Favorable Response of Chronic Refractory Immune Thrombocytopenic Purpura to Mesenchymal Stem Cells

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Seven patients with chronic refractory immune thrombocytopenic purpura (ITP) received adipose tissue-derived mesenchymal stem cells (AMSC) from haplo-identical family donors. The AMSC dose was 2.0×10^6 /kg. No side effects were noted after the AMSC infusions. Overall responses were reached in all patients and sustained response rate was 57.1% (4/7). The serum levels of transforming growth factor β 1 (TGF- β 1), interleukin (IL)-4, and IL-10 were significantly elevated, whereas those of interferon- γ (IFN- γ) and IL-2 were significantly decreased after AMSC administration, compared with those in the patients with active ITP. During follow-up, the cytokine profiles in patients maintaining sustained response remained stable compared with the post-treatment level, but IFN- γ and IL-2 levels were significantly increased, and those of TGF- β 1, IL-4, and IL-10 were significantly reduced again in relapsed patients. AMSC therapy seems to represent reasonable salvage treatment in severe, chronic refractory ITP by causing a shift in the Th1/Th2 cytokine balance to the same levels as normal controls.

Introduction

IMMUNE THROMBOCYTOPENIC PURPURA (ITP) is an acquired bleeding disorder characterized by autoantibody-mediated platelet destruction and impaired platelet production. Patients with chronic refractory ITP have the highest risk of death and disease-related or therapy-related complications [1,2]. Like in other autoimmune diseases, autoantibody production by B cells in ITP needs the help of T cells, as evidenced by its association with both T cell activation and T-B cognate interaction [3–6]. Almost all of these autoantigen-specific T cells are CD4⁺ Th cells, which may play very important roles in the pathogenesis of ITP [7–11]. It has become evident that Th1/Th2 cytokine mRNA ratios were significantly increased in ITP patients [12,13]. In addition, recent investigations have substantiated that chronic adult ITP is the manifestation of a Th1-polarized immune response and that the Th1 polarization could be corrected by high-dose dexamethasone or rituximab therapy, paralleled with the remission of ITP [14–16].

Mesenchymal stem cells (MSC) have been shown to exert in vitro immunosuppressive activities on activated T cells [17,18]. However, MSC from ITP showed an impaired proliferative capacity and a lower capability of inhibiting activated T-cell proliferation compared with healthy donors [19]. Just MSC from healthy donor can cause Th1 cells to decrease interferon- γ (IFN- γ) and cause the Th2 cells to increase secretion of interleukin (IL)-4 [20]. These suggest that the Th1 polarization in ITP may be corrected by MSC therapy and that MSC treatment might cause a shift in the Th1/Th2 cy-

tokine balance to the same levels as normal controls, leading to a more balanced Th1/Th2 cytokine profile response in vivo. So, healthy donor-derived MSC treatment may represent a novel therapeutic strategy for immune-mediated ITP. In this report, we describe our experience using adipose tissue-derived MSC (AMSC) to treat patients with particularly severe and refractory ITP.

Patients and Methods

AMSC preparation

After ethics committee at Henan Tumor Hospital approved the study and the healthy haplo-identical family donors gave written informed consent, AMSC were isolated, respectively, as previously described [21]. Briefly, subcutaneous abdominal adipose tissue obtained from the healthy donor was digested with 0.2% collagenase II (Sigma) for 30 min under constant shaking. After removal of the floating mature adipocytes and erythrocytes, the lower layer was centrifuged (200 g, 10 min). After successive filtrations through 100- and 70- μ m sieves, the cells were washed with phosphate buffered saline/2% fetal calf serum (FCS; Gibco Life Technologies) for 2 times and then plated in polystyrene flasks at a density of 2×10^6 /mL. Selective expansion medium contained 57% D-MEM/F-12 (Gibco), 40% MCDB-201 (Sigma), 2% FCS (Gibco), $1 \times$ insulin transferrin selenium (Gibco), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 10 ng/mL epidermal growth

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factor (Sigma), 10 ng/mL platelet-derived growth factor BB (Sigma), 100 U/mL penicillin, and 1,000 U/mL streptomycin (Gibco). Once adherent cells were more than 70% confluent, they were detached with 0.125% trypsin and 0.01% EDTA (Sigma) and expanded under the same culture conditions. The culture-expanded cells were assayed in a flow cytometer (FACSort; Becton Dickinson), and the data analyzed with Cellquest software (Becton Dickinson). As we previously described [21], these cells displayed fibroblast-like morphology and expressed fetal liver kinase, CD166, CD105, CD44, CD29, and HLA class I, but not CD34, CD45, CD14, or HLA class II. During the log phase of growth, the cells proliferated with a population doubling time of about 22 h. Before infusion, the cells were cultured negative for bacteria, mycoplasma, and fungi.

