International Immunopharmacology 15 (2013) 6–14

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The effect of Phloretin on human γδ T cells killing colon cancer SW-1116 cells

Sheng-Ping Zhu [a,1], Gang Liu [a,1], Xiao-Ting Wu [a,c], Fu-Xing Chen [b], Jun-Quan Liu [b], Zhong-Hai Zhou [b], Jian-Fu Zhang [c], Su-Juan Fei [a,*]

[a] Department of Gastroenterology, Affiliated Hospital of Xuzhou Medical College 99 West Huaihai Road, Xuzhou, 221002, Jiangsu, China
[b] Department of Central Laboratory, 97th Hospital of PLA, 226 Tongshang Road, Xuzhou, 221004, Jiangsu, China
[c] Department of Physiology, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou, 221002, Jiangsu, China

ARTICLE INFO

Article history:
Received 16 January 2012
Received in revised form 31 October 2012
Accepted 1 November 2012
Available online 19 November 2012

Keywords:
Phloretin
γδ T cells
Colon cancer SW-1116 cells
Killing activity
CD107a

ABSTRACT

Objective: To explore the effect and mechanism of Phloretin on human γδ T cells killing colon cancer SW-1116 cells.
Methods: γδ T cells were amplified in vitro from human peripheral blood mononuclear cells through isopentenyl pyrophosphate method (IPP). After cocultured different concentrations of Phloretin with γδ T cells or SW-1116 cells for 48 h respectively, MTT assay was used to test the growth curve of these two cells; Flow cytometry to test the expression of Granzyme B (GraB), perforin (PFP), CD107a and IFN-γ of γδ T cells; Lactate dehydrogenase (LDH) release assay to test the cytotoxic activity of the γδ T cells on SW-1116 cells; and Western blot to test the Wnt3a expression of the γδ T cells.
Results: After cultured with IPP for ten days, the percentage of γδ T cells increased from 3.31 ± 3.00% to 78.40 ± 10.30%. Compared to the control group, when the concentration of Phloretin increased from 2.35 μg/ml to 18.75 μg/ml, it could significantly proliferate the γδ T cell growth ($P<0.05$) and inhibit the growth of SW-1116 cells in dose–response, and the expression of GraB, PFP, CD107a and Wnt3a significantly increased ($P<0.05$). Significant positive relationships were observed among CD107a and PFP, GraB, cytotoxicity ($P<0.05$). The percentage of IFN-γ producing γδ T cells treated with Phloretin was significantly higher than control group.
Conclusion: Phloretin can enhance the killing effect of γδ T cells on SW-1116 cells; the mechanism may be that Phloretin could proliferate the γδ T cell growth, increase the expression of PFP and GraB, activate the Wnt signaling pathway, and produce higher level of IFN-γ. Indeed CD107a expression probably correlates quite well with antitumor activity.

1. Introduction

Cancer is a major public problem worldwide, although overall cancer especially colorectal cancer incidence rates decreased in the most recent time period in both men and women, cancer still accounts for more death than heart disease in persons younger than 85 years [1]. The treatments to cancer focused on surgery, radiotherapy and chemotherapy at present, but to the patients with advanced cancer, who cannot endure the radiotherapy and chemotherapy, how to improve the therapeutic effects and quality of life become the crucial treatment. With the development of antitumor immunotherapy, adoptive cellular immunotherapy becomes hot topic of the new treatments. Adoptive cellular immunotherapy is an alternative to chemotherapy and radiation, which can cause serious long-term side effects. The opportunity to use this type of treatment is especially important for children with cancer and other malignancies, because chemotherapy and radiation are even more toxic to them. Since γδ T cells were found bearing T cell receptor γ and δ chains in the 1980s, the crucial role of γδ T cells in immune regulation, antitumor immunosurveillance and primary immune response has continually been recognized [2]. γδ T cells can recognize nonpeptide antigens in a major histocompatibility complex (MHC) independent manner, exhibit potent MHC unrestricted lytic activity against different tumor cells in vitro, which is strongly suggested to be used in anticancer immunotherapy. γδ T cells account for only 0.15% to 5% of peripheral T lymphocytes but constitute a substantial fraction of T lymphocytes in the epithelial tissue of skin, intestine, esophagus, lung and genitourinary tract. γδ T cells have the ability to produce inflammatory cytokines involved in protective immunity against intracellular pathogens and tumors and to display strong cytolytic as well as bactericidal activities. This suggests a direct involvement of γδ T lymphocytes in immune control of cancer and infections. These observations have recently aided the development of novel immunotherapeutic approaches aimed at γδ T cell activation.

