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ATM–p53 pathway causes G2/M arrest, but represses apoptosis in pseudolaric acid B-treated HeLa cells

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ABSTRACT

Pseudolaric acid B (PAB) is a diterpene acid, isolated from the root and trunk bark of *Pseudolarix kaempferi Gordon* (Pinaceae). Previous studies demonstrated that PAB induced G2/M arrest and apoptosis in several cancer cell lines, but the relationship between G2/M arrest and apoptosis is still unclear. We examined the relevant signaling pathways for human cervical carcinoma HeLa cells treated with 1 μM PAB. Intriguingly, we found that activation of ATM–p53 signaling pathway by the treatment with 1 μM PAB played a protective role for the subsequent apoptosis. Although the treatment with 1 μM PAB up-regulated the expression of cyclin B1 and p-Histone 3 (mitotic markers) at 12 h, the expression decreased at 24 and 36 h along with the up-down expression of mitotic markers. The expressions of p-ATM and p-p53 that were involved in G2/M arrest increased at 12 h after treatment with PAB. However, a prolonged treatment with PAB (longer than 24 h) caused cell apoptosis. When the cells were arrested in G1 or S phase by the treatment with serum starvation, cytosine β-D-arabinofuranoside (Ara-C) or hydroxyurea (Hu), the apoptotic ratio induced by PAB decreased.

Introduction

Pseudolaric acid B (PAB)[1], a traditional Chinese medicine, is the principal biologically active compound (the chemical structure of PAB is shown in Fig. 1A) isolated from the root bark of *Pseudolarix kaempferi Gordon*. It exerts potent anti-fungal, -tumor, -fertility and -tubulin effects [1]. Previous studies revealed that PAB induced growth inhibition, cell cycle arrest and apoptosis in a variety of cancer cell lines [2,3]. PAB, a microtubule-binding drug, inhibits microtubule polymerization as well as colchicine does [4]. Therefore, molecular relationship between G2/M arrest and apoptosis induced by PAB has been investigated extensively. However, it has not been fully understood.

Microtubule-targeted drugs caused prolonged mitotic arrest of tumor cells, usually leading to cell death. Cells with an activated spindle checkpoint (SAC) escape from mitosis and initiate a new cycle with two fold content of DNA, a process termed mitotic slippage [5]. Cell death occurred during mitotic arrest or after mitotic slippage, and the eventual fate of cells suffering from mitotic slippage varied among different cell lines or various antimitotic drugs [6]. In some cases, apoptotic cell death occurs with morphological characteristics including blebbing, loss of cell membrane asymmetry and cell attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and caspase activation [7].

The ataxia-telangiectasia-mutated (ATM) protein kinase plays an important role in repairing damaged DNA [8]. The tumor suppressor protein, p53, which is a major target of ATM kinase, plays a critical role in regulating cell cycle progression or apoptosis after DNA damage [9]. In contrast to its role in promoting apoptosis during DNA-damaging stress, p53 contributes to cell survival during metabolic stress [10]. That is, transient p53 activation and cell-cycle arrest promotes cell survival by efficiently channeling depleted serine stores to glutathione synthesis [11]. The ATM–p53 signaling pathway not only promoted apoptosis [12],

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[1] *Abbreviations used:* PAB, pseudolaric acid B; MTT, methylthiazolyldiphenyl-tetrazoliumbromide; PI, propidium iodide; ATM, ataxia-telangiectasia-mutated; KU, KU55933; PFT, pifithrin-α; Hu, hydroxyurea; Ara-C, cytosine β-D-arabinofuranoside; siRNA, small interfering RNA.

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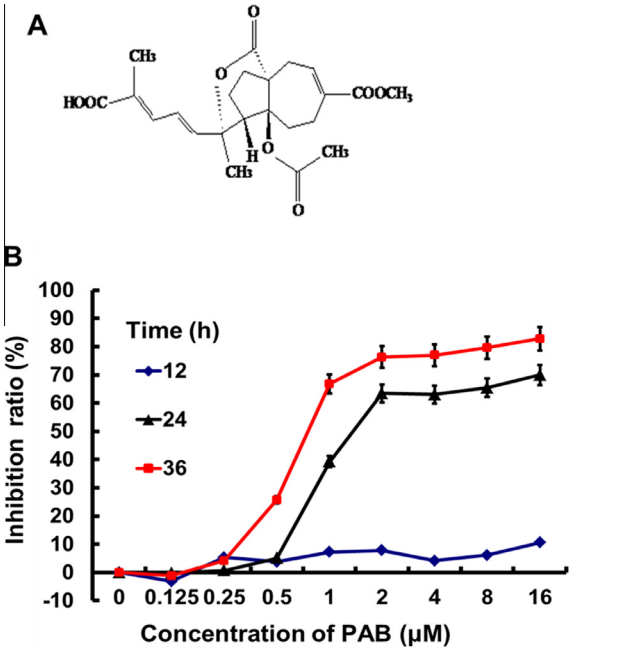

Fig. 1. The effect of PAB on the viability of HeLa cells. (A) The chemical structure of PAB. (B) The cells were cultured for 24 h and then incubated with different concentrations of PAB for 12, 24 and 36 h. Cell viability was determined by the MTT assay. The data are presented as the mean ± S.E.M. of three independent experiments.

but also limited apoptosis [13]. Taken together, the functions of ATM and p53 protein has remained to be fully clarified.

