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Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes

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ABSTRACT

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a quinone isolated from the roots of *Plumbago zeylanica* was recently reported to suppress the activation of NF-κB in tumor cells. NF-κB, a ubiquitous transcription factor, plays a central role in regulating diverse processes in leukocytes like cellular proliferation, expression of immunoregulatory genes and apoptosis during innate and adaptive immune responses. Consequently, plumbagin might affect the biological functions of leukocytes participating in various immune responses. The present report describes novel immunomodulatory effects of plumbagin. Plumbagin inhibited T cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) by blocking cell cycle progression. It also suppressed expression of early and late activation markers CD69 and CD25 respectively, in activated T cells. At these immunosuppressive doses (up to 5 μM), plumbagin did not reduce the viability of lymphocytes. Further, the inhibition of T cell proliferation by plumbagin was accompanied by a decrease in the levels of Con A induced IL-2, IL-4, IL-6 and IFN-γ cytokines. Similar immunosuppressive effects of plumbagin on cytokine levels were seen in vivo. To characterize the mechanism of inhibitory action of plumbagin, the mitogen induced IκB-α degradation and nuclear translocation of NF-κB was studied in lymphocytes. Plumbagin completely inhibited Con A induced IκB-α degradation and NF-κB activation. Further, plumbagin prevented Graft Versus Host Disease-induced mortality in mice. To our knowledge this is the first report showing the immunomodulatory effects of plumbagin in lymphocytes via modulation of NF-κB activation.

1. Introduction

For management of inflammation and survival of allograft, a broad range of immunosuppressive drugs like calcineurin inhibitors (cyclosporine A), adjuvants (azathioprine, mycophenolate mofetil, sirolimus) and steroids have been used [1]. These drugs, however, have undesirable side effects like metabolic derangements, toxicities, development of infections and cancers. One of the major complications in patients receiving repeated blood transplantation is Graft-versus-host disease (GVHD) which is characterized by undesired immune activation and proinflammatory cytokine production leading to tissue destruction. The objective remains to identify novel agents that can be used in combination with the current drugs to optimize treatment for acute inflammation and prolong graft survival while limiting the side effects.

Phytochemicals derived from traditional medicine have shown promising results as immunosupressors and immunomodulators [2]. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinone found in the plants of Droseraceae, Plumbaginaceae, Ancestrocladaceae and Dioncophyllaceae families, has been shown to possess potent anti-tumor activity [3]. It has been shown to inhibit the growth of Raji, Calu-1, HeLa and Wish cell lines in vitro [4]. A recent report showed that plumbagin induced cell cycle arrest and apoptosis in human melanoma A375.S2 cells through ROS/JNK pathway [5]. Further, plumbagin exhibited chemotherapeutic potential in BRCA1 mutated/defective ER-positive cancers [6]. Apart from its anti-cancer properties plumbagin has also been shown to act as a radio sensitizer in mouse Ehrlich melanoma cells [7].

Plumbagin has been shown to augment the bactericidal activity of macrophages at low concentrations whereas it had inhibitory effects at higher concentrations [8]. A recent randomized double blind study using plant based formulation containing plumbagin as one of the active components showed positive results in the management of chronic obstructive pulmonary disease in humans [9]. However, there are no reports on the immunomodulatory effects of plumbagin in lymphocytes. A thorough examination of the immunomodulatory properties of plumbagin will facilitate its use for application in human clinical trials.

It is well known that transcription factor NF-κB is central to a series of cellular processes like inflammation, cell proliferation and apoptosis and

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is of particular importance in modulating the expression of immunoregulatory genes [10]. Lately, plumbagin was shown to inhibit constitutive as well as inducible NF-κB activation and NF-κB regulated genes in tumor cells. It also inhibited DNA binding ability of NF-κB [11]. Several studies have shown that inhibition of NF-κB activation is a relevant strategy for alleviation of GVHD induced tissue damage [12,13]. It was shown that cardiac graft rejection was slower when the transplantation was performed in p50 and p52 deficient mice [14]. Further, the mice expressing transdominant IκB-α did not reject transplanted hearts from allogenic donors [15]. Based on these observations, we speculated that plumbagin may show immunomodulatory effects and might have significant clinical application in prevention of acute GVHD. To test this hypothesis, the immunomodulatory effects of plumbagin were studied in murine lymphocytes in vitro and in vivo and its ability to prevent induction of acute GVHD was tested in a mouse model.

2. Materials and methods

2.1. Chemicals

Plumbagin, RPMI 1640 medium, HEPES, ethylenediaminetetraacetic (EDTA), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), Nonidet P-40, polyethylene glycol (PEG) and propidium Iodide (PI) were purchased from Sigma Chemical Co. (USA). Carboxy fluorescein diacetate succinimidyl ester (CFSE) was procured from Molecular Probes, The Netherlands. Fetal calf serum (FCS) was obtained from GIBCO BRL. Concanavalin A was purchased from Calbiochem, USA. ELISA sets for detection of cytokines (IL-2, IL-4, IL-6 and IFN-γ) and fluorescently labeled antibodies and respective isotype controls were procured from BD Pharmingen (USA). Antibodies against IκB-α and β-actin were obtained from Cell Signaling Technologies (USA). CyQUANT cell proliferation assay kit was purchased from Molecular Probes, Invitrogen. All other chemicals were purchased from reputed local manufacturers.

