High-dose dexamethasone regulates interleukin-18 and interleukin-18 binding protein in idiopathic thrombocytopenic purpura

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ABSTRACT

To evaluate the effects of high-dose dexamethasone (HD-DXM) on the balance of interleukin-18 (IL-18) and its endogenous antagonist IL-18 binding protein (IL-18BP) in ITP patients, IL-18, IL-18BP as well as IFN-γ, IL-4 plasma levels and platelet counts were determined in 17 ITP patients receiving DXM 40 mg/day for four consecutive days and in 24 healthy subjects. Using RT-PCR, the mRNA expression of IL-18, IL-18BP, IFN-γ, IL-4, T-box (T-bet) and GATA-binding protein 3(GATA-3) were studied in all subjects. The *in vitro* effects of DXM on IL-18BP and IL-18 of peripheral blood mononuclear cells (PBMCs) were studied by ELISA. HD-DXM administration increased IL-18BP and reduced IL-18 expression significantly ($p<0.05$), which resulted in a downregulation of IL-18/IL-18BP ratio $p<0.05$). *In vitro*, DXM had a significant effect on secretion of IL-18BP while diminishing IL-18 release from cultures of PBMCs. These results suggest that downregulation of IL-18/IL-18BP might account for its clinical efficacy of HD-DXM in active ITP.

Key words: interleukin-18, interleukin-18 binding protein, high-dose dexamethasone, idiopathic thrombocytopenic purpura

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Introduction

Interleukin-18 (IL-18), a member of IL-1 cytokine family, is a potent interferon-γ (IFN-γ) inducing factor and is synthesized by Kupffer cells in the liver, pancreas, kidney, skeletal muscle, lung, osteoblasts, and keratinocytes.[1-3] IL-18 binding protein (IL-18BP) is a constitutively secreted protein able to bind IL-18 with high affinity, providing a potential mechanism whereby IL-18 activity is regulated.[4]

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder, characterized by early platelet destruction induced by autoantibodies directed against specific glycoproteins of platelet surface.[5,6] It has become evident that an increased Th1/Th2 ratio in the peripheral blood has been proposed to correlate with disease activity in ITP.[7-9] The expression of IFN-γ, a typical Th1 cytokine, is significantly higher in active ITP patients. Our previous study confirmed that the plasma and mRNA levels of both IFN-γ and IL-18 in active ITP patients were increased significantly compared with the normal controls. And the plasma concentrations of IL-18 correlated with IFN-α. We also observed IL-18BP was not significantly elevated in ITP patients, which resulted in an elevated ratio of IL-18/IL-18BP in patients with active disease. The balance of IL-18/IL-18BP plays a role in progression of ITP.[10]

Glucocorticoids (GC) have been widely recognized as the most appropriate first-line treatment for ITP, [11] even if the best therapeutic approach is still a matter of debate. Recently, high-dose dexamethasone (HD-DXM) in 4-day cycles has proven its clinical efficacy in the treatment of ITP.[12,13] However, the effects of HD-DXM on the balance of IL-18/IL-18BP remains unclear. In the present study, plasma levels as well as mRNA expression of IL-18, IL-18BP in peripheral blood mononuclear cells (PBMCs) and *in vitro* PBMC cultivation were measured to investigate possible effects of HD-DXM on IL-18 activity in ITP patients.

Design and Methods

Clinical study

Seventeen patients with newly diagnosed ITP (13 females and 4 males, age range 16–58 years, median 38 years) were

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assigned to receive DXM 40 mg/day for four consecutive days. All of the cases met the diagnosis criteria of ITP as previously described.[14] None of them had been treated with GC prior to first sampling. Peripheral blood samples were obtained from all patients prior to and two weeks after HD-DXM therapy (Table 1). A control group consisted of 24 healthy adult volunteers (18 females and 6 males, age range 18-65 years, median 40 years). The study was approved by the Medical Ethical Committee of Qilu Hospital, Shandong University, and informed consent was obtained from all patients.

Plasma and PBMC preparation

Plasma obtained from all subjects by centrifugation of heparinized peripheral blood samples were stored at –80°C until determination of cytokines. Mononuclear cells were isolated from heparinized blood by gradient centrifugation on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden), and were preserved at -80°C in aliquots for examination, and thawed only once to avoid degradation.

PBMC isolated from 8 ITP patients (Table 1) and 11 control subjects (8 women and 3 men) were adjusted to 1×10^6/mL in RPMI-1640 culture medium (Invitrogen, America), cultured at a density of 5×10^5 cells/well in a 48-well culture plate and incubated in humidified air in 5% CO2 at 37°C. The cells were treated with 10 μg/mL phytohemagglutinin (PHA) (Sigma, America) and DXM (0 nmol/L, 10 nmol/L, 25 nmol/L, 50 nmol/L, 100 nmol/L, 200 nmol/L) and cell supernatants were collected at 48 h for quantifying the secreted protein of IL-18 and IL-18BP using the ELISA method.

IL-18, IL-18BP, IFN-γ and IL-4 enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in the plasma and culture supernatants were measured using ELISA kits for IL-18 (Uscnlife, Missouri, TX, USA), IL-18BP (R&D Systems, Minneapolis, MN, USA), IFN-γ and IL-4 (Jingmei, Beijing, China) following the protocols recommended by the manufacturers.

Quantitative real time polymerase chain reaction analysis

Total RNA was isolated by Trizol (Invitrogen, America) according to the manufacturer's instructions. The amount of RNA was determined using the Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY, USA) and normalized to 1 μg/mL for each subsequent real time quantitative polymerase chain reaction (RT-PCR) process on an ABI PRISM®7500 Sequence Detection System (Applied Biosystems Foster City CA USA) by using SYBRw Green (Toyobo, Osaka, Japan) as a double-strand DNA-specific binding dye. The primers and annealing temperatures used for the amplification were as described before.[10] ABI Sequence Detection System software version 1.0 (PE Applied Biosystems, Warrington, UK) was used to determine the cycle number at which fluorescence emission crossed the automatically determined Ct value. All experiments were conducted in triplicate.

Table 1. Clinical characteristics and initial responses of ITP patients.

Patient N.	Sex/Age (years)	Bleeding symptoms	Platelet counts ($x10^9$/L) Pre-treatment	Post-treatment
1*	M/28	PT, GH	2	111
2	F/29	PT, EC,GH	12	81
3*	M/31	GH	16	273
4*	F/16	EC,EP,GH	1	39
5	F/27	GUH	5	209
6	M/58	PT,EC	10	123
7*	M/50	EC,GH	9	39
8*	F/43	PT,GH	9	243
9*	F/39	EP,GH	2	272
10	F/57	PT	9	50
11	F/22	PE, GH, GUH	2	88
12	F/29	PT,EP,GH	10	80
13	F/19	PT,EP	3	63
14*	F/38	PT, GUH	2	312
15	F/52	EP,GH	3	40
16*	F/48	EP	10	148
17	F/52	PT,EP	4	115
Median (min–max)	38 (16-58)		5 (1-16)[a]	111 (39-312)[b]

**Patients also in vitro PBMC cultivation. PT: petechiae; EC: ecchymoses; EP: epistaxis; GUH: genitourinary hemorrhage; GH: gingival hemorrhage. [a]p<0.01, ITP pre-treatment vs. normal control; [b]p<0.01, ITP post-treatment vs. pre-treatment.*

Statistical analysis

Data were expressed as mean ± SD. Statistical significance was determined by one-way ANOVA using SPSS Windows version 13.0. The numerical results of RT-PCR results were analyzed using a relative expression software tool (REST©).[15]

