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Original Article

Prediction of Clinical Outcome in Patients with Idiopathic Thrombocytopenic Purpura by Evaluating Bone Marrow Clot CD20$^+$ B Lymphocytes and Morphological Changes of Megakaryocytes

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The pathology of B-lymphocytes in the bone marrow of patients with idiopathic thrombocytopenic purpura (ITP) has not been well described, even though B-lymphocytes may be involved in the etiology of ITP. We retrospectively reviewed the medical records of 73 ITP patients between January 1997 and June 2005 with platelet counts of $< 50 \times 10^9/L$. Bone marrow clots were available for pathological review in 56 patients who were classified into 3 groups based on the results of the bone marrow clot examination : Group A (21 patients) had increased CD20$^+$ lymphocytes ($\geqq$ 1% of nucleated cells) and megakaryocytes with morphologic changes ; Group B (21 patients) had morphologic changes but no increase in CD20$^+$ lymphocytes ; and Group C (14 patients) had neither morphologic changes nor increased CD20$^+$ lymphocytes. Multivariate analysis showed that, compared to Group A, Group B had a significant prognostic factor ($p = 0.04$; odds ratio, 6.65 ; 95% confidence interval, 1.09 to 40.54) for achieving complete response, while Group C had a significant prognostic factor for any treatment response ($p = 0.04$; odds ratio, 14.26 ; 95% confidence interval, 1.08 to 188.02). Thus, ITP patients can be classified with different clinical outcomes based on immunohistopathological examination of bone marrow clots. 〔*J Clin Exp Hematopathol 48(1) : 11-15, 2008*〕

Keywords: CD20, B-lymphocyte, *Helicobacter pylori*, prednisolone

INTRODUCTION

Idiopathic thrombocytopenic purpura (ITP) is recognized as an immune-mediated disorder in which platelets are opsonized by autoantibodies and prematurely destroyed by the reticuloendothelial system.[1] The clinical outcome of ITP patients is variable. Approximately 25% to 30% of adult patients develop a chronic disease that becomes refractory to corticosteroids and splenectomy, as well as other available therapies.[1] However, the factors that predict the clinical outcome of ITP upon diagnosis are currently unknown.

Recently, several studies suggested that B-lymphocytes have an important role in the etiology of ITP. The presence of hyperplastic follicles in the white pulp in the marginal zone, which is formed by B-lymphocytes, was reported as a major pathologic feature of the spleen of ITP patients.[2] Clonal B-cell expansion has been reported in ITP[3] and anti-CD20 monoclonal antibody therapy has been reported to be useful in patents with refractory ITP.[4-6] However, the pathology of B-lymphocytes in the bone marrow has not been well described. In this study, we evaluated the pathological findings of the bone marrow clot using immunohistochemical methods and determined which factors would be useful for predicting outcome in ITP patients.

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PATIENTS AND METHODS

Study patients

Between January 1997 and June 2005, 73 adult patients with thrombocytopenia with a platelet count of < 50 x $10^9/L$ were diagnosed as having ITP in Toyohashi Municipal Hospital. Those patients underwent bone marrow aspirations at diagnosis. We do not conduct bone marrow biopsies unless the aspirated bone marrows do not contain sufficient materials for diagnosis. We reviewed the medical records of these patients. The diagnostic criteria of ITP were based on those previously described.[1] Patients with myelodysplasia in bone marrow clots were excluded from study patients. All medical procedures were performed with informed consent.

Definition of treatment response

As described in a previous report,[7] we defined a complete response (CR) as a platelet count of at least 150 x $10^9/L$ within 13 weeks after completion of therapy and a partial response (PR) as a doubling of the initial platelet count or a platelet count above 50 x $10^9/L$. We considered a platelet count that decreased to < 50 x $10^9/L$ after achieving CR or PR as a relapse, and patients who could not achieve CR or PR by primary and/or secondary treatment as being refractory.

Helicobacter pylori eradication

Helicobacter pylori (*H. pylori*) infection was assessed by the ^{13}C urea breath test. To eradicate *H. pylori*, lansoprazole (30 mg twice daily), clarithromycin (200 mg twice daily), and amoxicillin (750 mg twice daily) were given for 7 days. Eradication was assessed by repeating the urea breath test 1-3 months after treatment completion.

Pathological examination

All pathological evaluations were performed in a blinded fashion; a single experienced pathologist retrospectively reviewed all stored bone marrow clots without the patients' clinical characteristics and outcomes.

