control group (9.91±1.08) % (P< 0.01). The positive rate of HSCs SA-β-Gal staining in ASP group was (11.42±1.07) %, which was lower than that in model group (P < 0.05) (Fig. 1).

The ratio of cell cycle in HSCs

Compared with the control group, the proportion of HSCs in G_1phase increased significantly and the proportion of HSCs in S phase decreased significantly (P < 0.01). Compared with the model group, the proportion of HSCs in G_1phase decreased and that in S phase increased in ASP group (P < 0.05) (Table 1, Fig. 2)

Table 1.
The ratio of cell cycle and CFU-Mix number of HSCs ($\bar{x}$±s, n=8)

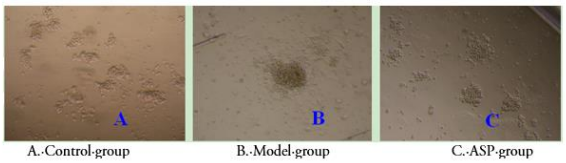
Group	G_1(%)	S (%)	CFU-Mix (each /10^4)
Control group	68.90± 4.29△	23.18± 3.90△	20.25 ±2.05
Model group	86.29± 2.54 *	9.49± 2.42 *	8.63 ±1.19
ASP group	72.48± 4.71△	20.72± 3.11△	19.25± 1.76

* $P<0.05$ Compared with the control group; △$P<0.05$ Compared with the model group

HSCs CFU-Mix formation ability

Compared with the control group, the number of CFU-Mix formed by HSCs in the model group decreased ($P < 0.05$). Compared with the model group, ASP can inhibit the decrease of CFU-Mix formed by HSCs ($P < 0.05$). (Table 1, Fig. 3)

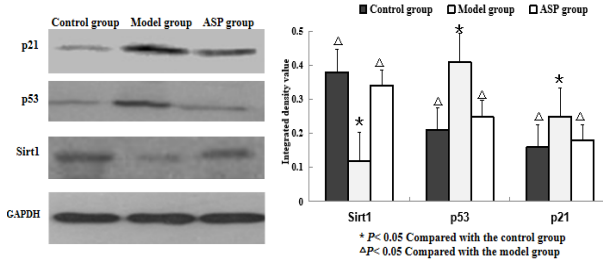
Figure.3
The capacities of CFU-Mix forming in HSCs（×100）



Expression of Sirt1, p53 and p21 protein in hscs

Western blot results showed that: compared with the control group, Sirt1 protein expression was decreased in the model group ($P< 0.01$), and the expression of p53 and p21 protein was significantly increased ($P< 0.01$).Compared with model group, Sirt1 protein expression was up-regulated in ASP group ($P< 0.05$), and p53 and p21 protein expression were down regulated ($P<$ 0.05). (Fig. 4).

Figure. 4
Expression levels of Sirt1, p53 and p21 proteins in HSCs



DISCUSSION

For a long time, China has entered an aging society due to the dual impact of family planning and the existing life expectancy extension. Aging and anti-aging have become the focus of attention in various fields. Human aging refers to a series of physiological or pathological phenomena[1]such as the stability of internal environment, the decline of stress ability and irreversible structural and functional decline of various cells, tissues and organs in the body with the increase of age[4]Aging itself is not a disease, but it is closely related to the occurrence of many senile diseases, such as Alzheimer's disease, cardiovascular disease, tumor, diabetes, etc[5].The anti-aging effect is mainly manifested in scavenging excessive free radicals, anti-lipid peroxidation, reducing DNA damage, inhibiting telomere shortening and regulating immune function[6, 7].With the continuous improvement of science and technology and medical level, traditional Chinese medicine has been proved to be able to regulate the molecular mechanism of aging from many aspects. It is characterized by syndrome differentiation, treatment and plays a unique advantage in health care and anti-aging.Holistic conceptsults showed that ASP may inhibit the aging of HSCs induced by X-ray[3].

At present, the classical signaling pathways related to cell aging mainly include p53-p21 and p16-Rb. However, the upstream and downstream regulatory genes involved are still unclear. Sirt1 is one of the most widely studied and deeply studied proteins in mammalian Sirtuins family. It is a homologue of yeast silencing information regulation gene, and contains a core catalytic domain of about 275 amino acids, which depends on NAD^+coenzyme to play deacetylase activity. The evidence supporting Sirt1 as a "longevity gene" mainly comes from calorie restriction (CR) test. Drosophila, nematode, yeast and human Cr models can prolong life through overexpression of Sirt1. The possible mechanism is that Sirt1 protein affects cell life through the following three pathways under heat and oxidative stress: on the one hand, Sirt1 affects cell life through regulating p53 activity. p53 is the first discovered substrate for deacetylation of Sirt1. When cells are under stress such as DNA damage, Sirt1 can deacetylate the 283rd lysine residue at the C-terminal of p53 protein, reduce the activity of p53 as transcriptional activator, and prevent apoptosis and/or growth inhibition induced by p53 pathway, thus prolonging cell life[8].

On the other hand, Sirt1 can resist oxidative stress by regulating the signal transduction pathway of forkhead transcription factor (FoxOs). Sirt1 can deacetylate FoxOs, an important transcription factor downstream of sirt1, so as to enhance the transcription activity of ROS detoxification enzyme, reduce the accumulation of ROS, and increase the activity of antioxidant enzymes such as superoxide dismutase (SOD), thus resisting cell aging and maintaining the stability of cell energy metabolism and genome[9, 10]. In the third aspect, Sirt1 regulates energy metabolism by stimulating the proliferation of metabolism-related gene peroxisome and activating receptor γ coactivator -1α(PGC-1α). It can catalyze the deacetylation of PGC-1α, induce the expression of gluconeogenic gene, and inhibit the activity of glycolytic gene, thus regulating insulin secretion by islet β cells[11, 12]. The expression of Sirt1 gene decreased in endothelial cells and macrophages of the elderly. The expression of Sirt1 in mouse embryonic fibroblasts and lung epithelial cells also showed age-related decrease. Compared with wild-type mice of the same age, Sirt1-deficient mice were smaller in size and aged faster[13, 14]. The above studies confirmed that Sirt1 is a protein that can maintain cell survival and regulate DNA damage repair in time. In this study, it was found that the positive rate of HSCs aging staining and the proportion of cells in G_1 phase increased with the increase of p53 and p21 protein expression, but the expression of Sirt1 decreased. ASP inhibited the positive rate of HSCs SA-β-Gal staining, the proportion of cells in G_1 stage and the increase of p53 and p21 protein expression in aging mice, and at the same time increased the expression of Sirt1 and the ability of mixed colony formation. It is speculated that ASP may activate the downstream p53 signal protein by increasing the expression of Sirt1, and deacetylate the 382nd position of p53 lysine, thus down-regulating its activity. The down-regulation of p53 further reduces the regulation of its downstream p21, thus reducing the inhibition of cellular protein expression, enabling HSCs to complete chromosome replication and proliferation from G1 phase to S phase, and relieving the inhibition effect of X-ray on proliferation and differentiation of HSCs, that is, increasing its self-renewal and multi-directional differentiation ability to delay the aging of HSCs[15, 16,17].

To sum up, ASP may inhibit p53/p21 pathway and delay the aging of mouse HSCs by regulating Sirt1. However, aging is a multi-factor and multi-link process. There may be more targets and regulatory mechanisms for ASP to affect Sirt1/p53/p21 pathway, which needs further exploration.

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