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Enhancement of CD3AK cell proliferation and killing ability by α-Thujone

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ABSTRACT

Thujone is a monoterpene ketone natural substance found mainly in wormwood and sage. Previous studies have shown that Thujone has various pharmacological effects, such as anti-tumor, analgesic, and insecticide. The effect of α-Thujone to human immune cells is still unknown. Our study focuses on investigating the effects and mechanism of α-Thujone to CD3AK (anti- CD3 antibody induced activated killer) cells proliferation and cytotoxicity to colon cancer cell lines. With cell proliferation and FCM assay, it is found that α-Thujone could significantly enhance CD3AK cell proliferation and expression of CD107a in a dose-dependent manner. The cytotoxicity to colon cancer cells detected by CCK-8 assay is also improved. The expressions of TNF-α and FasL detected with ELISA assay were not significantly changed. Mechanically, the study shows that α-Thujone could enhance the expression of p-ERK1/2 and p-Akt. In addition, α-Thujone has no cytotoxicity to HCT116 and SW620 cells proliferation. In a word, α-Thujone enhances CD3AK cell proliferation and cytotoxicity via the improvement of expression of CD107a, p-Akt and p-ERK1/2.

1. Introduction

In modern society, tumor is a great threat to human health. Colon cancer is the third one that happens most frequently in the world. It causes more than 500,000 deaths each year. So a therapeutic progress is required to reduce the risk of recurrence after surgery or to prolong survival of patients [1]. There is a growing evidence that indicates that the immune system may play an important role in the occurrence, growth and metastatic diffusion of tumors, for example up-regulation of B7-H1 and CTLA-4 by tumor [2,3,4,5,6], down-regulation of HLA-I [7], and induction of regulatory T cells [8]. Along with an in-depth research, adoptive immunotherapy of cancer including CD3AK cell, NK cell, γδT cell, and DC, has become the fourth most important therapy method, ranked after surgery, radiotherapy and chemotherapy.

Thujone is a kind of monoterpene ketone natural substance found mainly in wormwood and sage. It is commonly used for food, drinks, and herbal medicines, especially in vermouth. Recent studies have shown that Thujone has various pharmacological activities. Thujone is believed to have effects of analgesic, insecticide and anthelmintic [9]. In an in vitro study, Thujone could inhibit Melanoma A375 proliferation and induce apoptosis [10]. In an in vivo study, Thujone could inhibit tumor cell adhesion and invasion, and suppress Melanoma B16F-10 lung metastasis in C57BL/6 mice via regulating MMPs, VEGF and ERK-1 proteins [11]. Siveen KS also reported that α-Thujone could improve humoral and cell immunity in mice [12], but it's the effects on immunological cell is still unknown. In this study we focus on the activity of α-Thujone on CD3AK cell proliferation and its function.

2. Materials and methods

2.1. Reagents

(−)-α-Thujone was purchased from Sigma Aldrich in USA. FITC-anti-CD3, PerCP-Cy5.5-anti-CD8, APC-anti-CD107a, and APC-IgG1 were all obtained from BD Company in USA. The antibodies of GAPDH, Bcl-2, p-Akt, and p-ERK1/2 were obtained from Epitomics Inc. (Burlingame, CA). Human TNF alpha ELISA Kit SimpleStep (ab181421) and Fas Ligand(APTL) Human ELISA Kit (ab100515) were purchased from Abcam. RPMI-1640, MTT, McCoy's 5A and fetal bovine serum (FBS) were purchased from Gibco Company in USA. Anti-human CD3 monoclonal antibody and recombinant human interleukin-2 (rhIL-2) were secured from Xiamen Special Biological Engineering Co., Ltd. (Xiamen, China). CCK-8 kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Human AB type plasma was purchased from Xuzhou Blood station (Xuzhou, China).

2.2. Culture of colon cancer cells

HCT116 and SW620 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). HCT116 and SW620 cells were cultured in McCoy's 5A medium and RPMI-1640 respectively with 10% FBS at 37 °C in a 5% CO_2 atmosphere.

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2.3. Culture of CD3AK cells

Isolate peripheral blood mononuclear cells (PBMC) from 50 ml human healthy volunteers' peripheral heparin anticoagulant blood and were culture in RPMI-1640 with 10% FBS, 5% AB type plasma, anti-human CD3 (1 μg/L) and rhIL-2 (50 IU/L) at 37 °C in a 5% CO_2 atmosphere for 7 d. Cells were collected and washed twice by PBS, and then incubated with 20 μl FITC-anti-CD3 and 20 μl PerCP-Cy5.5-anti-CD8 in a dark place for 15 min. After being washed twice with PBS, cells were resuspended in 0.5 ml of PBS. The proportion of $CD3^+CD8^+$ was 81.19%.

2.4. CD3AK cell multiplication assay

CD3AK cells were seeded (2.5×10^5/ml) in a 96-well flat bottomed titer plate with different dilutions of α-Thujone (final concentration 0, 0.01, 0.04, 0.15, 0.58, 2.34, 9.38, 37.5, 150.0, 600.0 μmol/L) (N = 3) at 37 °C in a 5% CO_2 atmosphere for 20, 44, and 68 h;20 μl of CCK-8 was added and culture further for 4 h. The OD value was assessed by SEAC automatic enzyme immunoassay analyzer (Beijing XiYake Technology Co., Ltd.) at a wavelength of 450 nm.