Results and Discussion

In this study, oral HD-DXM was used in a single 4-day course as the initial treatment schedule in previously untreated ITP patients with active disease, and IL-18, IL-18BP as well as Th1, Th2 cytokines and transcription factors were profiled in these patients before and after HD-DXM treatment. All of the patients showed initial responses to four days of HD-DXM treatment, in accordance with previous reports.[11-13,16] The platelet counts of ITP patients after HD-DXM treatment (median 111×10^9/L, range $39\text{-}312\times10^9$/L) were significantly higher than that of pre-treatment groups (median 5×10^9/L, range $1\text{-}16\times10^9$/L, $p<0.01$) (Table 1). There was no significant association between clinical parameters (age, sex, platelet counts prior to and after HD-DXM) and the level of IL-18 or IL-18BP at any disease stage.

Previous data demonstrate that IL-18 and IL-18BP are constitutively expressed in humans and IL-18 is up-regulated in the active ITP patients.[10] IL-18BP has been shown to bind IL-18 with high affinity and effectively inhibit its biological activities by reducing induction of IFN-γ mediated responses *in vitro* and LPS-induced IFN-

γ production *in vivo*.[4] Regulating the balance of IL-18 and IL-18BP in ITP might provide a reasonable therapeutic strategy for ITP. We herein demonstrate that HD-DXM induces IL-18BP production and thereby regulates the balance of IL-18 and IL-18BP.

Administration of HD-DXM to ITP patients results in elevation of IL-18BP plasma levels. In biological accordance with this marked augmentation of the antagonist, IL-18 plasma levels steadily declined over the study period, resulting in a 47% decrease after HD-DXM therapy compared to pre-treatment values (Table 2). As demonstrated, IL-18 mRNA levels were reduced and IL-18BP mRNA levels decreased by three-fold after HD-DXM treatment. The effect of HD-DXM administration on IL-18 and IL-18BP plasma and mRNA levels consists in a substantial reduction of IL-

Table 2. Cytokine levels in ITP patients and controls (mean±SD).

Groups	Numbers of cases	IL-18 (pg/mL)	IL-18bp (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)
Controls	24	153.8±103.1	918.1±196.9	38.6±29.1	17.8±12.0
ITP(pre-treatment)	17	462.0±104.3*#	1025.8±250.2#	70.5±21.9*#	8.8±3.2*#
ITP(post-treatment)	17	245.9±77.9&	1430.8±222.3&	59.6±20.3&	16.6±7.9

**p<0.05, ITP (pre-treatment) compared with normal controls; #p<0.05, ITP (pre-treatment) compared with ITP (post-treatment); &p<0.05, ITP (post-treatment) compared with normal controls.*

