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Cancer Genetics and Cytogenetics 164 (2006) 90–91

CANCER GENETICS AND CYTOGENETICS

Letter to the editor

The translocation (4;12)(q31;q21) in myelofibrosis associated with myelodysplastic syndrome: impact of the 12q21 breakpoint

Myelofibrosis is sometimes associated with clonal hematopoietic stem cell disorders, including myelodysplastic syndromes (MDS), agnogenic myeloid metaplasia, and polycythemia vera. Nonrandom cytogenetic abnormalities have been found in myelofibrosis with myeloid metaplasia (MMM), but a single consistent abnormality such as Ph translocation in chronic myeloid leukemia has not been identified even though the new World Health Organization classification has proposed that abnormal activation of tyrosine kinase–dependent signal translocation pathways is essential in chronic myeloproliferative disorders. The frequent chromosomal changes in MMM are del(13q), del(20q), del(20p), and partial trisomy 1q [1]. Here we report on a case of MDS that finally evolved into myelofibrosis associated with t(4;12)(q31;q21).

A 70-year-old man complained of malaise in October 1994. Initial hematologic examination showed normocytic anemia: hemoglobin level of 87 g/L, a white blood cell count of 5.3 $\times$ 10^9/L, and a platelet count of 26.4 $\times$ 10^9/L. Upon physical examination, no organomegaly was observed. Bone marrow aspiration revealed slightly hypocellular marrow with trilineage dysplasia and 2% myeloblasts. Cytogenetic analysis of bone marrow cells revealed a normal male karyotype 46,XY (17 cells). He was diagnosed as having MDS refractory anemia (RA) and was treated conservatively. In May 2003, his hemoglobin decreased gradually and his liver and spleen enlarged progressively. Peripheral blood cells revealed dyserythropoetic changes such as anisocytosis, tear drop cells, and polychromasia with four erythroblasts in the blood cells. The bone marrow aspirate was a dry tap. Trephine biopsy revealed myelofibrosis with prominent multilineage dysplasia. Chromosomal analysis on peripheral blood cells revealed 46,XY,t(4;12)(q31;q21) (22 cells) (Fig. 1). Danazole therapy was initiated in association with transfusion as palliative therapy, but the latter did not appear to be effective. Thalidomide (200 mg) and prednisolone (20 mg/day, PO) were then administered on the basis of a diagnosis of MDS with myelofibrosis.

Balanced translocations in myeloproliferative disorders such as MMM are rare events. To our knowledge, balanced translocations have been reported in only 14 patients with myelofibrosis (Table 1). The translocation (4;12)(q31;q21) has not been reported in association with any myeloproliferative disorder other than MMM. Abnormalities at 12q21 and 12q24 alone or in combination with other abnormalities have been reported in several cases of myelofibrosis. Andrieux et al. [2] reported abnormalities at 12q21 or 12q24 in seven cases of MMM. They suggested that two different "hot spots" on 12q – 12q21 and 12q24 – might be involved in the malignant process of myelofibrosis. Breakpoints at 12q13~q21 or 12q24 subsequently have been identified in translocation-type aberrations in myelofibrosis. Our case demonstrates a clinical profile that may fit such a concept. The translocation (4;12)(q31;q21) was not detected during the MDS phase but appeared subsequently during evolution to myelofibrosis. Considering the long history of anemia and the recent marked splenomegaly in our case, we believe that 12q21 might be a secondary event rather than an initial aberration.

Recent studies have shown that the single point mutation of the cytoplasmic tyrosine kinase, JAK2, has a potential role in myeloproliferative disorders [3,4]. Baxter et al. investigated that a single point mutation (Val617Phe) in JAK2 was identified in 97% of patients with polycythemia

