Biochimica et Biophysica Acta 1800 (2010) 1268–1275

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Rhizoctonia bataticola lectin (RBL) induces mitogenesis and cytokine production in human PBMC via p38 MAPK and STAT-5 signaling pathways

Radha Pujari [a], Nagaraja N. Nagre [b], Vishwanath B. Chachadi [b], Shashikala R. Inamdar [b], Bale M. Swamy [b], Padma Shastry [a,*]

[a] National Centre for Cell Science, Ganeshkhind, Pune 411007, India
[b] Department of Biochemistry, Karnatak University, Dharwad 580003, Karnataka, India

ARTICLE INFO

Article history:
Received 7 July 2010
Received in revised form 9 September 2010
Accepted 16 September 2010
Available online 1 October 2010

Keywords:
PBMC
Proliferation
Signaling
MAPK
STAT-5
Cytokine

ABSTRACT

Background: Rhizoctonia bataticola lectin (RBL), purified from phytopathogenic fungus *Rhizoctonia bataticola* is highly mitogenic towards human peripheral blood mononuclear cells (PBMC). The lectin has sugar specificity towards N-glycans and binds to glycoproteins containing complex N-glycans (Nagre et al., Glycoconj J. 2010). In this study, we investigated the role of Mitogen Activated Protein Kinase (MAPK) and Signal Transducers and Activators of Transcription (STAT)-5 signaling in RBL-induced proliferation and production of Th1/Th2 cytokines.

Methods: Human PBMC were stimulated with RBL and proliferation was determined by tritiated thymidine incorporation assay, cytokine profiles by ELISA and activation of MAPK and STAT-5 by western blotting. RBL binding was monitored by immunofluorescence staining. Expression of IL-2Rα (CD25) was measured by flow cytometry.

Results: The binding and mitogenic activities of RBL were inhibited by glycoproteins– mucin, asialofetuin and fetuin. RBL stimulated expression of IL-2Rα and production of Th1/Th2 cytokines– IL-2, IFN-γ, IL-4 and IL-10. RBL-induced phosphorylation of ERK1/2 and p38 MAPK was detected at 1 h and 3 h respectively. Significant phosphorylation of STAT-5 (tyr^{694}) was observed at 12 h. Pharmacological inhibitors of p38 MAPK (SB203580) and JAK/STAT (AG490) but not ERK (PD98059) abrogated proliferation. RBL-induced expression of IL-2Rα and secretion of cytokines were drastically inhibited by SB203580 and AG490.

Conclusions: RBL-induced proliferation and production of Th1/Th2 cytokines are mediated via p38 MAPK and STAT-5 signaling.

General significance: RBL, a lectin with complex sugar specificity, is strongly mitogenic to human PBMC and stimulates the production of Th1 and Th2 cytokines. The results identified the signaling mechanism underlying the immunostimulatory activity of RBL.

1. Introduction

Lectins are carbohydrate binding proteins or glycoproteins of non-immune origin that are highly specific for sugar moieties and occur ubiquitously in plants, animals and microorganisms [1]. Lectins have been shown to function as recognition molecules in cell–molecule and cell–cell interactions in a variety of biological systems [1,2]. Lectins evoke an array of biological responses by binding to their specific cell surface glycoproteins [3]. They have proved to be useful tools for the investigation of carbohydrates on cell surfaces and for isolation and characterization of glycoproteins [4]. Over the past decade, fungal lectins have attracted wide attention due to their antitumor, antiproliferative and immunomodulatory activities [5–7].

Immunomodulatory properties of various lectins are determined by their capacity to induce proliferation, cytokine production and activation of immune effector cells [7]. Cytokines are classically divided into two groups. Th1 cytokines are produced by Th1 subset of cells and include IL-2, IFN-γ, IL-12 and TNF. Th2 cytokines– IL-4, IL-5, IL-6, IL-10, and IL-13– are products of Th2 helper cells. While Th1 cytokines promote cell-mediated responses, Th2 cytokines are involved in regulating humoral responses [8].