We conducted immunohistochemical examination as previously described.[8] Formalin-fixed bone marrow clots were embedded in paraffin, and serial sections were stained with hematoxylin and eosin as well as with a naphthol-ASD chloro-acetate esterase plus Giemsa double-staining (ASD-Giemsa) method. Immunohistochemical studies were performed using a streptavidin-biotin method. Mouse monoclonal antibodies, including CD20 (Dako, Glostrup, Denmark), were used as the primary antibodies. For the antibodies that required it, antigen retrieval was carried out with 0.5% trypsin digestion at 37°C for 30 min or with microwave treatment in 0.1M citrate buffer at pH6.0. Endogenous peroxidase activity was blocked with incubation in 0.3% hydrogen peroxide in methanol. Reactions were made visible with 3,3'-diaminobenzidine as a chromogen in 0.005% hydrogen peroxide, followed by counterstaining with Mayer hematoxylin.

Endpoint and statistical analysis

The primary endpoint of this study is to investigate whether the pathological change in the bone marrow clot which was obtained for the diagnosis of ITP have a prognostic impact of treatment outcome at 13 weeks after the completion of therapy or 6 months after diagnosis.

Fisher's exact test was used for analyses of categorical data. With respect to analyses of the distribution of continuous variables, one-way analysis of variance was used if homogeneity of variance was assumed; where homogeneity of variance could not be assumed, the Kruskal-Wallis test was performed. A two-tailed *p*-value of less than 0.05 was considered significant. We used multivariate logistic regression models to calculate the odds ratios of the potential risk factors for the outcome. All analyses were performed using Dr. SPSS II (SPSS, Chicago, IL).

RESULTS

Patients' characteristics

The bone marrow clots which were obtained at diagnosis contained sufficient nucleated cells for pathological review in 56 of 73 patients. Patients' characteristics are shown in Table 1. Median age of 56 patients was 66 years old (range, 16-86). Twenty-five patients are male and 31 were female. The pathological findings of the bone marrow are shown in Fig. 1. The median duration of follow-up was 15.6 months (range, 0.03-139.8).

Classification of study patients on pathological findings

Based on the pathological findings of the bone marrow clot, we classified the patients into 3 groups: Group A had morphologic changes of the megakaryocytes and increased $CD20^+$ lymphocytes ($\geqq$ 1% of nucleated cells); Group B had morphologic changes without increased $CD20^+$ lymphocytes (< 1% of nucleated cells); and Group C had neither morphologic changes of the megakaryocytes nor increased $CD20^+$ lymphocytes. The patients with megakaryocytes with morphological changes and no increase in $CD20^+$ lymphocyte are mostly classified into Group C. The morphological changes of megakaryocyte included a thick cytoplasmic rim, elongated cytoplasmic projections like a "corona" with the attachment of neutrophils on the top of the projections (Fig. 1b). The cytoplasmic projections occasionally are accompanied

Table 1. Clinical and pathological characteristics of the patients

Variable	Group A	Group B	Group C	*P*-value
No. of patients	21	21	14	
Age [median (range)]	58 (16-74)	71 (20-86)	72 (54-83)	< 0.01
Sex (male/female)	9/12	9/12	7/7	0.89
Laboratory findings at diagnosis				
White blood cell count [median (range)] ($10^9/L$)	6.10 (3.60-10.3)	5.24 (2.80-12.8)	5.66 (3.70-11.7)	0.61
Red blood cell count [median (range)] ($10^{12}/L$)	4.25 (3.51-5.39)	4.25 (2.15-5.42)	4.01 (2.31-5.11)	0.23
Hemoglobin [median (range)] (g/*dL*)	12.9 (9.0-15.7)	12.4 (6.2-15.8)	12.4 (7.3-16.3)	0.9
Platelet count [median (range)] ($10^9/L$)	13 (2-40)	15 (1-46)	26 (1-46)	0.51
Platelet associated-IgG [median (range)] (ng/10^7 cells)	200 (35.2-1094)	143 (48.5-1220)	116 (56.7-683)	0.76
Bone marrow findings at diagnosis				
Number of megakaryocyte per x400 field [median (range)]	8 (6-10)	6 (4-10)	2 (2-4)	< 0.01
$CD20^+$ lymphocytes in bone marrow (< 1%/1-5%/> 5% of nucleated cells)	1/7/13	2/15/4	10/3/1	< 0.01
Primary treatment				0.17
Prednisolone 1 mg/kg	8	4	1	
Prednisolone 0.5 mg/kg	8	12	6	
Observation without steroid therapy	5	5	7	
Treatment response of primary treatment (CR/PR/others)	7/7/7	12/2/7	6/5/3	NA
Treatment response of primary treatment within 2 weeks (CR/PR/others)	2/10/9	5/8/8	2/4/8	
Secondary treatment				1.00
Prednisolone and Azathioprine	1	1	0	
Splenectomy	0	1	0	
Treatment response (CR/PR)	0/0	2/0	0/0	
Helicobacter pylori infection				
Positive patients/assessed patients	5/10	5/8	4/8	1.00
Eradication	4	3	4	0.28
Treatment response (CR/PR/others)	1/1/2	2/0/1	4/0/0	0.33
Refractory patients	3	0	0	0.11
Relapsed patients	1	2	2	0.84