The aim of this study is to investigate the relationship between G2/M arrest and apoptosis by examining how ATM–p53 pathway functions in PAB-treated HeLa cells. The results with 1 μM PAB indicated the induction of G2/M arrest via ATM–p53 pathway and apoptosis. When HeLa cells were arrested in G1 or S phase, the apoptotic ratio decreased, indicating that G1 or S phase arrest limited PAB-induced apoptosis. Unexpectedly, inhibiting ATM–p53 pathway increased the apoptotic ratio at a low concentration of PAB, demonstrating that ATM–p53 pathway played a protective role for the survival of HeLa cells under the condition.

Materials and methods

Reagents

PAB and Taxol with 99% purity were obtained from China Institute of Biological Products (Beijing, China). PAB was dissolved in dimethylsulfoxide (DMSO) as a stock solution. The concentration of DMSO was kept below 0.1% in all the cell cultures so as not to have any detectable effect on cell growth. Methylthiazolyldiphenyl-tetrazoliumbromide (MTT), propidium iodide (PI), RNase A, acridine orange (AO), 2′,7′-dichlorofluorescin diacetate (H_2DCFDA), 2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester (Rhodamine 123), KU55933 (KU), pifithrin-α (PFT), cytosine β-D-arabinofuranoside (Ara-C), hydroxyurea (Hu), 3-methyladenine (3MA) and anti-LC3 antibody were purchased from Sigma Chemical (St. Louis, MO, USA). Primary antibodies against phosphorylated ataxia-telangiectasia mutated (p-ATM), p-p53, p-cdc25c, p21, cyclin B1, p-Histone 3, cdc2, caspase 3, capase 6, Beclin-1 and β-actin as well as horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SuperSignal® West Pico Chemiluminescent Substrate for horseradish peroxidase (HRP) was obtained from Thermo Scientific (Rockford, IL, USA).

Cell culture

HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI-1640 (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere. Logarithmically growing cells were used in all the experiments.

Growth inhibition assay

The growth on HeLa cells was measured using an MTT assay. The cells were seeded in 96-well cell culture clusters (Corning, NY, USA) at a density of 6×10^3 cells per well. After incubation for 24 h, the cells were treated with various doses of PAB for the time periods indicated. Thereafter, the cells were rinsed twice with PBS and incubated with 100 μl 0.5 mg/ml MTT solution at 37 °C for 3 h. After removing the supernatant, the formazan crystals formed by MTT were dissolved with 150 μl DMSO. Absorbance at 492 nm wavelength was measured using a microplate reader (Thermo Scientific Multiskan MK3, Shanghai, China). The inhibition of cell growth was calculated as follows:

$$\text{Inhibitory ratio}\,(\%) = (A_{492}, \text{control} - A_{492}, \text{sample}) / (A_{492}, \text{control} - A_{492}, \text{blank}) \times 100.$$

Quantification of the mitotic index

The mitotic cells are round and bright. The characteristic was used to measure the mitotic index. HeLa cells were treated with PAB for different time periods, and the cellular morphology was observed using phase contrast microscopy (Olympus, Tokyo, Japan). At least 200 cells per field of vision were counted for each sample in three random fields. Mitotic index was calculated as follows:

$$\text{Mitotic index}\,(\%) = \text{numbers of mitotic cells} / \text{numbers of whole cells} \times 100.$$

Analysis of cell cycle by flow cytometry

After the cells were treated with PAB for the time periods indicated, they were collected and fixed in 70% (v/v) ethanol at 4 °C overnight. Then fixed specimen were rinsed with PBS twice and stained with 0.5 ml of PI solution (PI 50 μg/ml and RNase 1 mg/ml) at 4 °C in the dark for 30 min. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Nuclear damage observed by AO staining

Acridine orange (AO) is a fluorescent, DNA-selective and RNA-binding dye. It is nowadays widely used in studies for nuclear changes in morphology of apoptotic cells. After incubation with PAB for the time periods indicated, the cells were stained with 20 μg/ml AO at 37 °C for 15 min and then observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of apoptotic cells by flow cytometry

The quantitative analysis of apoptotic cell death caused by PAB treatment was analyzed by using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China) following the manufacturer's protocol. The treated cells were harvested and rinsed with PBS twice, then stained with Annexin V-FITC