Patients

ITP was diagnosed in accordance with standard criteria and after other causes of thrombocytopenia were excluded. Seven adult patients with ITP having a platelet count less than $20 \times 10^9/L$ that persisted for at least 12 months with an inadequate or transient response to multiple therapies were treated with AMSC (Table 1). Patient 1 was described previously [22]; the information on this patient given here represents an update.

The control group consisted of 10 adult healthy volunteers (5 females and 5 males, age range 23–47 years, median 29 years). Platelet counts were ranged from 125 to $198 \times 10^9/L$, with the median count of $168 \times 10^9/L$.

Treatment regimen

A dose of 2×10^6 MSC/kg was given intravenously to each of the patients. No maintenance or other treatment modality was used. Initial response evaluation was made at the end of the first week after treatment initiation. We defined overall response as a platelet count level of $30 \times 10^9/L$ or higher and doubling of baseline maintained for at least 4 weeks to reflect the goals of treatment for this group of refractory patients [23,24]. Response was classified as sustained when it was stable for a minimum of 6 months. Relapse was defined as a drop in platelet count to below $30 \times 10^9/L$ and/or the need for ITP rescue treatments.

Laboratory assay

All of the serum samples were isolated from 10-mL ethylene diaminetetraacetic acid (Pharmacia)-anticoagulated peripheral blood by centrifuging at 2,000 rpm at room temperature for 20 min twice, and then stored at -20°C for future use. Serum samples were obtained before therapy and once a week after therapy. Determination of IFN- γ , IL-2, IL-4, IL-10, and transforming growth factor $\beta 1$ (TGF- $\beta 1$) levels in the serum was performed by an enzyme-linked immunoassay [15,25].

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation. The differences between 2 groups and patients before and after treatment were evaluated using unpaired and paired Student's *t*-test, respectively.

Results

Patient characteristics

The clinical characteristics of patients entered into our study are summarized in Table 1. The patients were 3 women and 4 men, with the median age of 32 years (range, 21–45 years). The median duration of ITP before AMSC treatment was 53 months (range, 14–74 months) and the median number of prior treatments was 6 (range, 4–8), which included splenectomy, prednisone, intravenous immune globulin, danazol, cyclophosphamide, vincristine, azathioprine, cyclosporine, and T-cell-depleted autologous peripheral blood stem cells transplantation. All patients had a history of major bleeding; these episodes were often transient but recurrent. Major hemorrhagic events included gastrointestinal or genitourinary bleeding, intrabuccal hemorrhagic vesicles, diffuse ecchymosis, prolonged epistaxis, and retinal hemorrhages.

Clinical therapeutic effect of AMSC

The results of AMSC treatment are shown in Table 1. Overall responses were reached in all patients at the end of the second week after the AMSC had been administered. Six (85.7%) patients achieved a platelet count $> 50 \times 10^9/L$ and 5 (71.4%) achieved a platelet count $> 90 \times 10^9/L$. The median platelet count on treatment was $92 \times 10^9/L$ (range, 45 – $104 \times 10^9/L$). The median time to response and the median time to maximum response were 13 days (range, 11–17 days) and 26 days (range, 21–34 days), respectively. No side effects were noted after the AMSC infusions. The median follow-up period was 13 months (range, 5–55 months) by now. Among all the 7 patients, 4 (57.1%) sustained response after a single course of AMSC without any further therapy during follow-up. Major bleeding episodes did not occur. The remaining 3 patients (patients 4, 6, and 7) had a relapse within 6 months after the first AMSC administration but responded to the second AMSC treatment. The time to the second response for patients 4, 6, and 7 was 11, 12, and 16 days, respectively, whereas the time to the second maximum response was 26, 27, and 31 days, respectively. Now they all have got sustained response for more than 8 months. Figure 1 depicts the platelet count response in 1 such representative patient (patient 7).

No side effects such as fever, chills, and respiratory symptoms were noted during or after the AMSC infusions. During the whole follow-up period, neither ectopic tissue formation nor other illnesses related to the AMSC treatment have been recorded in our group of patients.