Mitogen Activated Protein Kinases (MAPK) are important modulators of the signaling cascade initiated by mitogens in lymphocytes. The best studied components of these kinases implicated in polyclonal activation of T-cells and IL-2 secretion are p38 [9] and ERK1/2 [10,11]. Another major pathway involved in lymphocyte proliferation is the Signal Transducers and Activators of Transcription (STAT) pathway

Abbreviations: RBL, *Rhizoctonia bataticola* lectin; PBMC, Peripheral blood mononuclear cells; MAPK, Mitogen Activated Protein Kinase; ERK, Extracellular regulated kinase; JAK, Janus Kinase; STAT, Signal Transducers and Activators of Transcription; IL-2Rα, Interleukin-2 receptor alpha

* Corresponding author. Tel.: +91 20 25708108; fax: +91 20 25692259.
E-mail address: padma@nccs.res.in (P. Shastry).

0304-4165/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2010.09.003

[12]. STATs comprise a family of several transcription factors that are activated by a variety of cytokines, hormones and growth factors [13]. STATs are specifically phosphorylated at a single tyrosine residue by the Janus Kinase (JAK) family of tyrosine kinase and consequently dimerize and migrate to the nucleus where they regulate gene transcription [14].

We have earlier reported purification and characterization of a lectin designated RBL, isolated from a phytopathogenic fungus, *Rhizoctonia bataticola*. RBL is a tetramer with a subunit mass of 11 kDa, exhibiting complex sugar specificity recognizing mucin, fetuin and asialofetuin. Glycan array analysis revealed high affinity binding of RBL towards N-glycans and glycoproteins containing complex N-glycan chains. The lectin demonstrated high mitogenicity towards human PBMC [15]. The aim of the present study was to evaluate the immunomodulatory properties of RBL by analyzing the Th1 and Th2 cytokine profiles and investigate the role of MAPK and STAT-5 signaling pathways in RBL-induced response in normal human PBMC.

2. Materials and methods

2.1. Isolation and purification of RBL

Purification of the lectin and preparation of the fluorescein isothiocyanate (FITC)-labeled lectin was essentially according to the methods described previously [15].

2.2. Reagents and antibodies

Phycoerythrin (PE)-conjugated anti-human-CD3, -CD4, -CD8, -CD14 and anti-CD25 conjugated with FITC were purchased from BD Biosciences (USA). Western blotting analysis was done using the following antibodies— phospho-ERK1/2, phospho-STAT-5 (tyr^{694}), STAT-5 (Cell Signaling Technologies, Beverly, USA), Phospho-p38 (BD Bioscience, USA), ERK1/2 and p38 (Santa Cruz Biotechnologies, Santa Cruz, CA), and β-actin (MP Biomedicals, USA). IL-2 blocking antibody was obtained from R&D Systems (NE Minneapolis, USA). Species specific HRP-labeled secondary antibodies were procured from Biorad (USA). Pharmacological inhibitors SB203580, PD98059 and AG490 were purchased from Calbiochem (Darmstadt, Germany). Th1/Th2 cytokine ELISA kit was obtained from eBiosciences (USA). Phytohemagglutinin L (PHA-L), mucin, fetuin, asialofetuin, N-acetyl-D-galactosamine, β-D-glucose, sucrose, lactose and xylose were procured from Sigma (St. Louis, MO).

2.3. Isolation of PBMC

Human PBMC were isolated by density centrifugation of heparinized blood of healthy donors using Histopaque 1077 (Sigma Chemicals). Cells collected from interface were suspended in RPMI 1640 medium supplemented with heat inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and maintained at 37 °C under 5% CO_2 in humidified incubator. The study was approved by the ethics committee of NCCS.

