Biochemical Pharmacology 80 (2010) 218–225

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Obaculactone suppresses Th1 effector cell function through down-regulation of T-bet and prolongs skin graft survival in mice

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ARTICLE INFO

Article history:
Received 3 February 2010
Accepted 24 March 2010

Keywords:
Obaculactone
Allorejection
Th1 effector cell
T-bet
Treg cell

ABSTRACT

Allograft rejection is a predominantly Th1 immune response. In this study, we showed that obaculactone, a natural compound derived from citrus fruit, prolonged skin graft survival in mice when treated after but not before transplantation. Furthermore, obaculactone inhibited alloantigen-specific production of Th1 cytokine IFN-γ as well as proinflammatory cytokine IL-2, TNFα and IL-6. In parallel, IL-10 production was markedly up-regulated. Obaculactone significantly enhanced the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the $CD4^+$ splenocytes without any effect on their inhibitory function. *In vitro* and *in vivo* tests showed obaculactone down-regulated T-bet expression in Th1 effector cells. Taken together, the unique immunomodulatory properties might qualify obaculactone as a putative, therapeutic compound for the treatment of Th1-driven diseases, including transplant rejection.

1. Introduction

CD4 Th cells play a central role in the transplantation immunity [1]. An allograft is rejected by intragraft immune responses, including infiltration of alloreactive T cells into the graft and the activation of these cells by Th1-associated cytokines [2]. It has been proposed that a Th1 phenotype can promote allograft rejection via IFN-γ-induced activation of macrophage function. In contrast, the Th2 phenotype has been proposed to favor longer graft survival in some models [3]. Therefore, the hallmark Th1 cytokine IFN-γ is frequently used in the determination of the functional status of alloreactive T cells during the development of transplantation rejection [2,4,5]. Recently, the critical role of IL-17 in alloimmune responses adds an additional dimension to this complex network involving a battery of cytokines [3].

In order to prevent allograft rejection, most current immunosuppressive drugs nonspecifically target T-cell activation, clonal expansion or differentiation into effector cells. Despite their efficacy, the drugs carry the risks of adverse effects including kidney failure, cancer and infections. Calcineurin inhibitors such as cyclosporine A (CsA) which mainly inhibit CD4 Th cells are now importantly positioned as the cornerstone of immunosuppressive therapy in organ transplantation. Nevertheless, continual treatment has a significant adverse impact on renal function and cardiovascular disease, and extended long-term graft survival has not been achieved. Previous studies indicate that CsA is ineffective in inhibiting effector T cells while it can prevent naïve T cell activation [5]. The treatment is generally started before or at the time of transplantation with the goal of preventing acute rejection [6]. We hypothesized that it might be a more attractive strategy to selectively inhibit the functional response of pathogenic effector T cells to the allograft without damaging the normal immunocompetence.

Obaculactone (7,16-dioxo-7,16-dideoxylimondiol), also called limonin, is a triterpenoid dilactone extracted from citrus fruit. *In vivo*-tests have shown that obaculactone inhibits carcinogen-induced tumor growth in different organs [7,8]. In addition to anticancer properties, obaculactone shows anti-inflammatory activity by eliciting a suppressive effect on $CD4^+$ T cells [9]. Moreover, obaculactone down-regulates inducible nitric oxide synthase and cyclooxygenase-2, inhibits NF-κB p65 nuclear translocation, and is proposed capable of modulating $CD4^+$ T-cell function [9,10]. To address our hypothesis, we compared the effects on skin allograft rejection of obaculactone treatment after transplantation with those before transplantation. The results revealed obaculactone as a potential immunomodulatory agent which down-regulated the action of IFN-γ in Th1 effector T cells and up-regulated the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells during the ongoing alloresponses.

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doi:10.1016/j.bcp.2010.03.028

2. Materials and methods

2.1. Animals and agents

Male Balb/c (H-2^d) and C57BL/6 (H-2^b) mice, 6–8 weeks of age, were purchased from Experimental Animal Center of Jiangsu Province (Jiangsu, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. Obaculactone (98%) and CsA were purchased from Sigma (St. Louis, MO) and the stock solution was prepared with dimethyl sulfoxide. Anti-mouse antibodies against CD3, CD25 and CD28 were from BD pharmingen (Becton Dickinson, San Diego, CA), and antibodies against CD4, Foxp3 and IFN-γ from eBioscience (San Diego, CA). IL-12 was from PeproTech (PeproTech Inc., Rocky Hill, NJ). All other chemicals were obtained from Sigma.

2.2. HPLC analysis and structural elucidation

HPLC analysis was applied on a Waters 600 series HPLC system consisting of a Waters 600 pump, a 2487 UV detector, an online degasser and an LC Work Station equipped with Empower TM software. Obaculactone was applied to YMC-pack Pro C18 column (5 μm, 150 mm × 4.6 mm, YMC Co., Ltd., Japan) and eluted with methanol–water (47:53, v/v). The effluents were detected at 204 nm. Column temperature was set up at 25° and the flow rate was 1 mL/min. The mobile phase was degassed by ultrasonic and filtered though a 0.22 μm membrane filter (Advantec, Tokyo Roshi Kaisha, Ltd., Japan). Before sample analysis, the column was stabilized with mobile phase for at least 30 min.