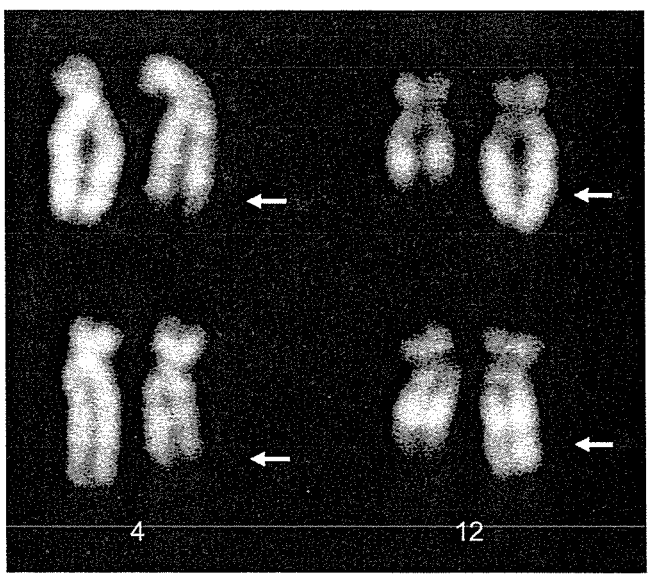

Fig. 1. Partial Q-banded karyotypes of unstimulated peripheral blood cells showing t(4;12)(q31;q21). Arrowheads indicate possible breakpoints of chromosomes 4 and 12.

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Table 1
Clinical features of well-documented cases showing balanced translocations in myelofibrosis

No.	Sex/age	Sample	Disease stage	Karyotypes of the abnormal clone	Report (reference)
1	M/43	BM	MMM at diagnosis	46,XY, t(4;12)(q33;q21)	Andrieux et al. [2]
2	M/75	BM	MMM at diagnosis	46,XY, t(5;12)(p14;q21)	Andrieux et al. [2]
3	F/67	BM	MMM at diagnosis	46,XX, t(1;12)(q22;q24)	Andrieux et al. [2]
4	M/58	BM	MMM at diagnosis	46,XY, t(7;12)(p11;q24)	Andrieux et al. [2]
5	F/44	BM	MMM at diagnosis	46,XX, t(1;12)(p21;q12)	Andrieux et al. [2]
6	M/ND	PB	MF at diagnosis	46,XY, t(6;12)(q13;q23)	Miller et al. [5]
7	M/59	PB	AMF on therapy	46,XY, t(1;4)(q32;q35)	Shah et al. [6]
8	M/76	ND	IMF at diagnosis	46,XY, t(1;12)(p31;q21)	Reilly et al. [1]
9	M/65	PB	IMF at blast crisis	46,XY, t(4;12)(q26;q15), t(5;12)(q13;q24)	Przepiorka et al. [7]
10	F/ND	PB	MF with AMM at diagnosis	46,XX, t(1;7)(p31;p22), t(10;20)(q26;q11)	Jean et al. [8]
11	M/64	BM	AMM at diagnosis	46,XY, t(8;12)(p23;q21)	Borrego et al. [9]
12	M/68	BM	CMPD with MF	46,XY, t(2;3)(p21;p26)	Herens et al. [10]
13	M/52	PB	MDS with MF	46,XY, t(5;12)(q33;p12-3)	Lerza et al. [11]
14	M/69	PB	MDS with MF	46,XY,t(4;12)(q31;q21)	Present case

Abbreviations: MF, myelofibrosis; BM, bone marrow; PB, peripheral blood; MMM, myelofibrosis with myeloid metaplasia; AMF, acute myelofibrosis; IMF, idiopathic myelofibrosis; AMM, agnogenic myeloid metaplasia; CMPD, chronic myeloproliferative disorder; ND, no description.

vera and in 50% of patients with idiopathic myelofibrosis. They suggested that these results have important implications for the classification, diagnosis, and treatment of these diseases and provide insight into their pathogenesis. However, the pathogenesis of half the patients with myelofibrosis has not been established yet. Other tyrosine kinases may be involved in chronic myeloproliferative disorders, including myelofibrosis. Of note is that translocation-type abnormalities in MF showed clustering breakpoints on chromosome 12 [i.e., 12q21 in 6/14 cases, 12q24 in 3/14 cases, and 12q12~q13 in 2/14 cases of myelofibrosis showing translocation-type abnormalities (Table 1)].

Our case confirms an association between myelofibrosis and abnormalities of chromosome 12, especially 12q21, regardless of the etiology of myelofibrosis. The role of the other chromosomal aberrations seen in myelofibrosis remains unclear, however, though we have not had a chance to investigate the JAK2 mutation. The present well-documented report of a case of myelofibrosis in MDS with balanced translocation (4;12)(q31;q21), in association with other well-characterized cases of myelofibrosis, should help in future analyses to identify the elusive genetic basis of this disease.