Phloretin is a type of natural phenols, which can be found in the apples, pears and a variety of vegetable juice. Phloretin with glucose transporter inhibitory activity has been shown in vitro anti-tumor

* Corresponding author. Tel.: +86 516 856280 2211.
E-mail address: feisj1031@hotmail.com (S.-J. Fei).
[1] Sheng-Ping Zhu and Gang Liu contributed equally and thus share first authorship.

1567-5769/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2012.11.001

activity on tumor cells and in vivo inhibition of tumors in experimental animal models [3]. It not only can induce apoptosis in human liver cells by inhibiting of glucose transport or through the mitochondrial pathway [4,5], but also could inhibit the growth, invasive, migration and adhesion ability of human liver cells and reduce the rate of colony formation in a dose-dependent manner [6]. However the potential in vivo immunomodulatory activity of Phloretin has not been completely elucidated. In order to explore the effect of Phloretin on the human immune cells, we incubated γδ T cells with Phloretin in vitro to study possible anti-tumor immune responses, and finally demonstrated that Phloretin enhances tumor immunosurveillance against colon cancer SW-1116 cells by regulating γδ T cells. Our results suggest that Phloretin may be a useful complementary therapy for treating colon cancer.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal antibody fluorescein isothiocyanate (FITC)-conjugated-anti-TCR γδ, phycoerythrin (PE)-conjugated-anti-Granzyme B (GraB), PE-conjugated anti-perforin (PFP), APC conjungated anti-CD107a, APC conjungared anti-IFN-γ and control IgGs were all purchased from BD Pharmingen. Phloretin were purchased from Sigma Company. Mouse anti-Wnt3a (3A6) was obtained from Santa Cruz Biotechnology, Inc. Recombinant human interleukin-2 (rhIL-2) was from TEBAO Biotechnology Co., Ltd. Xiamen. Isopentenyl pyrophosphate (IPP) and trypsin were purchased from Gibco Company.

2.2. Human γδ T cell purification and culture

Human blood samples were obtained from 6 healthy volunteer donors with an average age (27.35 ± 5.14) years (approved according to the policies of the Xu Zhou Medical College in accordance with the Declaration of Health Ministry of China). Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by centrifugation over a Ficoll-Hypaque density gradient for 30 min at 1500 rpm. The purified PBMCs were then incubated in RPMI1640 medium (Gibco, USA) supplemented with 100 ml/l fetal calf serum (FCS, Gibco, USA), 50 ml/l human AB serum, 2 μg/l isopentenyl pyrophosphate (IPP) and 100 IU/ml rhIL-2, for 10 days maintained at 37 °C, 5%CO_2. On day 10 of culture, cells were harvested, and purified populations of the γδ T cells were obtained by staining with anti-TCRγδ-FITC and goat anti mouse-IgG1, κ-FITC that served as isotype control. The stained cells were analyzed on Flow cytometry (BD, USA) as described elsewhere. Cell viability was determined using trypan blue exclusion.

2.3. Colon cancer SW-1116 cell culture

Human colon cancer cells (SW1116) obtained from Shanghai Cell Institute, Chinese Academy of Sciences were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified 5% CO_2 incubator at 37 °C. And then replaced the medium every two days, subculture of SW-1116 cell line was performed by enzymatic digestion (trypsin/EDTA solution:0.05/0.02%). At the end, the harvested SW1116 cells were centrifuged for 3 min at 800 rpm; the supernatants were carefully removed, pulsed with PBS to be resuspended at the final concentrations for experiment.