2.3. Skin transplantation and treatment

Full-thickness dorsal skin (1 cm^2) from C57BL/6 or BALB/c donor mice was transplanted on the dorsal flank area of BALB/c recipient mice and secured with a plastic adhesive bandage for 7 days [11]. Graft survival was assessed by daily visual inspection in a masked fashion. Rejection was defined as >70% necrosis of the transplanted skin surface, which gives the appearance with desiccation and shrinkage. The survival curve was monitored until the mice in all groups rejected grafts. Two treatment protocols were performed. In one protocol, different dosages of obaculactone or CsA (10 mg/kg) were intraperitoneally administered for 4 days (day −4, −3, −2, −1) before skin transplantation (day 0). In another protocol, these agents were given for 4 days (day 3, 4, 5, 6) after transplantation.

2.4. Histology

Skin grafts were harvested on day 7 after transplantation. Skin tissues were embedded in paraffin, and cut into 5 μm of sections and stained with hematoxylin and eosin.

2.5. Cytokine analysis by ELISA and CBA assay

For cytokine analysis, splenocytes harvested at day 20 after transplantation from recipients were restimulated by mitomycin C (Sigma) – treated donor spleen cells. The cell-free supernatants of individual wells were removed after 72 h. IFN-γ and IL-10 were measured by a specific ELISA kit from eBioscience according to the manufacture's instruction. Cytokine levels of IL-2, IL-17, TNFα, IL-6 and IL-4 were determined using Cytometric Bead Array (CBA) cytokine assay kit according to the manufacture as recommended by BD pharmingen.

2.6. Intracellular staining

For the intracellular IFN-γ staining, freshly isolated spleen cells were activated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (0.5 μg/ml) for 4 h in the presence of 1 μg/ml monensin (eBioscience). Cultured cells were stained with anti-CD3-PerCP. After permeabilization cells were stained by phycoerythrin (PE) conjugated anti-IFN-γ mAb or isotype control. Samples were analyzed by flow cytometry on a FACScan (Becton Dickinson).

Regulatory T cells were detected by Mouse Regulatory T cell Flow Cytometry staining Kit (ebioscience). According to manufacture's protocol, freshly isolated spleen cells were stained with PerCP-labelled anti-CD4 mAb and fluorescein isothiocyanate-labelled anti-CD25 mAb. After washing, these cells were then fixed and stained subsequently with PE-labelled anti Foxp3 mAb or PE-labelled rat IgG 2a mAb as nonspecific isotype control.

2.7. Regulatory T cell isolation and suppression assays [12]

A single-cell suspension was obtained by passing spleens cells through 38 μM cell strainers, and erythrocytes were lysed by Tris–NH_4Cl buffer. Regulatory T cells were isolated by $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Milteny, Germany). According to manufacture's protocol, $CD4^+$ T cells were pre-enriched by depletion of non-$CD4^+$ T cells in a cocktail method. For selecting $CD25^+$ and $CD25^-$ subsets, $CD4^+$ cells were labeled with CD25-PE, followed by anti-PE microbeads, and then loaded onto a MACS column. Positively selected $CD4^+CD25^+$ cells were >95% pure on flow cytometric analysis.

To determine the inhibitory activity, purified $CD4^+CD25^+$ or control cells were added, at indicated ratio, into the reporter $CD4^+CD25^-$ T cells (2×10^5/well), which were stimulated by anti-CD3 mAb (1 μg/ml) and anti-CD28 mAb (1 μg/ml) in triplicate in 96-well plates, and maintained in RPMI-1640 complete medium for 3 days in 5% CO_2 in air. [^{3}H] TdR (1 μCi/well) was added for the final 6 h, and incorporation of [^{3}H] TdR was assessed by liquid scintillation counting.

2.8. Western blotting

In brief, cells were washed with phosphate-buffered saline and lysed in the lysis buffer containing Triton X-100. After 10,000 × g centrifugation for 10 min, the protein content of the supernatant was determined by a BCA™ protein assay Kit (Pierce, Rochford, IL). The protein lysates were separated by 10% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% nonfat milk for 1–2 h at room temperature. The blocked membrane was incubated with the indicated primary antibodies. Primary antibodies used were against T-bet (1:1000 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA) and GAPDH (1:1000 dilution, Santa Cruz Biotechnologies), and the secondary antibody were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (1:10,000, KPL, Gaithersburg, MD). Protein bands were visualized using Western blotting detection system according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA).

2.9. Quantitative RT-PCR

RNA was extracted from cells using Trizol Reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reversely transcribed to cDNA. The primer sequences used in PCR were as follows: GAPDH, 5′-AACGACCCCTTCATTGAC and 3′-CACGACTCATACAGCACCT, T-bet, 5′-CTCAGGTGGCTGGCTTTC and 3′-ATTCGTTCCTGCCGCTTA, The PCR cycle conditions were: 94 °C for 30 s, 58 °C for 30 s, and