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Promotion proliferation effect of a polysaccharide from *Aloe barbadensis* Miller on human fibroblasts in vitro

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ABSTRACT

A polysaccharide fraction was isolated from fresh *Aloe barbadensis* Miller leaves, which can promote the wound healing of the superficial II scald model mice. The monosaccharide composition and linkage determination were investigated by methylation and GC–MS, acetylation and GC, ^{13}C NMR and DEPT. The results show that its glycosyl components contain D-glucose, D-galactose, D-xylose in a molar ratio of 5:5:1, and the API consists of a backbone of →2)-α-D-Gal*p*-(1 → 2)-α-D-Glc*p*-(1→, having a branch of α-D-xylofuranosyl residue that is (1 → 3) linkage at *O*-3 of α-D-galactopyranosyl residue. It was found that the API could enhance proliferation of the human fibroblasts in vitro. The mechanisms of promotion proliferation were studied preliminarily.

1. Introduction

Aloe barbadensis Miller, one of Asphodelaceae plants, has been used as a folk medicine for many centuries. In some rural areas of China, *A. barbadensis* Miller, as well as *Aloe ferox* Mill and *Aloe vera* L var. *chinensis* (Haw.) Berger, is often used to treat burns and incised wounds. It is also recorded officially as a medicine herb for the treatment of constipation and some ringworm in Chinese Pharmacopoeia (2005).

In recent years, there are many reports on its components and clinical applications. Two kinds of substances have been isolated from fresh leaves of *Aloe*. One is anthraquinones used for their cathartic effects, and the other is polysaccharide, which can be used for the topical treatment of skin burns and incised wounds [1,2]. These reports have promoted researches on the chemical, biochemical and pharmacological properties of *Aloe*, as well as on other medical uses [3–10]. However, up to nowadays, there are few reports on the pharmacological mechanisms of polysaccharides isolated from *Aloe* on promoting wound healing.

In our experiments, a new polysaccharide (API) was isolated from the fresh leaves of *A. barbadensis* Miller and identified as a (1 → 2) linkage galactoglucan with a branch of α-D-xylofuranosyl residue at *O*-3 of α-D-galactopyranosyl residue. Further studies showed that the API could promote the wound healing of the superficial II scald model mice. Since fibroblast plays a very important role in the course of wound healing, and the proliferation of fibroblast is the event of great relevance in the wound healing process [11,12]. It was assumed that the API obtained in our experiment could speed up the proliferation of fibroblasts. Therefore, the aim of this study was to identify the structure of API and confirm the promotion proliferation effect of the API on human fibroblasts and its pharmacological mechanisms.

2. Experimental

2.1. Chemicals and materials

The fresh leaves of *A. barbadensis* Miller were collected from Fuzhou in China. L-Glutamine, penicillin, streptomycin, amphotericin, trypsogen, calf serum (CS), fetal bovine serum (FBS), Dulbecco's minimum essential medium (DMEM), and dimethyl sulfoxide (DMSO) were purchased from Gibco (USA). 3-(4,5-Dimethy1-2-thiazoyi)-2,5-diphenyi1-2*H*-tertazolooum bromide (MTT) and glutaraldehyde (GA) were obtained from the second agent factory of Shanghai (China). Tritiated thymidine (^{3}H-TdR) and tritiated proline were purchased from the Institution of Atomic Energy (Beijing, China).

2.2. Preparation and characterization of the API

Full size mature leaves were cut from the fresh *A. barbadensis* Miller and the rind was discarded. The colorless gel mesophyll

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(about 9000 ml) was homogenized and centrifuged with a rate of 3000 rpm. The supernatant was collected and precipitated with 95% (v/v) ethanol, and then lyophilized. 3.5 g of crude polysaccharide was obtained. The obtained polysaccharide were dissolved in 500 ml of water and deproteinized according to the previous reports [13]. The deproteinized crude polysaccharide solution was precipitated with 95% (v/v) ethanol for 12 h at 4 °C. The precipitate was lyophilized, and about 1 g of polysaccharide was obtained. The purified polysaccharide was dissolved again in 200 ml of water and dialyzed against water through a 10,000 Da MWCO membrane (Spectrum, Gardena, CA, USA) for 48 h at room temperature. The dialyzed solution was again precipitated with 95% (v/v) ethanol for 12 h at 4 °C. The precipitate was collected and dissolved in 50 ml-distilled water, followed by loading onto a Sephadex G-200 column. The column was eluted with distilled water. Fractions containing carbohydrates were detected by the phenol–sulfuric acid assay method, collected and precipitated with 95% (v/v) ethanol. The fraction was then separated by a DEAE-sepharose (DFF-100, Pharmacia) column with distilled water as the eluant. After lyophillization, 0.5 g of API (white solid) was obtained. The molecular weight of API was estimated by comparison with retention times of high-molecular weight dextran standards (Sigma) according to the literature [14,20–21].

The structure of API was estimated using GC, GC–MS and ^{13}C NMR. In order to obtain monosaccharide residues of API, a complete acidic hydrolysis was performed, followed by acetylation. The product was dried and dissolved in 0.5 ml $CHCl_3$. The solution was then analyzed using gas chromatography with glucose, mannose, galactose, and xylose as reference substances. Glycosyl linkage analyses were performed using the traditional methods [14–15,20–21].