2.2. Animal maintenance

Six to eight week old inbred Swiss male mice, weighing approximately 20–25 g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed at 23 ± 3 °C with a 12/12 hour light/dark cycle and were given mouse chow and water ad libitum. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed.

2.3. Treatment with plumbagin

A 100 mM solution of plumbagin was prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. In all in vitro experiments, cells were treated with plumbagin for 4 h in 2-mercapto ethanol (ME) free RPMI medium and were further stimulated with Con A (10 μg/ml) without washing the cells. DMSO was used as vehicle control in vitro. In all in vivo experiments, mice were injected i.p with plumbagin in 25% PEG (2 mg/kg body weight) in a 0.2 ml volume. The mice in control group were treated with an equal volume of vehicle (25% PEG in saline).

2.4. Proliferation assay

Splenocytes were obtained by squeezing the spleen through a nylon mesh in a petri plate containing RPMI medium. The RBC were lysed by brief hypotonic shock. Splenocytes were stained with CFSE (20 μM, 5 min, 37 °C) and washed three times using ice-cold RPMI medium containing 10% FCS, 100 IU/ml penicillin and 100 mg/ml streptomycin (complete medium, CM). Two million splenocytes were treated with plumbagin (50 nM to 5 μM, 4 h) and were stimulated with Con A (10 μg/ml) for 72 h at 37 °C in 2 ml RPMI with 10% FCS in a 95% air/5% CO_2 atmosphere. Vehicle treated cells served as a control. Cell proliferation was measured by dye dilution in a flowcytometer (BD FACSAria). Percent daughter cells that showed a decrease in CFSE fluorescence intensity were calculated using BD FACSDiva software and were expressed as daughter cells.

For ex vivo assay of proliferation, splenocytes were isolated from vehicle or plumbagin treated mice (2 mg/kg body weight, 24 h). Two million splenocytes were stimulated with Con A (10 μg/ml) in 2 ml RPMI WITH 10% FCS for 72 h at 37 °C. Proliferation was estimated from the change in the total DNA content in each well using CyQuant assay (CyQUANT NF Cell Proliferation Assay Kit) according to manufacturer's protocol. Fluorescence signals were read from a 96-well plate using a plate reader (Fluostar Optima, BMG Labtech) with excitation at 485 nm and emission at 530 nm. Results were expressed as mean fluorescence intensity ± SEM (four replicates per experiment).

2.5. Estimation of cell cycle and apoptosis

The percentage of cells in different phases of cell cycle (G1, S + G2/M) and percentage of apoptotic cells was estimated by flowcytometry. For cell cycle analysis splenocytes were treated with plumbagin (50 nM to 5 μM, 4 h) and stimulated with Con A (10 μg/ml) for 72 h at 37 °C in RPMI medium supplemented with 10% FCS. Vehicle treated cells served as a control. To study the effect of plumbagin on cell viability, two million splenocytes were treated with plumbagin (5 μM) for 24 h and 72 h at 37 °C in 2 ml RPMI medium supplemented with 10% FCS. Vehicle treated cells served as a control. At the end of incubation period cells were washed with PBS and incubated with 1 ml of staining solution (0.5 μg/ml propidium iodide, 10 μg/ml ribonuclease A, 0.1% sodium citrate and 0.1% Triton X-100) overnight [2]. A total of 20,000 cells were acquired in Partec PAS III flow cytometer and analyzed using FloMax® software. The pre G_1 population represented the apoptotic cells. Undivided cells were in G_1 phase of cell cycle (2n DNA content). The population showing more than 2n DNA represented cells in S + G2/M phase of cell cycle. RN1, RN2 and RN3 in flowcytometric histograms stand for Region 1 (hypodiploid/apoptotic cells), Region 2 (cells in G0/G1 phases of cell cycle) and Region 3 (Cells in S/G2/M phases of cell cycle) respectively.

2.6. Measurement of cytokine secretion

The concentration of IL-2, IL-4 and IFN-γ and IL-6 in the supernatant of control vehicle treated cells and cells stimulated with Con A (10 μg/ml) for 24 h after plumbagin (50 nM to 5 μM, 4 h) treatment was estimated using cytokine ELISA sets (BD Pharmingen, USA).

2.7. Antibody staining

Splenocytes were treated with plumbagin (5 μM and 2.5 μM, 4 h) and were further stimulated with Con A (10 μg/ml). After 24 h, cells (0.5×10^6) were stained with PE conjugated CD25 antibody or CD69 antibody and 20,000 cells in each group were acquired in a flowcytometer (Partec PAS III) [16]. The frequency of CD25+ and CD69+ cells in each treatment group was determined using FLOMAX ® software.

2.8. Western blot analysis

Splenocytes were treated with plumbagin (5 μM, 4 h) and were stimulated with Con A (10 μg/ml) for 1 h at 37 °C and cytosolic extract prepared as explained in Sandur et al. [11]. Vehicle treated cells served as a control. Briefly, the cells were washed with ice-cold phosphate-buffered saline and suspended in 0.1 ml lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml