Biomedicine & Pharmacotherapy 92 (2017) 316–323

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Pentoxifylline attenuates cytokine stress and Fas system in syngeneic liver proteins induced experimental autoimmune hepatitis

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ARTICLE INFO

Article history:
Received 8 March 2017
Received in revised form 15 May 2017
Accepted 17 May 2017

Keywords:
Experimental autoimmune hepatitis
Syngeneic liver antigen
Complete Freund's adjuvant
Tumor necrosis factor alpha/tumor necrosis factor alpha receptor 1
Fas/Fas ligand
Pentoxifylline

ABSTRACT

Background: Apoptosis is a hallmark in the pathogenesis of autoimmune hepatitis (AIH). Cytokine stresses and extrinsic apoptotic pathway have been implicated in this type of hepatic injury. Pentoxifylline plays an important role in controlling inflammation and apoptosis in different autoimmune diseases.
Aim: To assess the protective effect of pentoxifylline for 30 days against pro-inflammatory cytokines as tumor necrosis factor-alpha (TNF-α), interferon-gamma (INF-γ) and mediators of extrinsic apoptotic pathway involving TNF receptor 1 (TNFR1) and its ligand TNF-α and Fas receptor and its ligand (FasL) in experimental autoimmune hepatitis (EAH) model.
Methods: EAH was induced by intraperitoneal injection of syngeneic liver antigen emulsified in complete Freund's adjuvant (CFA) in male C57BL/6 mice. Five groups of mice were used: two control groups; Control PBS group and Control CFA group, EAH group and two EAH + pentoxifylline treated groups in doses (100 or 200 mg/kg/d, given by oral gavage). Serum transaminase, pro-inflammatory cytokines (TNF-α and interferon-γ) and hepatic caspase-8 and 3 activities were evaluated. Signs of autoimmune hepatitis were confirmed by liver histology. In addition, hepatic TNFR1, Fas and FasL mRNA expression were assayed.
Results: Serum transaminase levels and signs of AIH observed in EAH mice were significantly reduced by pentoxifylline. Upregulated serum TNF-α, IFN-γ, hepatic caspase-8 and 3 activities and TNFR1, Fas and FasL mRNA expression in liver tissues in EAH group were significantly downregulated by pentoxifylline.
Conclusion: Pentoxifylline protects against syngeneic liver antigen induced hepatitis and associating apoptosis through attenuating the exaggerated cytokine release and extrinsic apoptotic pathway. Thus, this may represent a new therapeutic strategy for hepatitis.

1. Introduction

As a chronic inflammatory disease of the liver, the current therapeutics of autoimmune hepatitis (AIH) remain unsatisfied as the pathogenic mechanisms of AIH have not yet been elucidated. Misdirected immune response against self-liver antigens or foreign antigens that resemble self-liver antigens is believed to be a major pathogenic mechanism in AIH and referred as molecular mimicry [1].

The basic features observed in AIH including interface hepatitis, hypergammaglobulinemia and serum autoantibodies suggest an aggressive cellular immune response directed towards hepatocytes [2]. Dysregulated cellular immune response associating AIH particularly involves predominating Th1 responses and impairment of crucial lymphocyte subset, regulatory T-cells, that normally retain immune-tolerance to autoantigens [3].

Imbalance in cytokines homeostasis plays an important role in the pathogenesis of chronic inflammatory diseases. Pro-inflammatory cytokines, produced by different immune cells, contribute to the onset and progress of autoimmune inflammation [4]. Release of cytokines in the liver is an indication of local hepatic immune responses [5].

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) is a key mediator of cellular immune response and inflammation. TNF-α is released from Th1 cells as well as macrophages and critically contributes in immune mediated hepatitis [6] and inflammatory liver diseases [7]. Interferon-gamma (IFN-γ), a Th1 driven cytokine, promoted hepatic inflammation aggravating liver damage [8] and overexpressed in association of TNF-α in autoimmune hepatitis [9].

Persistent pro-inflammatory cytokine stresses promote hepatocyte apoptosis by overwhelming hepatocyte survival [10] which

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http://dx.doi.org/10.1016/j.biopha.2017.05.084
0753-3322/

is considered the principal pathway of hepatic loss in AIH. Regulation of the apoptotic process is complex and may be triggered through the activation of so-called death receptors which belong to the TNF receptor family proteins [11]. Among these, the TNF receptor 1 (TNFR1) with its ligand TNF-α and Fas with its ligand FasL, that resemble the extrinsic apoptotic pathway, are implicated in several liver diseases including hepatitis [12,13]. Receptor-ligand binding targets activation of caspase-8 initiating a cascade that eventually activates the executioner caspases-3 promoting cell death.

