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Pirfenidone restricts Th2 differentiation in vitro and limits Th2 response in experimental liver fibrosis

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ABSTRACT

Polarized T helper type 2 (Th2) response is linked with fibrosis. Here, we evaluated the effect of the anti-fibrotic agent pirfenidone on Th type 1 (Th1) and Th2 responses. For in vivo testing; Wistar rats were made cirrhotic by intraperitoneal administration of thioacetamide. Once hepatic damage was established, pirfenidone was administered intragastrically on a daily basis during three weeks. Gene expression of Th marks was evaluated by RT-PCR and Western blot assays from liver homogenates. Pirfenidone therapy induced down-regulation of Th2 transcripts and proteins (GATA3 and IL-4), without affecting significantly Th1 genes expression (T-bet and IFN-γ). We found that the activated form of p38 MAPK (identified by Western blot) was reduced by pirfenidone treatment, which is consistent with the anti-Th2 activity observed. Pirfenidone reduced GATA3 nuclear localization without modifying its DNA binding activity (evaluated by electrophoretic mobility shift assay). For in vitro testing; human naive CD4+ T cells were cultured in either Th1 or Th2 polarizing conditions in the presence of pirfenidone and flow cytometric analysis of intracellular synthesis of IFN-γ and IL-4 was conducted. Pirfenidone impaired development of Th2 subpopulation. In conclusion, pirfenidone is capable of impairing Th2 differentiation and limits Th2 profibrogenic response. The mechanism involves p38 inhibition and regulation of GATA3 expression and translocation.

1. Introduction

Liver fibrosis is an aberrant wound healing process characterized by the accumulation of extracellular matrix proteins in response to chronic liver injury. Recruitment of leukocytes takes place following hepatic injury and persists during the process. This chronic inflammatory reaction is characterized by a large infiltrate of mononuclear cells including T lymphocytes (Wynn, 2008). CD4+ T-helper (Th) lymphocytes are divided into four major subsets based on their expression profile of transcription factors and secreted cytokines: 1) Th1 interferon-γ (IFN-γ) producing cells, 2) Th2 expressing interleukins 4, 5 and 13 (IL-4, IL-5 and IL-13), 3) regulatory T cells that produce IL-10 and transforming growth factor beta (TGF-β), and 4) Th17 that synthesize IL-17.

Th lymphocytes have an important role in the progression of fibrotic disease in several tissues including the liver (Wynn, 2004). Fibrogenesis is strongly linked with the development of a dominant (polarized) type 2 CD4+ T-cell response (T helper 2 or Th2 response), whereas anti-fibrotic activity has been described when type 1 CD4+ T-cell response (Th1 response) dominates (Wynn, 2008).

The Th differentiation process is complex. The T-box transcription factor T-bet is the central regulator of Th1 differentiation (Szabo et al., 2000) and directly activates transcription of IFN-γ gene (Lee et al., 2004; Shnyreva et al., 2004; Szabo et al., 2000), and also blocks Th2 differentiation and production of the Th2 cytokines (Hwang et al., 2005; Usui et al., 2006). On the other hand, the zinc-finger transcription factor GATA-binding protein 3 (GATA3) determines Th2 cell differentiation and selectively activates promoters of IL-4, IL-5 and IL-13 genes through chromatin remodeling (Lavenu-Bombled et al., 2002; Yamashita et al., 2004).

The anti-fibrotic agent pirfenidone (5 methyl-1-phenyl-2-(1H)-pyridone) has proven effectiveness for preventing and resolving the accumulation of fibrous tissue in pulmonary and liver fibrosis (Armendariz-Borunda et al., 2006; Garcia et al., 2002; Iyer et al.,

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1995; Oku et al., 2008; Raghu et al., 1999; Simone et al., 2007). In addition to pirfenidone anti-fibrotic properties, anti-oxidant (Giri et al., 1999; Salazar-Montes et al., 2008) and anti-inflammatory activities have been documented (Oku et al., 2002).

In the context of Th response, pirfenidone was found to inhibit the responder frequency of TCR-stimulated CD4+ cell total proliferation in vitro and in vivo and to diminish significantly Th cytokines in the bronchoalveolar lavage fluid from animals in airway chronic allergen challenge with ovalbumin (Hirano et al., 2006; Visner et al., 2009).

In this study, we have demonstrated an immunoregulatory effect of pirfenidone on Th differentiation in vitro and in Th2 response in vivo. Essentially, our data showed that pirfenidone impaired Th2 development, limited Th2 cytokine production, reduced expression of GATA3 and partially blocked its translocation to the nucleus. A decline in Th2 response due to pirfenidone treatment resulted in a significant improvement on fibrosis in vivo.

2. Materials and methods

2.1. Animals and pirfenidone dosing

Male Wistar rats (150–180 g) were randomized into three experimental groups (each n = 10), as follows: 1) Non-TAA or control, 2) TAA or fibrotic and 3) TAA-pirfenidone or pirfenidone. Thioacetamide (TAA) induced liver fibrosis was achieved using a dose of 200 mg/kg of this hepatotoxic agent, administrated intraperitoneally at 3 times per week for 7 weeks, as previously described (Li et al., 2002; Neef et al., 2006). At the end of TAA intoxication regimen, the group on TAA-pirfenidone was given the anti-fibrotic agent (200 mg/kg) orally by gavage, daily during three weeks (Garcia et al., 2002). Liver extraction from all groups was performed after pirfenidone treatment was concluded. With the purpose of monitoring the expression of Th and fibrosis markers in TAA-induced liver fibrosis model, additional rats were intoxicated with TAA as described above and were sacrificed at weeks 1, 4, 7 and 10 (each n = 5) after TAA administration started. Immediately after sacrifice, representative liver sections were excised and either fixed with 4% buffered paraformaldehyde for histological examination or frozen at −70 °C for molecular analysis.