CR, complete response; PR, partial response; NA, not applicable

with the presentation of complement molecules (C3 and C4). It is speculated that those morphological changes are caused by the mechanism of antibody dependent cellular cytotoxicity and those changes are specific to ITP (unpublished data provided by Ito M, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan).

Pathological findings in ITP were shown in Fig. 1. The patients were classified into those 3 groups as follows; Group A (n = 21), Group B (n = 21), and Group C (n = 14) (Table 1).

Treatments of ITP

Treatment for ITP was shown in Table 1. Primary treatment consisted of prednisolone in 39 of 56 patients. CR was achieved in 25 patients, and PR was achieved in 14 patients after primary treatment. Spontaneous CR and PR without treatment occurred in 3 and 4 patients, respectively. Twenty-six patients underwent the ^{13}C urea breath test. Results of the ^{13}C urea breath test and *H. pylori* eradication was shown in Table 1.

Pathological findings and treatment responses

Results of the multivariate analysis are shown in Table 2. The pathological findings were significant prognostic factors for treatment responses. Compared to Group A, Group B status was a significant prognostic factor ($p = 0.04$; odds ratio, 6.65; 95% confidence interval, 1.09 to 40.54) for achieving CR, and Group C status was a significant prognos-

Table 2. Prognostic factor for treatment response

Factor	Prognostic factor for complete response					Prognostic factor for any treatment response				
	P-value	Odds ratio	95% confidential index			*P*-value	Odds ratio	95% confidential index		
Gender (female vs. male)	0.76	0.81	0.21	−	3.17	0.19	3.08	0.58	−	16.42
Pathology (Group B vs. Group A)	0.04	6.65	1.09	−	40.54	0.95	1.05	0.20	−	5.57
Pathology (Group C vs. Group A)	0.09	6.35	0.76	−	53.12	0.04	14.26	1.08	−	188.02
Age ($\geq$ 65 vs. < 65 years)	0.60	1.48	0.34	−	6.38	0.83	1.19	0.24	−	5.85
Primary treatment (0.5 mg/kg of PSL vs. no therapy*)	0.05	5.42	0.98	−	30.02	0.01	12.00	1.84	−	78.17
Primary treatment (1 mg/kg of PSL vs. no therapy*)	< 0.01	65.90	4.13	−	1050.39	0.01	112.46	2.90	−	4365.71
Hemoglobin (> 10 vs. $\leq$ 10 g/*dL*)	0.56	1.87	0.23	−	15.50	0.26	6.52	0.25	−	169.47

*Those included *H. pylori* eradication.