2.4. MTT assay

Purified γδ T cells and SW1116 cells were respectively plated in 96-well plates in quintuplicate at a density of 1×10^6 cells/ml, 0.2 ml/well, incubated in a humidified 5% CO_2 incubator at 37 °C for 24 h. Cells above were then incubated in the presence of Phloretin at final concentrations of 0, 2.35, 4.70, 18.75, 37.50, and 75.00 μg/ml at 37 °C, 5% CO_2 for another 48 h. 20 μl of MTT (5 mg/ml) was added to each well. After incubation at 37 °C, 5% CO_2 for 4 h, the supernatants were removed carefully, and 150 μl of DMSO was added to each well. The cells were then shocked for 10 min in the dark. The OD assessed by SEAC automatic enzyme immunoassay analyzer (Beijing Xi Yake Technology Co., Ltd) at a wavelength of 540 nm was mathematically analyzed to evaluate cell proliferation rate and cytostatic rate.

2.5. Detection of intracellular cytokine and antigen

Purified γδ T cells cultured for 10 days were plated in 6-well plates in quintuplicate at a density of 1×10^6 cells/ml (3 ml/well), incubated in a humidified 5% CO_2 incubator at 37 °C for 24 h, and then incubated in the presence of Phloretin at final concentrations of 0, 2.35, 4.70, 18.75, 37.50, and 75.00 μg/ml at 37 °C, 5% CO_2 for another 48 h. The wells were incubated in the absence of Phloretin as the control group. The cells were collected and centrifuged at 2000 rpm for 5 min, washed once by PBS, and the concentration adjusted to 1×10^{10} cells/ml for subsequent immunofluorescent staining of intracellular cytokine or antigen.

2.5.1. Expression of GraB and PFP of γδ T cells

20 μl of anti-TCR-γδ FITC was added to each well, where γδ T cells in 50 μl of medium contain 0.1% azide. They were mixed thoroughly and incubated for 30 min in the dark at 4 °C. And then 100 μl fixation buffer was added into each well, incubated in the dark at 4 °C for 15 min, washed twice with PBS, and centrifuged for 5 min at 1500 rpm; the supernatants were carefully removed. The cells were permeabilized with 100 μl 0.5% saponin. The cells were then stained either with PE-anti-PFP antibody or PE-anti-GraB antibody, or their PE-IgG istotype control antibody. They were placed in the dark at 4 °C for 15 min, then 3 ml of PBS was added, centrifuged at 2000 rpm for 5 min, the supernatants were removed, and 0.5 ml of PBS was added to resuspend the cells, finally analyzed by FCM.

2.5.2. Expression of CD107a

Purified γδ T cells incubated with various concentrations of Phloretin were fixed and permeabilized as described above and subsequently stained with anti-TCRγδ-FITC antibody and either APC Mouse Anti-Human CD107a or APC Mouse IgG1 isotype control antibody, incubated for 20 min in the dark at 4 °C, washed with 2 ml of PBS, centrifuged at 2000 rpm for 5 min, the supernatants were removed, resuspended cells with 0.5 ml of PBS, and then were tested with FCM.

2.5.3. Expression of IFN-γ

Cultured γδ T cells were stained with FITC-anti-TCRγδ antibody followed by fixation and permeabilization for intracellular IFN-γ staining, using APC-anti-IFN-γ. APC-conjugated mouse IgG1 was used as an isotype control. Gating was performed on γδ T cells and the percentage of IFN-γ producing cells was reported.

2.6. LDH release assay

Purified γδ T cells cultured for 10 days were plated in 6-well plates in quintuplicate at a density of 1×10^6 cells/ml (3 ml/well), incubated in a humidified 5% CO_2 incubator at 37 °C for 24 h, and then incubated in the presence of Phloretin at final concentrations of 0, 2.35, 4.70, 18.75, 37.50, and 75.00 μg/ml at 37 °C, 5% CO_2 for another 48 h. The wells were incubated in the absence of Phloretin as the control group. γδ T cells that have been pretreated or not with Phloretin were resuspended at the final concentration of 2×10^9 cells/ml, and 100 μl was then added to round-bottom polystyrene tubes together with human colon cancer SW-1116 cells (100 μl), to obtain E:T ratios of 10:1. The cells were incubated for 6 h at 37 °C, in 5% CO_2, and then centrifuged at 1500 rpm for 10 min, supernatants were collected. LDH release assay was executed according to Weimann et al. [7]. Briefly,