All animals were fed *ad libitum* and received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23 revised 1985).

2.2. Determination of gene expression for IL4, IFN-γ, T-bet, GATA3, α1(I) collagen and TGF-β1 by quantitative real-time RT-PCR

RNA was isolated from liver homogenates with Trizol reagent (Invitrogen, Carlsbad CA, USA). Reverse transcription was performed with 2μg of total RNA, using M-MLV reverse transcriptase (Invitrogen, Carlsbad CA, USA). Then, 2μl of cDNAs was subjected to real-time PCR using Rotor Gene Termocycler under the following conditions: 2 min/50 °C, 10 min/95 °C, and 45 cycles of 15 s/95 °C and 1 min/60 °C. Specific probes designed to align with IL4, IFN-γ, T-bet, GATA3, α1(I) collagen and TGF-β1 rat mRNAs were acquired from Applied Biosystems (Carlsbad CA, USA). Gene amplification was normalized against 18S rRNA expression. Relative quantification by $2(-\Delta\Delta CT)$ method was carried out comparing to control group as internal calibrator (Livak and Schmittgen, 2001; Yuan et al., 2006).

2.3. Analysis of activation of p38 MAPK and Th1 and Th2 proteins in liver homogenates

Western blot assays were conducted to analyze Th1 and Th2 protein expressions, as well as to identify the active form of P38 MAPK (phosphorylated p38 MAPK) from liver homogenates. Proteins were extracted from 400 mg of liver using lysis buffer (50 mM Tris–HCl pH 8.0, 150 nM NaCl, 0.02% NaN_3). After centrifugation at 13,000 rpm/5 min/4 °C supernatant was collected and quantified by Bradford assay. Briefly, 20–25 μg of total proteins was separated by 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules CA, USA). Blocking was carried out using 3% nonfat dry milk for 2 h; primary antibody dilution was 1:500 for GAPDH (loading control), IFN-γ, IL-4 and phosphorylated p38 antibodies and 1:800 for GATA3 and T-bet antibodies (Santa Cruz Biotechnology, Santa Cruz CA, USA). Antibody binding was revealed with a secondary anti-antibody diluted 1:5000–1:6000 using BM Chemiluminescence kit (Roche Diagnostics, Indianapolis IN, USA). Densitometric analysis was realized with a Kodak 1D 3.5 Image analyzer (Eastman Kodak Co., Rochester NY, USA).

2.4. Assessment of T-bet and GATA3 translocation

Western blot assays of cellular fractions (nucleus and cytoplasm) were performed to evaluate translocation of T-bet and GATA3. Cytoplasmic and nuclear extracts were isolated from liver biopsies according to Andrews and Faller (1991). Briefly, 20 μg of cytoplasmic or nuclear extract proteins was used on each western blot assay performed as previously described in Section 2.3. GAPDH was used as cell fractionation control.

2.5. Evaluation of T-bet and GATA3 DNA binding activity

A LightShift Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) Kit (Pierce, Rockford IL, USA) was used to detect DNA–protein interactions. Briefly, double-stranded oligonucleotides corresponding to the specific DNA binding consensus sequences for GATA3 and T-bet transcription factors were biotin-end labeled with a Biotin 3′ End DNA Labeling Kit (Pierce, Rockford IL, USA) and then incubated with 1 μg of nuclear protein obtained as described in a previous section. For identification of DNA-bound protein, a binding reaction included the specific antibody, Anti-T-bet or Anti-GATA3 (Santa Cruz Biotechnology, Santa Cruz CA, USA). These reactions were then subjected to gel electrophoresis on a native 5% polyacrylamide gel and transferred to a nylon membrane. Biotin end-labeled DNA was detected using the streptavidin horseradish peroxidase conjugate and the chemiluminescent substrate. Densitometric analysis was realized with a Kodak 1D 3.5 Image analyzer (Eastman Kodak Co., Rochester NY, USA).

2.6. Histological examination

Hepatic sections were randomly taken from the right, median and left lobes of rat livers and immediately fixed by immersion in 4% paraformaldehyde diluted in phosphate saline buffer (PBS), dehydrated in graded ethylic alcohol and embedded in paraffin. Sections (5 mm thick) were stained with Masson's trichrome technique. Then, 20 random fields from each liver section were analyzed with light-microscopy (20X) using a computer-assisted morphometric analyzer (Image Proplus, Bethesda MD, USA). The percentage of liver tissue affected by fibrosis was determined calculating the ratio of connective tissue to the whole area of the liver (Garcia et al., 2002).

2.7. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll Paque Plus (Amersham, Piscataway NJ, USA) density gradient from healthy donors following informed consent. Naive CD4+ T cells were positively purified from PBMCs. Briefly, PBMCs were incubated at 4 °C for 30 min with a mixture of anti-CD4 PE-Cy7 and anti-CD45RA-APC (eBioscience, San Diego CA, USA). After washing twice in buffer [phosphate-buffered saline (PBS)], cells were resuspended