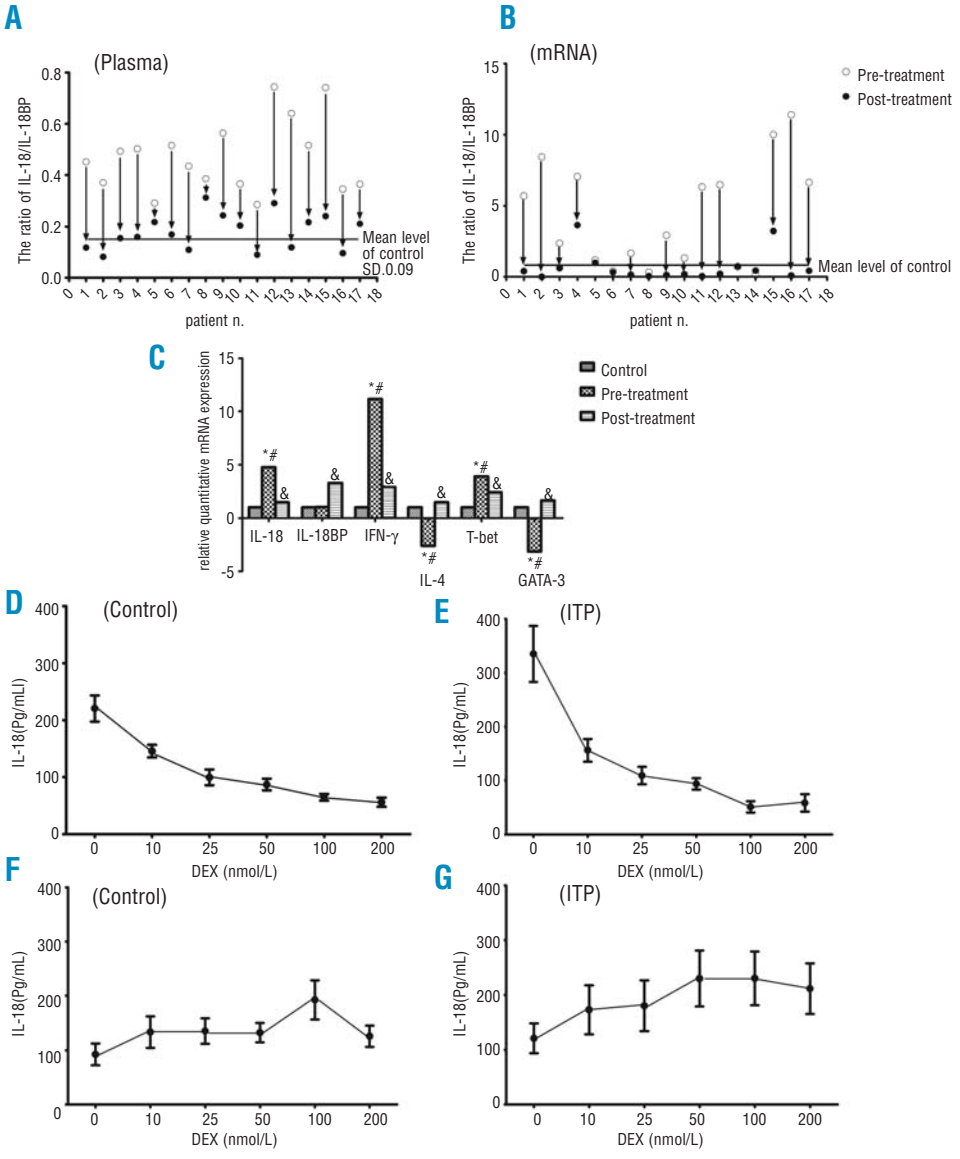

Figure 1. (A) and (B) Calculated IL-18/IL-18BP level four days after 40 mg/day DXM administration of 17 patients with newly diagnosed ITP. IL-18/IL-18BP ratio was downregulated after HD-DXM therapy. (Data are presented for individual patients). (C) Relative mRNA expressions of IL-18 and IL-18BP and other cytokines in active ITP patients, HD-DXM treated patients and healthy controls. mRNA expressions of IL-18, IL-18BP and other cytokines and transcription factors in freshly isolated human PBMCs were quantified by RT-PCR.**p*<0.05, ITP (pre-treatment) compared with normal controls; #*p*<0.05,ITP (pretreatment) compared with ITP (post-treatment); &*p*<0.05, ITP (post-treatment) compared with normal controls. (D)-(G) IL-18 and IL-18BP release from DXM-stimulated PBMCs. PBMC derived from 11 healthy volunteers and 8 ITP patients were cultured with or without DXM. IL-18 and IL-18BP release into supernatant were assayed by ELISA. Administration of DXM results in reduction of IL-18 (pg/mL) secretion in a dose-dependent manner (D and E) and elevation of IL-18BP (pg/mL) levels to their highest levels at 100 nmol/L (F and G).

18/IL-18BP (Figue 1 A-C). However, we did not observe any correlation between IL-18BP and IL-18 levels in the present study.

We have demonstrated that DXM down-regulates IL-18 release into the supernatant of cultured PBMCs after 48 hours of culture (Figue 1D and E).DXM-untreated control PBMCs secreted 220.4±72.4 pg/mL IL-18 into the supernatant. Addition of DXM resulted in a 70% decrease (55.9±25.3 pg/mL, $p<0.05$, 200 nmol/L, Figure 1D). Similar results were obtained in cultures of PBMCs derived from ITP patients, where DXM (200 nmol/L) reduced IL-18 secretion from 335.0±137.7 pg/mL to 58.3±42.7 pg/mL ($p<0.05$, Figure 1E). The expression of IL-18 in PBMC cultures from ITP patients was higher than those in the normal control group ($p<0.05$). These *in vitro* data correspond to the presented *in vivo* data and might outline the cellular basis of DXM effects on IL-18.

We also observed an increased induction of IL-18BP by DXM-treated PBMCs *in vitro*. As shown in Figure 1F and G, DXM had a significant effect on secretion of IL-18BP from healthy and ITP patients PBMC cultures. DXM appeared to induce an increase of IL-18BP in all conditions. However, the amount of IL-18BP released in the cell supernatants was comparable between the two sample groups and, generally, much lower than those observed in the plasma. However, stimulation with DEX did not result in changes on IL-18 and IL-18BP mRNA levels (*data not shown*).

To exclude the possibility that induction of IL-18BP is secondary to DXM-induced IFN-γ release, we determined IFN-γ levels after DXM treatment *in vitro*. IFN-γ levels were just at the detection limit of the assay (*data not shown*). IFN-γ levels were not elevated during the course of HD-DXM treatment *in vivo*, thereby excluding the possibility that induction of IL-18BP is secondary to IFN-γ. From this point of view, suppression of the Th1 cytokine IL-18 and induction of IL-18BP by HD-DXM is favorable for the resolution of disease.

Evidence exists showing that an imbalance of Th1 versus Th2 polarization in favor of Th1 cell subsets appears to be a key pathogenic mechanism in ITP and the clinical efficacy of HD-DXM in ITP depends on the correction of Th1 polarization. In this study we have also demonstrated other Th1 and Th2 cytokines and transcription factors that regulate the differentiations of Th1 and Th2 cells (Figure 1C). In accordance with previous reports,[7-9] elevated IFN-γ/IL-4, IL-18/IL-4 and T-bet/GATA-3 ratio were observed in patients with active ITP compared with the control group, which further implied a Th1-dominated cytokine profile in ITP with active disease. After HD-DXM treatment all fell to the normal range. HD-DXM therapy for ITP could cause a shift in the Th1/Th2 cytokine balance, leading to a more balanced Th1/Th2 cytokine profile response *in vivo*.

GC was known to affect cytokine synthesis in T cells by binding to and activating cytoplasmic GC receptors. The receptor-corticosteroid complex then translocates to the nucleus, where it regulates the transcription of target genes through several mechanisms. GC may directly inhibit Th1 cytokine production in T cells and potentially enhance Th2 cytokine synthesis by inhibiting IL-12 production in antigen-presenting cells.[17-19] The present observation that IL-18 and IFN-γ plasma and mRNA levels were reduced after HD-DXM treatment is in agreement with these data. The HD-DXM-mediated Th1/Th2 cytokine profile alterations observed in this study might be the results of a downregulation of IL-18 and other Th1 cytokines by induction of IL-18BP while permitting the production of Th2 cytokines. As ITP is a heterogeneous and complex autoimmune disease, several abnormalities involving the cellular mechanisms of immune modulation have been described.[5,6] The precise mechanisms of HD-DXM await further clarification.

Taken together our findings suggest that the reversal of IL-18/IL-18BP in ITP is driven by IL-18BP upregulation after HD-DXM treatment. The role of IL-18 and IL-18BP remains to be determined, but they are possibly involved in the clinical efficacy of HD-DXM in ITP treatment.

Authorship and Disclosures

SN: experimental design, data analysis and manuscript writing; ZX: contributed to data analysis and writing of the manuscript; WQ, WC contributed to the statistical analysis; QP: data analysis; PJ: data analysis and writing of the manuscript; HM: obtained funding, experimental design and writing of the manuscript. All authors revised the manuscript critically and approved the final version to be published.

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