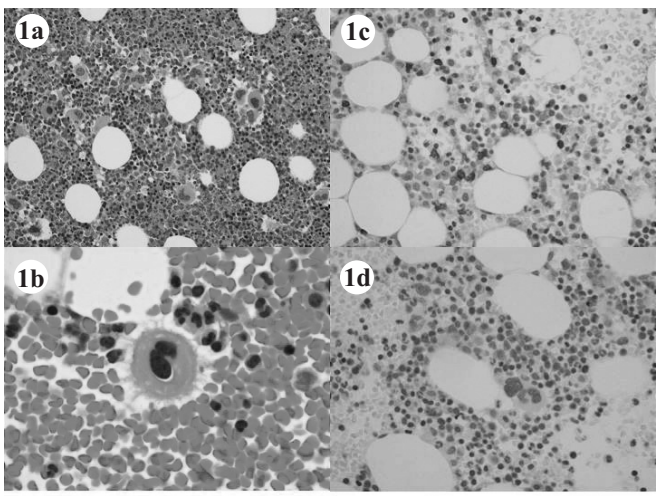

Fig. 1. Immunohistological analysis of bone marrow clots. (***1a***) Bone marrow histopathological findings of an ITP case. Increased numbers of megakaryocytes are randomly distributed in the marrow particle. (Bone marrow clot, HE stain, x20). (***1b***) Megakaryocyte of an ITP case. Mature megakaryocyte shows a thick cytoplasmic rim, elongated cytoplasmic projections like a "corona" with the attachment of neutrophils on the top of the projections. No dysplastic findings are observed. (Bone marrow clot, HE stain, x400). (***1c***) Bone marrow findings of an ITP case. About 5% of cells observed are $CD20^+$ B-cells in the high power view. (Bone marrow clot, CD20 immunostaining x100). (***1d***) Bone marrow findings of normal control. Less than 1% of cells observed are $CD20^+$ B-cells in the high power view. (Bone marrow clot, CD20 immunostaining, x200).

tic factor for any treatment response (p = 0.04 ; odds ratio, 14.26 ; 95% confidence interval, 1.08 to 188.02).

The 3 patients who were refractory to any form of treatment were all in Group A. One of the three patients died of cytomegalovirus pneumonia 18 months after diagnosis. The remaining 2 patients have been treated supportively and are alive 36 and 90 months after diagnosis, respectively.

DISCUSSION

We have shown that patients with ITP can be classified into 3 groups with different clinical outcomes based on immunohistopathological examination of the bone marrow clot. Group A patients, who included all refractory patients, had the worst treatment outcome. This suggests that the presence of $CD20^+$ lymphocytes in the bone marrow is related to an unfavorable outcome.

We suggested the importance of investigating on the B-cell clones. Previous studies suggested that the B-cell clones in ITP might produce antiplatelet antibodies[9,10] and involve in the activation of macrophage Fc-receptor function and the clearance of IgG-coated platelets.[4,11] However, the role of B-cells is still controversial. The increased B-cell clones might be the result of B-cell dysregulation.[3] Further studies on the role of those clones will allow a proper interpretation of these findings.

The morphological changes seen in the megakaryocytes have been found to be an important factor for patient outcome. However, the pathophysiology of these changes is unknown because those morphological findings have not been studied previously.[12,13] The adherence of antiplatelet antibodies and complement to megakaryocytes may have induced these changes. Further study is needed to determine the precise pathophysiology.

Interestingly, the patients with the remarkable increase of $CD20^+$ lymphocytes and morphologic change of megakaryocytes are younger than the patients with less pathological changes. This indicates that the etiology and optimal management of ITP might differ between younger and older adults. However, further large-scale multicenter studies are necessary to make proper interpretations. The median age was high and the patients were not predominantly female, which is inconsistent with previous studies of ITP in adults.[14-16] The patients' background might have affected the result, thus further large-scaled studies are warranted.

Recent reports have suggested that there is an association between *H. pylori* infection and ITP.[17,18] In this study, the rate of *H. pylori* infection was comparable in all groups. However, the number of patients who underwent the ^{13}C urea breath test was small. Furthermore, this study included patients with platelet counts of $< 50 \times 10^9/L$, for whom we normally use steroids as the primary therapy. Studies with more patients are required, and we are planning to evaluate immunohistopathological findings of bone marrow clots in patients with platelet counts of $> 50 \times 10^9/L$, in whom *H. pylori* eradication could be used as the primary treatment.

The present study provided novel information on developing a risk-based strategy for treating ITP. However, this study has its limitations, as it was a small, retrospective study in which unrecognized biases may have affected the results. Heterogeneity between different specimens from the same patient was not investigated. A single observer evaluated the marrow samples ; the inter-observer variation is unknown. However, to our knowledge this is the first study to demonstrate that the pathological evaluation of $CD20^+$ lymphocytes and the morphologic changes seen in the megakaryocytes could be useful prognostic factors for patient outcomes. Further studies in larger scale are warranted. Studies of the association of *H. pylori* infections and immunohistopathological findings in the bone marrow clot are also required. Furthermore, it might be worth studying the immunohistopathological changes after treatment for ITP. In conclusion, an approach based on pathological examination appears to be useful in establishing a risk-based strategy for treating ITP patients.

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