2.4. Analysis of lectin binding

Peripheral blood mononuclear cells were incubated with FITC-conjugated RBL (1 μg/100 μl) for 60 min at 4 °C. After washing, the cells were visualized by confocal laser scanning microscope (CSLM), 510Meta, Ziess (USA). To determine the sugar specificity of RBL, FITC-labeled RBL was incubated with 10 μg/100 μl of mucin, fetuin, asialofetuin, and 200 mM of N-acetyl-D-galactosamine, β-D-glucose, sucrose, lactose and xylose for 1 h and this lectin–sugar complex was used for staining the cells.

Binding of RBL to PBMC subpopulations– CD3+ (T-cells), CD4+ (helper T-cells), CD8+ (cytotoxic T-lymphocytes) and CD14+ (monocytes)– was determined by flow cytometric analysis. PBMC were treated with 3% BSA for blocking and stained with FITC-labeled RBL and PE-labeled CD markers. Data was acquired for 10,000 events on BD FACS Calibur cytometer (Becton Dickinson, San Jose, CA) and analyzed using cell quest-pro software. Unstained cells processed similarly were used as negative control.

2.5. Proliferation assay

The dose and time kinetics of RBL stimulation of PBMC were determined in our previous studies [15]. PBMC were stimulated with 1.25 μg/ml of RBL and PHA for 72 h. During the last 18 h, tritiated thymidine 1 μCi/well (Board of Radiation and Isotope Technology, India) was added and the stimulation was measured as counts per minute (CPM). To determine the significance of sugar specificity, RBL was incubated with 100 μg/ml of mucin, asialofetuin, fetuin and 200 mM N-acetyl-D-galactosamine and tritiated thymidine incorporation assay was performed. PBMC treated with lectin alone was used as control.

To determine the effect of IL-2 blocking on RBL-induced mitogenic response of human PBMC, IL-2 secretion was neutralized using specific blocking antibody (0.1–10 μg/ml) followed by stimulation with RBL and tritiated thymidine incorporation assay was performed. Percent proliferation was calculated considering proliferation induced by RBL alone as 100%.

To identify the signaling pathways activated upon RBL stimulation, PBMC were seeded in 96 well tissue culture plate (10^5 cells/well) in serum free media and incubated with 25 μM of SB203580 (p38 inhibitor), PD98059 (ERK inhibitor) and AG490 (JAK/STAT inhibitor) prior to RBL treatment.

2.6. Flow cytometry analysis

PBMC were stimulated with RBL (1.25 μg/ml) and harvested at 6 h, 12 h, 24 h and 48 h. The cells were stained with FITC-labeled anti-CD25 antibody and flow cytometry analysis was performed. To assess the role of p38 and STAT-5 signaling on CD25 expression, PBMC were treated with pharmacological inhibitors of p38 (SB203580) and JAK/STAT pathway (AG490) for 1 h prior to stimulation with RBL and the expression of CD25 was determined by flow cytometry.

2.7. Cytokine ELISA assay

Culture supernatants were collected from PBMC stimulated with RBL (1.25 μg/ml) for different time intervals up to 48 h and analyzed for IL-2, IFN-γ, IL-4 and IL-10 secretion using cytokine sandwich ELISA kit (eBiosciences), following the manufacturer's protocol. Conditioned medium from unstimulated PBMC was used as control.

To study the signaling pathways involved in RBL-induced Th1/Th2 cytokine secretion, PBMC were pretreated for 1 h with SB203580 (25 μM) and AG490 (25 μM) before stimulation with RBL. The cell free supernatants collected after 24 h were analyzed for IL-2, IFN-γ, IL-4 and IL-10 by ELISA.

2.8. Western blotting

PBMC were stimulated with RBL for different time intervals up to 48 h. At specific time intervals the cells were lysed using RIPA lysis buffer (120 mM NaCl, 1.0% Triton X-100, 20 mM Tris–HCl, pH 7.5, 100% glycerol, 2 mM EDTA, protease inhibitor cocktail, Roche, Germany) and total protein was electrophoresed on 10% SDS-polyacrylamide gels and blotted onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% BSA, the blots were probed with antibodies to phospho-ERK1/2, -p38 and -STAT-5 (tyr^{694}). The bands were visualized by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce,