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Elevated Semaphorin 5A correlated with Th1 polarization in patients with chronic immune thrombocytopenia

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ABSTRACT

Background: Primary immune thrombocytopenia (ITP) is an immune-mediated disorder in which cellular immunity deficiency and disturbed cytokine profiles have been found. Semaphorin 5A (Sema5A) has been showed to be implicated in cellular immune response. We aimed to evaluate the role of Sema5A in patients with chronic ITP.
Methods: Plasma levels of Sema5A, T helper (Th) cytokines (interferon [IFN] -γ,interleukin [IL]-4,IL-17A) were determined by enzyme-linked immunosorbent assay (ELISA) in ITP patients and healthy controls. Using real-time quantitative polymerase chain reaction (RT-PCR), mRNA levels of Sema5A and its receptor plexin-B3, plexin-A1 in peripheral blood mononuclear cells(PBMCs)were studied in all subjects. Specific anti-platelet autoantibodies were measured by the Pak Auto method. The dynamic change of plasma Sema5A and mRNA levels of its receptors was measured in 9 patients after effective therapy.
Results: Plasma Sema5A levels were significantly increased in active patients with chronic ITP compared to patients in remission and healthy controls. Elevated levels of Sema5A were found positively correlated with higher levels of plasma IFN-γ, IFN-γ/IL-4 ratio and negatively correlated with lower levels of plasma IL-4, platelet counts in ITP patients. The mRNA plexin-B3 was decreased in active ITP patients and inversely correlated with plasma Sema5A levels. Additionally, plasma levels of Sema5A and IFN-γ were reduced with up-regulation of plexin-B3 expression after effective treatment.
Conclusions: Our data demonstrated elevated plasma Sema5A in chronic ITP patients might be involved in Th1 polarization by down-regulating receptor plexin-B3 expression and correlated with disease activity.

1. Introduction

Primary immune thrombocytopenia (ITP) is an immune-mediated platelet disorder in which autoantibody-coated platelets are removed from the blood by monocytic phagocytes, resulting in a remarkable decrease of platelet count, accompanied by impaired platelet production[1,2]. Although autoreactive B lymphocytes secreting antiplatelet antibodies are considered as the primary immunologic defect in ITP, dysfunctional cellular immunity, such as T-cell dysregulation and T-cell-related cytokine abnormalities play central roles in the initiation and development of ITP [3–5].

ITP has traditionally been classified as a T helper (Th) 1-mediated disease with a shift of Th1/Th2 balance, characterized by Th1 cytokine polarization [4,6]. Th1 response is characterized primarily by the presence of cytokines interleukin (IL)-2, interferon (IFN)–γ and tumor necrosis factor (TNF)-α, whereas Th2 response produces IL-4, IL-5, IL-6, IL-10 and IL-13 [7,8]. Assessment of IFN-γ and IL-4 production is always utilized in order to determine the Th1/Th2 ratio analysis in ITP patients [9,10]. Recent studies revealed that the abnormal Th17 cells were also involved in the pathogenesis of ITP by producing IL-17A and other related proinflammatory cytokines [11,12]. As circulating cytokines reflect the activation status of ongoing immune processes, the evaluation of serum cytokines is a reliable surrogate marker of disease activity in autoimmune diseases. Reversal of Th1/Th2 imbalance and recovery of altered levels of Th17 cells may thus well be the underlying mechanism for the effectiveness of a variety of immunomodulatory therapy for ITP [8,13].

Semaphorins are a large family (classified into eight subclasses) of secreted and membrane bound proteins originally discovered in the nervous system, which are involved in repulsive axon guidance during nervous system development [14,15]. Several members of the semaphorin family, the so-called “immune semaphorins,” are essentially involved in immune cell regulation [16] and also implicated in the pathogenesis of autoimmune disorders [17–19]. Semaphorin 5A (Sema5A), a secreted member of this family, comprises transmembrane protein that exhibits a unique extracellular domain containing 7 thrombospondin specific repeats in addition to the sema domain [20].

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Receptors for Sema5A are plexin proteins, including plexin-A1 and plexin-B3 [21,22]. Further studies [23,24] revealed Sema5A as immune semaphorin for its role in innate immune responses by inducing the expression of TNF and IL-8 genes.

Owing to the significant role of secreted Sema5A in innate immunity, increasing attention has been placed on its immune regulator in autoimmune disease. A recent study showed that significantly elevated levels of secreted Sema5A were detected in the serum of patients with rheumatoid arthritis (RA) and other autoimmune diseases compared with control serum [25]. In addition, soluble Sema5A strongly increased T cell and NK cell proliferation and induced the secretion of Th1/Th17 proinflammatory cytokines [25]. Accordingly, Sema5A stimulation caused significant up-regulation of T-bet and retinoic acid receptor–related orphan nuclear receptor [25]. Taken together, these findings suggested Sema5A was involved in immune responses by mediating both cellular response and cytokine production. However, its role in chronic ITP has not been determined yet.

In the present study, plasma levels of Sema5A, T helper cell-related cytokines and mRNA expression of Sema5A as well as its receptors in peripheral blood mononuclear cells (PBMCs) were determined in ITP patients. Furthermore, we investigated the possible pathogenic role of Sema5A in chronic ITP.