Cytokine profile changes in ITP patients

Compared with the normal controls, the pretreatment serum levels of IFN- γ and IL-2 were significantly increased ($P < 0.05$) in patients with active ITP, whereas IL-4, IL-10, and TGF- $\beta 1$ levels were considerably decreased ($P < 0.05$) (Table 2). After AMSC treatment, both of IFN- γ and IL-2 levels were significantly decreased ($P < 0.05$) and normalized, whereas the levels of IL-4, IL-10, and TGF- $\beta 1$ were significantly increased ($P < 0.05$). There was no significant difference between the treated patients and the normal controls ($P > 0.05$). The cytokine profiles in patients maintaining sustained

TABLE 1. PATIENTS' CHARACTERISTICS BEFORE AND AFTER ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS TREATMENT

Patient No.	Age/gender	Duration of disease (months)	Previous treatments	AMSC donor Age/gender	AMSC dose ($\times 10^6/\text{kg}$)	Platelet counts ($\times 10^9/\text{L}$)		Bleeding ^b		Time to maximum response (days)	Overall response	Response duration (months)
						Before therapy	After therapy ^a	Before therapy	After therapy			
1	21/M	43	P, V, Az, C, IVIg, S, CyA, AutO-PBSCT	Haplo/44/F	2	9	51	Major skin, retinal	Minor skin, mucosal	34	Yes	Yes, 55
2 ^c	34/F	71	P, V, Az, C, IVIg, S, CyA	Haplo/F/29	2	12	92	Major skin, menorrhagia	No	21	Yes	Yes, 19
3	45/M	62	P, IVIg, A, D, De, S	Haplo/F/42	2	5	103	Major skin, retinal	No	23	Yes	Yes, 13
4	32/F	53	P, D, V, Az, IVIg, S	Haplo/F/37	2	11	91	Major skin, mucosal	No	25	Yes	Yes ^d , 8.5
5 ^c	29/M	37	P, D, V, Az, IVIg, S	Haplo/M/32	2	15	104	Major skin, intestinal	No	26	Yes	Yes, 12
6	22/F	74	P, De, V, S, CyA	Haplo/F/48	2	3	45	Major skin, menorrhagia	Minor skin, mucosal	27	Yes	Yes ^d , 10
7	38/M	14	P, Az, S, V	Haplo/M/34	2	10	99	Major skin, mucosal	No	31	Yes	Yes ^d , 8.7

Patient 1 was described previously [15].

^aPlatelet count measurements were done at the end of the second week after AMSC treatment initiation.

^bMajor skin indicates diffuse ecchymosis; mucosal, intrabuccal hemorrhagic vesicles, or prolonged epistaxis; and intestinal and menorrhagia, gastrointestinal, and genitourinary bleeding, respectively.

^cThe patients had psoriasis, which occurred before immune thrombocytopenic purpura.

^dThese 3 patients had a relapse within 6 months after the first AMSC administration but responded to the second AMSC treatment, and they all have got sustained response for more than 8 months so far.

M, male; F, female; P, prednisone; V, vincristine; Az, azathioprine; C, cyclophosphamide; IVIg, intravenous immunoglobulins; S, splenectomy; CyA, cyclosporin A; PBSCT, peripheral blood stem cells transplantation; D, danazol; De, dexamethasone; Haplo, HLA haploidentical related donor; AMSC, adipose tissue-derived mesenchymal stem cells.

FIG. 1. Platelet count achieved after AMSC in a representative patient (patient 7) with chronic refractory ITP. Day 0 = the first dose of AMSC infusion. AMSC, adipose tissue-derived mesenchymal stem cells; ITP, immune thrombocytopenic purpura.

response remained stable compared to the post-treatment level ($P > 0.05$). On the contrary, IFN- γ and IL-2 levels increased, and IL-4, IL-10, and TGF- β 1 levels reduced again in the relapsed patients ($P < 0.05$) (Table 2).

Discussion

Patients with chronic refractory ITP have an associated mortality of 10%–30% from bleeding or perhaps, more frequently, toxicities of therapy [1,2]. Currently, the options for the management of these patients are limited, although the recent introduction of thrombopoietin-receptor agonist offers considerable promise [26,27]. Rituximab is an emerging new agent for the treatment of several autoimmune disorders and, in particular, many reports highlighted the effect of rituximab in refractory ITP [28–30], but this strategy is not practical just because most patients with refractory ITP in China cannot afford it.