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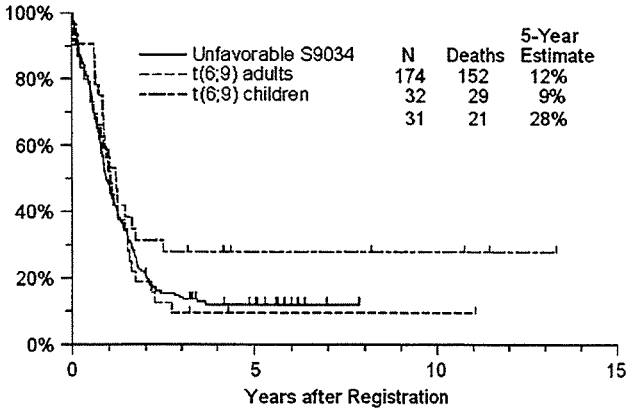

Figure 1 Estimated probabilities of overall survival of t(6;9) adult and pediatric cases compared with SWOG/ECOG S9034/E3489 younger adult AML study[8] unfavorable risk cytogenetics subgroup. Table 1 describes the t(6;9) patients included in this analysis.

perhaps molecular monitoring among the various centers or cooperative groups involved.

In closing, we report the largest t(6;9) patient cohort to date confirming and refining consistent t(6;9) pathological features including increased basophilia, single or multilineage dysplasia in adult patients, variable FAB morphology, a CD13+, CD33+, CD38+, CD45+, and HLA-DR+ immunophenotype, and a high incidence (71%) of FLT3 internal tandem duplications. Furthermore, the low incidence of secondary aberrations may be important for targeted therapeutic options in the future. Like all retrospective studies, data collection procedures were discordant among the Groups, complicating the analyses; nevertheless, our study objective is to raise awareness of this very poor risk AML subtype that tends to be more common in younger patients. Accordingly, t(6;9) AML may warrant a prospective multi-center investigation of aggressive and/or novel therapeutic strategies, including allogeneic SCT for patients with an HLA-matched donor, and perhaps because of the presence of CD33 and FLT mutations, the addition of anti-CD33-based therapies plus a FLT3 inhibitor for patients without suitable donors. Finally, to facilitate scientific collaboration to accomplish such an initiative, we support the proposal of adding AML with t(6;9)(p23;q34), DEK/NUP214, as a separate disease entity to the World Health Organization (WHO) Classification of Hematological Malignancies.[3,9]

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Reply to Kremer M *et al.*, The JAK2 V617F mutation occurs frequently in myelodysplastic/myeloproliferative diseases, but is absent in true myelodysplastic syndromes with fibrosis

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The current approach to detect JAK2 V617F mutation in patients with chronic myeloproliferative disorders (CMPD) revealed the molecular category of this disease entity and related diseases.[1,2] Kremer *et al.*[3] found JAK2 V617F mutation in three of four patients with myelodysplastic (MDS)/myeloproliferative (MPD) diseases, whereas none of 25 patients with MDS with myelofibrosis showed the mutation. They further stated that

MDS/MPD patients with the JAK2 V617F mutation showed clinical features somewhat resembling MPD, including splenomegaly, leukocytosis, and/or thrombocytosis, and morphologic features of CMPD in marrow biopsy.[3] They suggested that detection of the JAK2 V617F mutation could be a help to identify the borderline between typical MDS and atypical MPD.Myelodysplastic syndrome with myelofibrosis is believed to be a heterogeneous entity; some patients show myelofibrosis at the time of MDS diagnosis and are closely associated with acute myeloid leukemia with megakaryoblastic nature, or 'acute myelodysplasia with myelofibrosis'.[4] On the other hand, some MDS patients show myelofibrosis at a later stage of the disease.[5] Moreover, some patients with MPD have clinico-hematologic features resembling MDS at the first manifestation: some of them progress into MPD several years later. The WHO criteria may not cover all of these heterogeneous entities, therefore, we should probably diagnose such patients tentatively at the present juncture. For example, late appearing myelofibrosis in MDS patients should not be categorized as MDS/MPD at the first manifestation.

