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Halofuginone suppresses T cell proliferation by blocking proline uptake and inducing cell apoptosis

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ABSTRACT

Inactivation of T cells is a widely used strategy for immunosuppression. Halofuginone (HF) is an anti-protozoal agent for treating parasites in veterinary medicine, and has been demonstrated to inhibit collagen type 1 synthesis, T helper 17 cell differentiation and cytokine production in activated T cells. The present study was designed to examine the biological effects of HF against T cell receptor and interleukin (IL)-2 stimulated T cell proliferation. T cell proliferation in cultured murine splenocytes was determined by methylthiazol tetrazolium assay. Cell apoptosis was mainly determined by fluorescence-activated cell sorting with Annexin-V and 7-aminoactinomycin D staining. Here, we showed that HF significantly suppressed T cell proliferation in naïve splenocyte cultures in response to alloantigen or anti-CD3 antibody (IC_{50}, 2–2.5 nM; $P < 0.0001$), or in activated T cell cultures in response to IL-2 (IC_{50}, 16 nM; $P < 0.0001$) in a dose-dependent manner. HF did neither attenuate IL-2 production in anti-CD3 antibody activated T cells nor disrupt STAT5 signaling in IL-2-stimulated T cells, but its anti-T cell proliferation was correlated with an increase in cell apoptosis and a decrease in proline uptake in culture medium. Further experiments showed that proline supplement in cell culture medium significantly prevented HF-mediated suppression of T cell proliferation and cell apoptosis. In conclusion, these data suggest that HF interferes with proline incorporation or uptake, resulting in apoptosis via amino acid starvation response in T cells in the response to antigen/mitogen or IL-2 stimulation.

1. Introduction

Of total lymphocytes in the blood circulation in the human body, 45% to 78% are T cells [1,2], and these cells play a central role in cell-mediated immunity as well as humoral immunity; therefore, specific inactivation of T cells, particularly effector T cells, can become an attractive immunosuppressive strategy for transplant rejection and autoimmune diseases. Indeed, anti-CD3 monoclonal antibody as a selective immunosuppressive agent against T cells has been used for successfully treating transplant rejection and autoimmune diseases for years [3–6]. However, it also has many side effects resulting from either its excessive immunosuppressive activity or activation of immune system [7].

Halofuginone (HF) is a synthetic halogenated derivative of febrifugine, a natural quinazolinone alkaloid that can be found in the Chinese herb *Dichroa febrifuga* (Chang Shan), and has been used for treating parasite infection in veterinary medicine, such as in dairy calves to prevent cryptosporidiosis [8–10] and in poultry against coccidiosis [11,12]. Many experimental studies have revealed that HF is a collagen type 1 synthesis inhibitor or an anti-fibrotic agent; HF inhibits TGF-β-mediated collagen type 1 synthesis in fibroblasts [13–15], and the treatment with HF prevents dermal fibroblast [14], liver fibrosis [16], extracellular matrix deposition in diabetic nephropathy [17], and capsular fibrosis [18]. Interestingly, it has also been demonstrated that in vitro HF inhibits human Th 17 cell differentiation [19], and cytokine production (i.e. TNF-α, IFN-γ, IL-4, IL-13 and TGF-β) in activated T cells [20], and in vivo treatment with HF reduces the severity of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [19], and delayed-type hypersensitivity (HTH) response [20]. All these studies may suggest the potential of HF for immunosuppression against T cells and of anti-fibrosis. However, the effect of HF on T cell proliferation has not been investigated yet. The objective of the present study was designed to examine its anti-T cell proliferative activity, and the mechanism(s) by which HF inhibited T cell proliferation.

2. Materials and methods

2.1. Cells and reagents

A single cell suspension of splenocytes was prepared by gently crushing the spleens of mice in phosphate buffered saline (PBS) in a Cell strainer (BD — Canada, Mississauga, ON, Canada), followed by removal of erythrocytes by a brief incubation (~4 min) with lysis buffer

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(0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA, pH 6.8). After washing with PBS again, splenocytes containing ~20% T cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (complete RPMI medium), and grown at 37 °C in a humidified atmosphere of 5% CO_2. HF was purchased from the Toronto Research Chemicals Inc. (Catalogue number: H102500, Toronto, ON, Canada). L-Proline (proline) was obtained from the Life Technologies Inc. (Burlington, ON, Canada). Anti-mouse anti-CD3 antibody was purified from anti-mouse CD3 hybridoma ascite [21]. Recombinant mouse IL-2 (rmIL-2) was purchased from BD Pharmingen (San Diego, CA, USA).

2.2. Preparation of TCR stimulated naïve T cells

Naïve T cells in splenocyte preparations were primarily activated by anti-CD3 antibody or alloantigens in one way mixed lymphocyte reaction (MLR). In anti-CD3 antibody-stimulated T cell cultures, 2×10^5 cells of naïve splenocytes in 100 μL of RPMI complete medium per well in 96-well microplates were incubated with anti-CD3 antibody (2 μg/mL) for 48 h. MLR was a culture of splenocytes (2×10^5/well) from BALB/c mice mixed with mitomycin C (Sigma-Aldrich Canada, Oakville, ON, Canada)-pretreated splenocytes (1×10^5/well) from C57BL/6j (B6) mice in 96-well U-bottom microculture plates (Corning Inc, Corning, NY). In the MLR, the splenocytes from B6 mice as allogeneic stimulators were prepared by pretreated the cells (1×10^6 cells/mL) with 50 μg/mL mitomycin C at 37 °C for 30 min, followed by extensively washed with PBS. The controls for basal levels of responder proliferation (splenocytes from BALB/c mice) were the cultures without stimulator cells. Cultures were maintained in RPMI-1640 complete medium for 48 h at 37 °C in 5% CO_2. T cell proliferation both in anti-CD3 antibody stimulated cultures and MLR as well as IL-2 stimulated cultures was quantitatively measured using methylthiazol tetrazolium (MTT) assay as described below.