Chronic ITP is an autoimmune disorder in which activated Th cells and different Th-cell cytokines might play an important role [3–15]. So, T-cells depletion might be a fundamental condition for a positive outcome of transplantation in ITP, similar to that observed in other autoimmune diseases submitted to autologous transplantation, but in different reports its success has been variable [31–35]. In addition, it has been described that depletion of CD8 $^{+}$ T cells with anti-CD8 monoclonal antibodies and complement did not reduce the proliferative capacity of the responding peripheral blood mononuclear cells from patients with ITP, indicating that CD4 $^{+}$ T helper cells may be responsible for the response [7].

A recent study assessed the intracellular IL-4 and IFN- γ production in CD4 $^{+}$ T lymphocytes activated by phorbol 12-myristate 13-acetate and ionomycin in patients with ITP, and found that the Th1/Th2 ratio in the untreated group was significantly higher than that in the control group [14]. In accordance with previous reports [14,36,37], the serum levels of IFN- γ , IL-2, IL-4, and IL-10 in our patients further implied a Th1-dominated cytokine profile in ITP with active disease. In addition, our data show that the significantly lower level of TGF- β 1 observed in the patients registered in this study should be associated with a downregulated Th3 response in active ITP patients, but it significantly elevated after AMSC treatment, so this suggests that Th3 may play an important role in bystander immune suppression in ITP [38,39]. More interestingly, we found that AMSC therapy for ITP could cause a shift in the Th1/Th2 cytokine balance to the same levels as normal controls, leading to a more balanced Th1/Th2/Th3 cytokine profile response in vivo [14,40,41]. Furthermore, our data displayed that in the patients who maintained the response could also sustain their cytokine profiles very much resembling their post-treatment pattern; however, the cytokine profiles tended to get back to the baseline values in the relapsed patients. These data indicated that relapsed ITP rooted in recurring of the Th cytokine imbalance, and may need repeated or additional treatment.

In addition, in ITP patients, MSC have a reduced proliferative capacity and a lower inhibitory effect on activated T-cell proliferation compared with MSC from healthy donors [19]. These abnormalities suggest a role of MSC malfunction in the physiopathology of the disease and may have

TABLE 2. PLASMA CYTOKINE LEVELS CHANGE IN IMMUNE THROMBOCYTOPENIC PURPURA PATIENTS

Cytokines (pg/mL)	Normal control (n=10)	Pretreatment (n=7)	Post-treatment (n=7)	Response duration (n=4)	Relapsed (n=3)
IFN- γ	11.07 $\pm$ 3.28	20.53 $\pm$ 5.53 ^a	11.88 $\pm$ 3.93 ^b	12.10 $\pm$ 1.73	22.65 $\pm$ 7.64 ^{c,d}
IL-2	8.37 $\pm$ 5.35	25.19 $\pm$ 9.08 ^a	11.70 $\pm$ 4.73 ^b	11.54 $\pm$ 5.45	24.78 $\pm$ 5.92 ^{c,d}
IL-4	12.61 $\pm$ 5.81	5.30 $\pm$ 3.87 ^a	11.65 $\pm$ 4.96 ^b	11.50 $\pm$ 3.48	4.43 $\pm$ 2.50 ^{c,d}
IL-10	8.64 $\pm$ 2.90	3.40 $\pm$ 2.04 ^a	8.11 $\pm$ 2.78 ^b	8.84 $\pm$ 2.16	3.83 $\pm$ 1.53 ^{c,d}
TGF- β 1	7.50 $\pm$ 2.97	1.72 $\pm$ 0.74 ^a	5.17 $\pm$ 1.60 ^b	6.48 $\pm$ 2.56	1.67 $\pm$ 0.97 ^{c,d}

^a $P < 0.05$, pretreatment versus normal control.

^b $P < 0.05$, post-treatment versus pretreatment.

^c $P < 0.05$, relapsed versus response duration.

^d $P < 0.05$, relapsed versus post-treatment.

IL, interleukin; IFN- γ , interferon γ ; TGF- β 1, transforming growth factor β 1.

therapeutic implications. Finally, in vivo, in humans, autologous and allogeneic MSC are safe to infuse with no acute adverse events and no long-term MSC-associated adverse events [21,22,42–44]. In conclusion, we hypothesized that MSC therapy seems to represent reasonable salvage treatment in severe, potentially life-threatening, refractory ITP.

Acknowledgments

The authors would like to thank all patients for their cooperation. This study was supported by grants from the National Natural Science Foundation of China (No. 30900637) and the National Natural Science Foundation of China (No. 81070398).

Author Disclosure Statement

No competing financial interests exist.

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Received for publication May 07, 2011

Accepted after revision June 14, 2011

Prepublished on Liebert Instant Online June 28, 2011