In our current study published in *Leukemia*,[6] we did not have the chance to identify the JAK2 V617F mutation during the MDS phase lacking myelofibrosis. We detected the mutation in the myelofibrosis phase; therefore, we considered that the difference of the JAK2 V617F mutation,[6] unlike the contention of Kremer *et al.*,[3] might mainly be owing to different diagnostic categorization. Owing to the lack of a concrete diagnostic definition of MDS with myelofibrosis, we cannot completely eliminate the possibility of overestimating 'true' MDS with myelofibrosis, but we do not know the biological or definite clinical significance of the disease, and such an overestimation may cause some confusion concerning the data on the JAK2 V617F mutation.

Eventually, as Kremer *et al.*[3] also suggested, MDS/MPD might be one domain of MDS, but probably only a part of even those may show the JAK2 V617F mutation, as about 90% of polycythemia vera and 50% of essential thrombocythemia or primary myelofibrosis carry this mutation. Therefore, we can only say that the JAK2 V617F mutation might be one diagnostic tool for the identification of MPD. An accumulation of conditions overlapping MDS to ascertain degree, including a compilation of the MPD-like clinico-hematologic features, in combination with the detection of the JAK2 V617F mutation, might be required to resolve the problematic topology of MDS with myelofibrosis.

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Chromosomal aberrations in leukaemia cells may delete tumour target antigens of stem cell-based immunotherapy

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A novel approach to eliminate residual disease after human leucocyte antigen (HLA)-matched stem cell transplantation (SCT) for leukaemia is tumour-specific adoptive immunotherapy. Excellent immunotherapeutic targets for adoptive immunotherapy are the haematopoietic system-specific minor-histocompatibility antigens (mHags) HA-1 and HA-2, which are expressed on all normal and malignant haematopoietic cells.[1,2] HA-1 and HA-2 epitopes are presented on the cell surface in the binding groove of HLA-A2 molecules. Immunotherapy with HA-1 or HA-2-specific cytotoxic T lymphocytes (CTLs) is restricted to leukaemia patients positive for the immunogenic mHag HA-1 or HA-2 alleles, that is HA-1H or HA-2V, respectively. The mHag status of patients is routinely determined by allele-specific genomic polymerase chain reaction (PCR) on peripheral blood mononuclear cells (PBMCs).[2] Generally, leukaemic cells have the same mHag allelic patterns as the PBMCs. Most leukaemias, however, have karyotypic abnormalities,[3] some of which affect mHag encoding genomic regions (e.g. 19p13.3 harbouring the HA-1 gene[4] or 7p12-13 harbouring the HA-2 gene[5]).

Here, we describe an isochromosome 7 causing loss of HA-2 CTL recognition of leukaemic cells in a patient with pre-B acute lymphoblastic leukaemia. The patient was typed on PBMCs to be heterozygous for both HA-1 (H/R) and HA-2 (V/M) (Figure 1e and f). The leukaemia karyotype was 46,XX,i(7)(q10),der(19)t(1;19)(q23;p13). Hereby, the HA-1 encoding region on one chromosome 19 and the HA-2 encoding region on one chromosome 7 were deleted (Figure 1a). The bone marrow of the patient contained 90% leukaemic blasts. We separated these CD10 positive leukaemia cells from nonleukaemic cells with magnetic beads and used them as targets in a chromium release

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Stromal-cell-derived factor-1/CXCL12-induced chemotaxis of a T cell line involves intracellular signaling through Cbl and Cbl-b and their regulation by Src kinases and CD45

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Abstract

Stromal-cell-derived factor-1α (SDF-1α/CXCL12) is a potent chemoattractant for T cells. We report that Cbl family members, Cbl and Cbl-b, are tyrosine-phosphorylated after SDF-1α/CXCL12 stimulation of Jurkat T cells. Enhanced phosphorylation of Cbl and Cbl-b was regulated by src family kinases, and perhaps Fyn. Activated Cbl and Cbl-b interacted with Crk-L, Zap-70, Nck, PLC-γ and Fyb after SDF-1α/CXCL12 stimulation, implicating association of these proteins in SDF-1α/CXCL12 actions. SDF-1α/CXCL12 did not induce tyrosine phosphorylation of Cbl or Cbl-b in Lck-deficient T cell line J.CaM1.6 or CD45-deficient T cell line J45.01. Thus, Lck Src kinase and tyrosine phosphatase CD45 are likely involved in regulating activation of Cbl family members. A functional role for Cbl and Cbl-b in migration was demonstrated by the decrease in SDF-1/CXCL12-induced migration in a T cell line in which transfected small interfering RNA for Cbl and Cbl-b decreased expression of Cbl and Cbl-b, but not MAPK activity. SDF-1α/CXCL12-induced chemotaxis was greatly reduced in the CD45-deficient T cell line. Our results implicate CD45, Cbl, Cbl-b, src kinases and potentially other associated proteins as mediators of SDF-1α/CXCL12-induced cell migration of Jurkat T cells.