2. Materials and methods

2.1. Patients and controls

The study was approved by the Institutional Review Board and written informed consent was obtained from each study subject. We enrolled 31 chronic ITP patients in active disease presented consecutively at our hospital between September 2013 and October 2014. The diagnosis of chronic ITP was based on criteria reported previously [26,27]. All individuals with histories of infectious, autoimmune diseases or previous tumors were excluded. The clinical parameters of the ITP patients were collected and summarized in Table 1. Nineteen ITP patients in remission (12 females and 7 males, age range 24–74years, median 43 years) were also enrolled, and the platelet counts ranged between 104 and 287×10^9/l with a median of 167×10^9/l. We also selected healthy persons visiting for health examinations during the same period as age- and sex- matched controls. The normal control group consisted of 21 adult healthy volunteers (11 females and 10 males, age range 32–64 years, median 47 years). We followed up on 9 active patients who responded to treatment in order to analyze the dynamic change of Sema5A (Table 2).Treatment response was evaluated according to the following criteria [26]: complete response (CR) was defined as a platelet count ≥100×10^9/l and absence of bleeding; response (R)was defined as a platelet count ≥30 but ≤100×10^9/l and a doubling from baseline and absence of bleeding.

2.2. Plasma and PBMCs preparation

Plasma obtained from centrifuged peripheral blood samples anticoagulated with ethylenediamine tetraacetic acid (EDTA) was stored at −80 °C until assay. PBMCs were separated from EDTA anticoagulated whole blood by density gradient centrifugation using Ficoll-Hypaque (1.077 g/ml).

2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used for measuring levels of Sema5A in plasma (Jiyinmei, Wuhan, P.R. China) and levels of IFN-γ, IL-17A, IL-4 in plasma(Neobioscience Technology, Shenzhen, P.R. China), both according to the manufacturers' instructions. The lower limits of detection were 0.1 ng/ml for Sema5A, 1 pg/ml for IL-4, 8 pg/ml for IFN-γ and IL-17A.

Table 1
Clinical characteristics of active ITP patients.

Patient No.	Sex/Age (years)	Course of disease (months)	Platelet counts ($\times 10^9$/l)	Bleeding symptoms	Major previous therapy
1	F/35	24	15	PT	GC,IVIg, VCR
2	M/41	13	28	PT	IVIg,GC
3	M/60	14	6	PT,EC	IVIg, TPO
4	F/52	72	14	OH,GUH	GC
5	F/46	13	10	GH	GC
6	M/51	20	21	PT,OH	IVIg,GC,TPO
7	F/47	24	37	PT	GC,TPO, IVIg
8	M/42	13	9	PT,EC	GC,DNZ
9	M/61	14	20	PT,OH	None
10	M/69	16	21	PT	IVIg,GC,VCR
11	F/26	24	13	PT,EC,OH	None
12	M/48	18	38	PT	GC,TPO
13	F/31	36	18	PT	GC,IVIg,VCR
14	F/51	16	16	PT	GC
15	F/59	24	13	PT,EC,EP	None
16	F/52	48	41	PT	GC
17	M/67	21	30	PT	GC,TPO
18	F/23	27	27	PT	GC
19	F/54	13	23	PT	None
20	F/78	31	15	GH	IVIg,TPO,GC
21	F/59	40	1	PT,EC,OH	GC,TPO
22	F/24	13	7	PT	None
23	F/29	27	3	PT,EC	GC
24	F/42	60	5	PT	GC,VCR,DNZ
25	M/69	38	8	PT,EC	GC
26	F/54	48	11	PT	GC
27	F/68	13	37	PT,GH	None
28	M/55	25	26	PT	None
29	M/62	61	32	PT	GC
30	F/59	93	11	PT,EC	GC,IVIg,VCR,TPO
31	F/61	20	9	PT,EP	GC,TPO
Median Range	52(23–78)	24(13–93)	15(1–41)		

EC, ecchymose; GH, gingival hemorrhage; EP, epistaxis; OH, oral hemorrhage; PT, petechiae; GUH, genitourinary hemorrhage; GC, glucocorticoid; IVIg, intravenous immunoglobulin; DNZ, danazol; VCR, vincristine; TPO, thrombopoietin.

2.4. RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and converted to cDNA using the PrimeScriptTM RT-PCR Reagent Kit (Takara, Japan) according to the manufacturer's instructions. The mRNA expression of Sema5A, plexin-B3, plexin-A1 and β-actin (endogenous control) was quantified by RT-PCR using SYBR Green (Applied Biosystems, Foster City, CA, USA) as a double-strand DNA-specific binding dye on an ABI-7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The sequences of the amplification

Table 2
Clinical features of active ITP patients on treatment.

Patients No.	treatment	Time to response (days)	Platelet counts $\times 10^9$/l	
			Pre-treatment	post-treatment
1	GC	13	15	130
3	GC,DNZ	13	6	82
5	GC, RTX	24	10	145
6	GC,TPO	10	21	117
7	GC,IVIg	10	37	180
11	GC	9	13	80
20	IVIg,TPO	10	15	105
22	GC	23	7	56
31	IVIg, TPO	13	9	110
Median range		13(9–24)	13(6–37)	110(56–180)

GC, glucocorticoid; IVIg, intravenous immunoglobulin; DNZ, danazol; TPO,thrombopoietin; RTX, rituximab.