2.5. Colon cancer cell multiplication assay

HCT116 and SW620 cells were seeded (2.5×10^4/ml) in 96-well flat bottomed titer plate and incubated with different dilutions of α-Thujone (final concentration 0, 0.01, 0.04, 0.15, 0.58, 2.34, 9.38, 37.5, 150.0 μmol/L) for 68 h at 37 °C in a 5% CO_2 atmosphere. 20 μl of MTT (5 mg/ml) was added and cells were cultured further for 4 h. The supernatants were discarded carefully, and 150 μl of DMSO was added to each well. The OD value was assessed by SEAC automatic enzyme immunoassay analyzer (Beijing XiYake Technology Co., Ltd) at a wavelength of 570 nm.

2.6. Detection of CD107a expression

CD3AK cells were cultured in 6-well plate at the density of 2.0×10^5 cells/ml with α-Thujone for 72 h and harvested by centrifugation. Cells were incubated with 20 μl FITC-anti-CD3, 20 μl PerCP-Cy5.5-anti-CD8 and 10 μl APC-CD107a in a dark place at 4 °C for 15 min. Washed twice by PBS and resuspended in 0.5 ml of PBS, cell were then detected by FCM.

2.7. Detection of FasL and TNF-α expression

The supernatant of CD3AK cells cultured in 6-well plate with α-Thujone for 72 h were collected and stored at −20 °C. The expressions of FasL and TNF-α were detected strictly according to the ELISA kit instructions.

2.8. 2.8 CD3AK cell cytotoxicity to colon cancer cells

CD3AK cells, cultured with α-Thujone for 72 h were harvested as effector cells. HCT116 and SW620 cells, collected at logarithmic growth phase are taken as target cells. Mix CD3AK cells with cancer cells in a 96-well round bottomed titer plate(effector: target ratio = 20:1), and at the same time set effector cell wells, target cell wells and blank wells respectively with 3 wells in each group. Mixed cells were cultured at 37 °C in a 5% CO_2 atmosphere for 24 h. Add 20 μl of CCK-8 and culture further for 4 h. The value of OD can be assessed by SEAC automatic enzyme immunoassay analyzer (Beijing XiYake Technology Co., Ltd.) at a wavelength of 450 nm. Cytotoxicity was calculated with the following formula:

$$\text{Cytotoxicity} = \left[1 - \frac{OD(mixed) - OD(effector)}{OD(t\ \arg\ et)}\right] \times 100\%.$$

2.9. Western blot

CD3AK cells cultured with α-Thujone for 72 h were collected and washed twice with cold PBS. Proteins were extracted and separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane. After being blocked with 1% BSA in TBST, the membranes were incubated overnight with primary antibodies for GAPDH, Bcl-2, p-Akt and p-ERK1/2. The membranes were incubated with an alkaline phosphatase peroxidase-conjugated secondary antibody. Detection was performed by the BCIP/NBT Alkaline Phosphatase Color Development Kit. Bands were recorded with a digital camera.

2.10. Data analysis

Values were expressed as mean ± S.D. Statistical analysis was performed by SPSS 16.0. Multiple-group analysis was conducted by one-way ANOVA. Significance was accepted at p-values < 0.05.

3. Results

3.1. Enhancement of CD3AK cell proliferation treated by α-Thujone

We confirmed the effects of α-Thujone on CD3AK cell proliferation by treating cells with α-Thujone at different doses (0, 0.01, 0.04, 0.15, 0.58, 2.34, 9.38, 37.5, 150.0, 600.0 μmol/L) for 24 h, 48 h, 72 h. The result shows that α-Thujone had no effect on cell proliferation at 24 h, enhanced CD3AK cell proliferation at 48 h at the dose of 0.04 and 0.15 μmol/L, and significantly improved cell proliferation at the dose of no more than 9.38 μmol/L. Meanwhile, α-Thujone inhibits cell proliferation at 600.0 μmol/L (Fig. 1). We concluded that α-Thujone can improve CD3AK cells proliferation in a dose-dependent manner. Therefore we choose the dose of no more than 37.5 μmol/L of α-Thujone treating for 72 h in the following study.

3.2. No effect on colon cancer cell proliferation treated by α-Thujone

Biswas has reported that Thujone inhibited A375 melanoma cell proliferation. We wonder whether α-Thujone has the same effect on colon cancer cells. We confirm the effects of α-Thujone on colon cancer cell proliferation by treating cells with α-Thujone at different doses for 72 h. As shown in Fig. 2, there was no obvious effect on the proliferation of HCT116 and SW620 cells.

3.3. Improvement of CD107a expression treated by α-Thujone

According to the result of CD3AK cell viability, α-Thujone with doses ranging from 37.5 to 0.01 μmol/L was selected in the following research. As shown in Fig. 3, α-Thujone could significantly increase the expression of CD107a in a dose dependent manner, especially at the dose of 0.15 μmol/L.

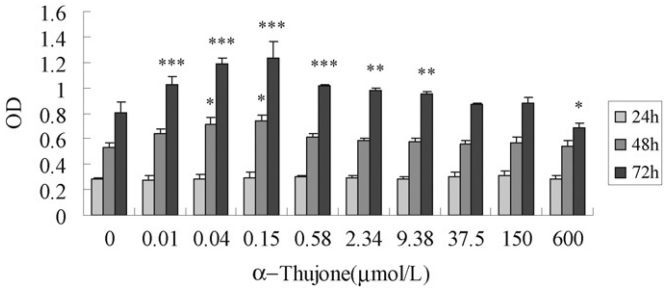

Fig. 1. Proliferative responses of CD3AK cells by α-Thujone. CD3AK cells were treated with α-Thujone at concentrations of 600, 150, 37.5, 9.38, 2.34, 0.58, 0.15, 0.04, 0.01 and 0 μmol/L for 24 h, 48 h and 72h. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ VS 0 μmol/L group at the same time period.