Keywords: T lymphocytes; Jurkat cells; Chemotaxis; SDF-1/CXCL12; Signal transduction; Cbl; Src kinases; Lck; CD45

Introduction

Stromal-cell-derived factor-1 (SDF-1/CXCL12) is a CXC chemokine produced by bone marrow stromal cell types [1]. CXCR4, a receptor for SDF-1/CXCL12, is widely expressed by a variety of hematopoietic cell types, including T cells [2]. SDF-1 is chemotactic for human lymphoid, myeloid and CD34 positive progenitor cells and has been implicated in cell migration of human and murine hematopoietic cells (reviewed in [3,4]). Lymphopoiesis and myelopoiesis are markedly reduced in CXCR4 and SDF-1/CXCL12-deficient mice [5].

Towards a better understanding of events mediating SDF-1/CXCL12 effects, a number of investigators have begun to elucidate the intracellular signals triggered by SDF-1/CXCL12 and the role these proteins may play in SDF-1/CXCL12-induced cellular activities. The full range of intracellular signals regulating SDF-1/CXCL12 actions remains to be elucidated. Among a number of intracellular proteins implicated in SDF-1/CXCL12 actions are Cbl [6] and CD45 [7]. Cbl is tyrosine-phosphorylated and associates with PI3 kinase, Crk-L and 14-3-3 when cells are stimulated with SDF-1/CXCL12 [6]. The protein tyrosine phosphatase CD45 was shown to differentially regulate CXCR4-mediated chemotaxis as well as MAP kinase activation by regulating activities of focal adhesion components and other effectors downstream of the T cell receptor [7]. Cbl is a 120 kDa phosphoprotein expressed in the cytoplasm of many cell types. Cbl is the cellular homologue of the v-Cbl oncogene and was first cloned from a recombinant murine retrovirus that

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causes B-cell lymphomas and myelogenous leukemia in mice [8]. Multiple proline-rich stretches are found in the C-terminal domain of Cbl, enabling it to bind to several src homology 2/3 (SH2/SH3) adaptor proteins, such as Grb2 and Nck [9–11]. In addition, the C-terminus of Cbl contains several tyrosine phosphorylation sites. Tyrosine 700, 731 and 774 have been identified as major phosphorylation sites of Cbl by protein tyrosine kinases (PTKs) [12]. Cbl-b is a distinct Cbl-related gene with similar overall domain structure to Cbl [13]. Cbl is a major target of tyrosine phosphorylation in response to stimulation through a wide variety of cell surface receptors [14]. Cbl-deficient mice manifest tissue hyperplasia and abnormal duc formation in mammary glands, and Zap-70 is hyperphosphorylated in the thymocytes [15,16]. Overexpression of Cbl in mast cells suppresses Syk tyrosine kinase activity [17]. The aims of this study were to confirm and extend roles for Cbl and CD45 and determine a role for Cbl-b and associated proteins in the intracellular cascade of events set off by SDF-1α/CXCL12 actions in induction of chemotaxis.

Materials and methods

Reagents and antibody

Recombinant human SDF-1α/CXCL12, anti-phosphotyrosine monoclonal antibody (mAb, 4G10) and anti-Crk-L mAb were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-Cbl antibody (Ab), anti-Cbl-b Ab, GST agarose, GST-fyn agarose and protein A/G agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine mAb (PY20), anti-Cbl mAb, anti-Nck mAb anti-Zap-70 mAb, anti-Fyb mAb and anti-PLC-γ mAb were obtained from Transduction Laboratories (Lexington, KY). Anti-phospho-Cbl Ab was from Cell Signaling Technology (Beverly, MA). Src kinase inhibitor PP2 was from Calbiochem-Novabiochem Corporation (San Diego, CA). Other reagents were from Sigma (St. Louis, MO).