2.3. Preparation of human fibroblast line

A small piece of healthy skin, which was collected in a surgical operation with the permission of the volunteer, was rinsed with isotonic Na chloride, and epidermis and hypoderm were then discarded under asepsis conditions. The dermis specimen was minced into approximately 1 mm^3 fragments and cultured in 10 ml DMEM supplemented with 15% (v/v) heat-inactivated FBS (including 1% L-glutamine and 1% penicillin/streptomycin/amphotericin) in an incubator containing 5% CO_2 at 37 °C. After 24 h, the media was changed with 5 ml of primary culture media. The media was then changed every 2 days until fibroblasts were visualized under light microscopy to be growing outward from the explanted tissue. At that time, the tissue was removed. With sufficient outgrowth of fibroblasts, cells were subcultured into 75 cm^2 culture flasks. After the sixth passage, the fibroblasts were seeded at the density of 5×10^4 cells/ml in 96-well plates. The cells were cultured in DMEM supplemented with 15% (v/v) heat-inactivated FBS (including 1% L-glutamine and 1% penicillin/streptomycin/amphotericin) in an incubator containing 5% CO_2 at 37 °C for 24 h. The cells were then treated with the API of different concentrations (25, 50, 100, 200, and 400 μg/ml, respectively) as API experiment groups and with water as control groups for 48 h, respectively.

2.4. Observation of changes of the cell microstructure

After continuously exposed to API for 48 h, the fibroblast suspensions were centrifuged at 1000 rpm, and the supernatant was removed. The fibroblasts were collected, and then examined by using an electronic microscope (H7500, Hitachi, Japan).

2.5. Determination of LDH leakage rate

After treatment with API at the different concentrations for 48 h, the supernatant was removed, and the fibroblasts in each well were treated with the 20 μl MTT (5 mg/ml) for 4 h and then with 150 μl DMSO. The LDH leakaged from the cells was determined by a full-automatic biochemical analyzer (AU640, OLYMPUS, Japan). The results were recorded as LDH_1. The cells were digested with 0.25% trypsogen and destroyed by ultrasonic wave. The LDH in the cells were determined and recorded as LDH_2. The LDH leakage rate (LDHLR) was calculated by using the equation of $LDHLR = LDH_1/LDH_2$ [16].

2.6. Determination of cell proliferation

Cell viability was assayed by a routine MTT method [17]. After continuously exposed to API for 48 h at the different concentrations, the fibroblasts in each well were treated with the 20 μl MTT (5 mg/ml) for 4 h and then with 150 μl DMSO. After that, 100 μl of 0.04 M HCl–isopropanol solution was added and mixed thoroughly. Within 30 min, the color change was recorded by spectrophotometry with the microplate reader (MRX, Dynex technologies, Chantilly, VA) at 570 nm. Optical density (OD) value was calculated for statistic analysis.

In order to examine the effect of API on the cell population-doubling day, the fibroblasts were seeded at the density of 5×10^4 cells/ml in 96-well plates and cultured continuously for 8 days with the treatment of API at different concentrations (25, 50, 100, 200, and 400 μg/ml, respectively). During the course of cultivation, some wells were selected to count the number of the cells for every day, and the experiments were repeated six times. The number of population-doubling day (PD/d) was calculated with the equation of $PD/d = (\lg N_1 - \lg N_0)/(d \times \lg 2)$, in which N_1, N_0, and d were the number of the cells at a selected time point, the initial cell number, and the incubation days, respectively. The growth curves were prepared by plotting the average of the cells against the incubation period.

2.7. Test of the effect of API on cell DNA and collagen synthesis

After cultivation for 48 h with the treatment of API at the different concentrations, the effect of the API on the DNA synthesis and collagen synthesis of fibroblasts were examined by the ^3H-TdR incorporation method and ^3H-proline incorporation method, respectively [18]. The counts per minute (CPM) values were assayed by using a liquid scintillation counter (Beckman).

3. Results

3.1. Characterization of API

Some aloe polysaccharides have been isolated and studied by several scholars [9]. However, in our study, a new aloe polysaccharide (API) was obtained with an average molecular weight of 150 kDa. It is white, soluble in water and insoluble in ethanol, and has a specific rotation $[\alpha]_D^{20} = -37.5°$ in water. The total soluble sugar content of API was beyond 97% by the phenol–sulfuric acid method with glucose as standard.

Monosaccharide composition of API was determined by GC [19–22]. The results show that the main saccharide residues are D-glucose (45.6%), D-galactose (44.1%), D-xylose (8.7%), D-fructose (0.1%) and D-6-deoxy-mannose (0.2%). Conventional methylation analysis and GC–MS showed four peaks including the 3,4,6-tri-*O*-methyl-galactopyranoside, 3,4,6-tri-*O*-methyl-glucopyranoside, 4,6-di-*O*-methyl-galactopyranoside and 2,3,4-tri-*O*-methyl-xylofuranoside derivatives.

The ^{13}C NMR and DEPT spectra of API are presented in Fig. 1. The chemical shifts are recorded in Table 1, which show API to be a complex polysaccharide with three signals in the anomeric region; those signals at δ 101.2, 100.9, and 100.4 were from the anomeric