Pentoxifylline (PTX), a nonspecific phosphodiesterase inhibitor, is widely used for the treatment of vascular disorders. Its anti-inflammatory and anti-apoptotic properties may favorably influence the course of diverse experimental inflammatory and autoimmune disorders [14–16]. Moreover, PTX, as an inhibitor of TNF-α synthesis, has been tested successfully in different hepatic disease states involving this pro-inflammatory cytokine. Importantly, TNF-α plays a key role in the pathophysiology of hepatitis with discrete etiologies involving AIH. Some reports suggested that some patients with AIH either alone or associated with other autoimmune diseases may respond well to anti-TNF therapy. However, despite these encouraging anecdotes, other reports have recorded AIH as a side effect of anti-TNF therapies [17,18]. Regarding non-alcoholic steatohepatitis (NASH), clinical trials have pointed that although PTX ameliorated NASH in most individuals, but it worsened steatosis, inflammation and fibrosis in a few patients [19,20]. This agrees with experimental studies that demonstrated beneficial effect of PTX on NASH [21,22], yet others showed that PTX doesn't prevent or even aggravate the hepatic injury [23,24].

The notion that inhibition of pro-inflammatory cytokines production that initiate inflammatory response ending with cell death raises the concern to explore new therapies to provide new applications in the clinic in the setting of AIH. Beside the unresolved controversy about the potential effect of PTX in the context of AIH, and that few studies were conducted on immunological induced model of hepatitis. In attempt to bridge this gap, this study was conducted to examine the effects of PTX in a model of experimental autoimmune hepatitis (EAH) induced by syngeneic liver antigen immunization in C57B/6 mice that closely replicates human AIH. Additionally, to the author's knowledge, this was the first report to investigate PTX's effect on extrinsic apoptotic pathway in immunological induced model of hepatitis.

2. Materials and methods

2.1. Animals

Seven-week-old male C57BL/6 mice (weighing 18–25 g) were obtained from Theodor-Bilharz Research Institute (Cairo, Egypt). All mice were housed in an animal room with temperature (22–24 °C) and lighting (12 h light–dark cycle) control with free access to water and pellet chow throughout the experiment. Acclimatization for 7 days was allowed before initiation of the experimental protocol. All procedures were in accordance with the EU Directive 2010/63/EU for animal experiments and was approved by ethical Committee, Faculty of Medicine, Ain Shams University.

2.2. Drugs and chemicals

PTX (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline. Doses of PTX were chosen based on previous study [21]. Phosphate Buffered Saline (PBS) and complete Freund's adjuvant (CFA) were purchased from Lonza Walkersville Incorporation, USA and Medico, Difco Laboratories, USA respectively.

2.3. Experimental procedure

2.3.1. Antigen preparation and induction of EAH

Antigen preparation followed the method of Lohse and his colleagues [25]. Briefly, livers from C57BL/6 mice were perfused with PBS to prepare syngeneic liver antigens. Livers were homogenized on ice and centrifuged at 150 × *g* for 10 min. Thereafter, the supernatant was collected and re-centrifuged for 1 h at 100 000 × *g* and the resultant supernatant called S-100 was used for immunization.

EAH was induced as described previously [26]. Intraperitoneal injection of freshly prepared syngeneic S-100 antigen at a dose of 0.4 ml antigen (containing 2.5 mg S-100 antigen in 0.2 ml of PBS were emulsified in an equal volume of CFA) every other day of total 3 injections in the sequence of day1, day3 and day5.

2.3.2. Animal groups and treatment regimen

Forty male C57BL6 mice were randomly distributed among 5 groups: (i) Control PBS group: received 0.2 ml PBS. (ii) Control CFA group: received 0.2 ml CFA. (iii) EAH group: received S-100 antigen as previously described (iv) EAH + PTX 100 mg/kg/d group: as EAH group with concomitant PTX (100 mg/kg/d). (v) EAH + PTX 200 mg/kg/d group: as EAH group with concomitant PTX (200 mg/kg/d).

Saline, the vehicle of PTX, was administrated by gavage in all groups for 30 days. PBS and CFA were administrated every other day of total 3 intraperitoneal injections in control PBS and CFA groups respectively. PTX, dissolved in its vehicle, was given by gavage concomitantly with the start of immunization till the end of 30 days in PTX treated groups. At the end of the experiment, blood and liver samples were taken for biochemical analysis, PCR analysis and histopathological study (Fig. 1).

2.4. Biochemical measurements

Retro-orbital blood samples were collected and centrifuged at 3000 rpm for 15 min to obtain serum. Then, animals were sacrificed to collect hepatic tissue samples.

2.4.1. Liver function tests

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using kits purchased from Diamond Diagnostic (Cairo, Egypt) following the manufacturer's instructions.

2.4.2. Serum cytokine measurement

Serum TNF-α and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) kits purchased from R and D systems (Minneapolis, MN, USA).

2.4.3. Caspase activities determination

Caspase-8 and caspase-3 activities in hepatic tissue homogenate were evaluated using caspase-8 and caspase-3 colorimetric assay kits (Enzo Life Sciences, Farmingdale, USA) per the manufacturer's instructions. Briefly, liver tissue was homogenized in cell lysis buffer, and then homogenates were centrifuged for 1 min at 10,000 g. Then, 100 μg protein of the supernatant was incubated with Ac-IETD-pNA and Ac-DEVD-pNA substrates for caspase-8 and caspase-3 and reaction buffer at 37 °C for 1.5 h. The caspase activities were assessed according to the absorbance recorded at 405 nm.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

RNA expression of hepatic TNFR1, Fas, FasL were examined using RT-PCR. Using RNAzol B (TEL-TEST, Friendswood, TX, USA),