2.3. Preparation of IL-2 stimulated activated T cells

Splenocytes were harvested from naïve B6 mice, and were cultured in the presence of 4 μg/mL of Concanavalin (Con) A (Sigma-Aldrich Canada) in complete RPMI 1640 medium at 37 °C in 5% CO_2. Con A is a lectin that binds to glycoproteins on the cell surface, resulting in T cell proliferation, activation and IL-2 production. After 3 days of incubation, cells were harvested and maintained in RPMI 1640 medium containing low serum (0.5% FBS) for 18 h in order to synchronize T cells to G1 phase of the cell cycle. The resting T cells were used as activated T cells after dead cells were removed by a gradient centrifugation through a layer of Histopaque-1083 (Sigma-Aldrich Canada) at 320 *g* for 10 min. The viable cells on the top of the gradient were collected and washed with PBS. These isolated cells contained >98% T cells based on the flow cytometric assay of the presence of CD3 antigen.

2.4. Determination of cell proliferation and growth

Cell proliferation or an increase in viable cell number in cultured splenocytes or activated T cells was measured using MTT assay. In brief, 10 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Canada) was added to each well (100 μL of cell culture), and incubated at 37 °C for 4 h. The formazan crystals in viable cells were then dissolved in 100 μL/well of dimethyl sulfoxide (DMSO, Sigma-Aldrich Canada). The absorbance of the color in each well was quantified as absorbance unit (AU) at 560 nm wavelength using ELx808 Ultra Microplate Reader (BioTek, Winooski, VT, USA). The percentage of inhibition in cell proliferation in drug-treated cultures against nondrug-treated control was calculated as follows: Inhibition (%) = (Drug-treated − Nondrug-treated) / Nondrug-treated × 100%.

2.5. Determination of apoptosis by flow cytometry

Apoptosis of splenocytes or T cells in cultures was measured by fluorescence-activated cell sorter (FACS) analysis following the manufacturer's protocol (BD Biosciences, Mississauga, ON, Canada), in which Annexin-V conjugated with phycoerythrin (Annexin-V-PE) staining was for early apoptosis and 7 amino-actinomycin D (7-AAD) for late apoptosis. Briefly, splenocytes or T cells (1×10^6 cells/well in 1 mL of complete RPMI medium) in 24-well plates were treated with the indicated concentrations of HF in complete RPMI medium for 48 h, followed by staining with Annexin-PE and 7-AAD for 15 min in dark. The proportions of cell population undergoing early apoptosis and/or late apoptosis were quantified by a flow cytometry, and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The levels of IL-2 transcript were determined using RT-PCR kits following the provided protocol (Invitrogen, Carlsbad, CA, USA). Briefly, splenocytes (1×10^6 cells/well) in 24-well plates were stimulated with 2 μg/mL of anti-CD3 antibody in the presence or absence of HF for 24 h, followed by total RNA extraction using TRIzol Reagent (Invitrogen), and 5 μg of total RNA was used for cDNA synthesis in reverse transcription, followed by PCR amplification with appropriate cycle number and annealing temperature using specific primers (IL-2: sense, 5′-AAC CTG AAA CTC CCC AGG AT, and antisense, 5′-TCC ACC ACA GTT GCT GAC TC; internal control glyseraldehyde-3-phosphate dehydrogenase [GAPDH]: sense, 5′-ATC ACT GCC ACC CAG AAG ACT G, and antisense, 5′-CCC TGT TGC TGT AGC CGT ATT C). All the PCR products were visualized in 1% agarose in Tris-acetate-EDTA (TAE) buffer containing 0.5 μg/mL of ethidium bromide, and the expression levels (the size and density of the band) of IL-2 transcripts were measured using a densitometry, and were presented as ratio units (RU) to GAPDH transcript from the same RT product.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Secreted levels of IL-2 in culture supernatants were measured using Mouse IL-2 ELISA kits according to the manufacturer's instruction (*e*Bioscience, San Diego, CA, USA). Briefly, splenocytes (1×10^6 cells/well) in 24-well plates were stimulated with 2 μg/mL of anti-CD3 antibody in the presence or absence of HF for 24 h, and the culture supernatants were collected by centrifugation. The secreted IL-2 level in each well was normalized by its number of total viable cells. Data were presented as the amount of total IL-2 protein per million cells.

2.8. Proline measurement

The concentrations of proline in culture supernatants were quantitatively measured using a high-performance liquid chromatography (HPLC) as described previously [22]. In brief, 1×10^6 cells/well in 1 mL of complete RPMI medium in 24-well plates were activated by anti-CD3 antibody in the absence or presence of 5 nM HF. After 6 or 24 h of treatment, 50 μL of culture supernatant sample was mixed with 150 μL of acetonitrile, followed by the incubation with coupling reagent (TEA/distilled water/phenyl isothiocyanate/methanol: vol/vol ratio of 1:1:1:7) for 20 min at room temperature. The mixture solution was dried under vacuum at room temperature using a Centrivap Centrifugal Vacuum Concentrator (Labconco, Kansas City, MO, USA). The dried samples were redissolved in 100 μL of 10 mM ammonium acetate, and pelleted the undissolved particles by centrifugation. The proline-phenyl isothiocyanate in the supernatants was then quantified by