Cell culture, transfection and infection

Human leukemic T cell line Jurkat, Lck-deficient T cell line J.CaM1.6 and CD45-deficient T cell line J45.01 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The J.CaM1.6 cell line is a derivative mutant of Jurkat, and J45.01 cell line is a CD45-deficient variant of the E6-1 clone of Jurkat. These cell lines were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan UT) with 1% penicillin/streptomycin in a humidified incubator at 37°C.

Immunoprecipitation and Western blot analysis

Jurkat, J.CaM1.6 and J45.01 cells were factor-starved overnight and treated with 100 ng/ml SDF-1α/CXCL12 (a pre-established maximal concentration for induction of chemotaxis for Jurkat and other T cells) at the indicated times and washed once with ice-cold phosphate-buffered saline (PBS). Cells were lysed in lysis buffer containing 20 mM Tris–HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 μM EDTA; 10 μg/ml leupeptin; 100 mM sodium fluoride; 2 mM sodium orthovanadate; 1% NP-40, for 20 min on ice. The lysates were centrifuged at 12,000 rpm for 20 min at 4°C. Protein content of lysates was determined with protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein of cell lysates were boiled with 2× SDS sample buffer for 5 min. For immunoprecipitation, cell lysates were incubated at 4°C overnight with the indicated precipitating Ab. Immunoprecipitates were collected using 40 μl of protein A/G agarose for 2 h at 4°C. After washing four times in lysis buffer, immunocomplexes were eluted and boiled with 5 min in 2× sample buffer. Proteins or immunocomplexes were loaded onto polyacrylamide gels (Biowhittaker, Rockland, ME) and then transferred to polyvinylidine difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked by 3% skim milk PBS–Tween 20 (PBST) or 1% BSA PBST and probed with the indicated primary antibody at appropriate dilution for 2 h at room temperature (RT) or 4°C overnight. Blots were probed with secondary antibodies conjugated horseradish peroxidase and developed using the

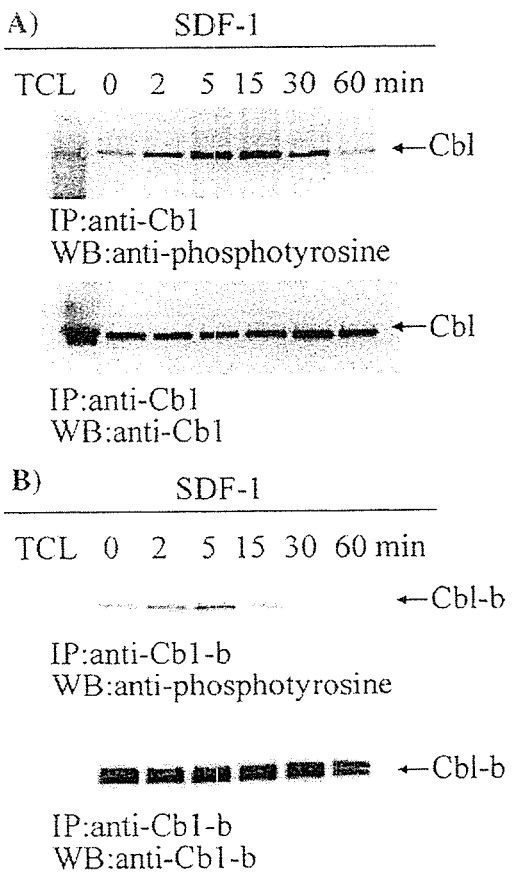

Fig. 1. SDF-1/CXCL12 enhances tyrosine phosphorylation of Cbl and Cbl-b. Jurkat cells were left unstimulated (−) or stimulated with SDF-1α/CXCL12 (100 ng/ml) for 5 min. Total cell lysates (TCL) were immunoprecipitated with anti-Cbl Ab (A) or anti Cbl-b Ab (B). Immunoprecipitates (IP) were immunoblotted with anti-phosphotyrosine Ab, anti-Cbl Ab (A) or anti Cbl-b Ab (B). WB: Western blot. Results shown in panels A and B are representative of at least 3 complete experiments.

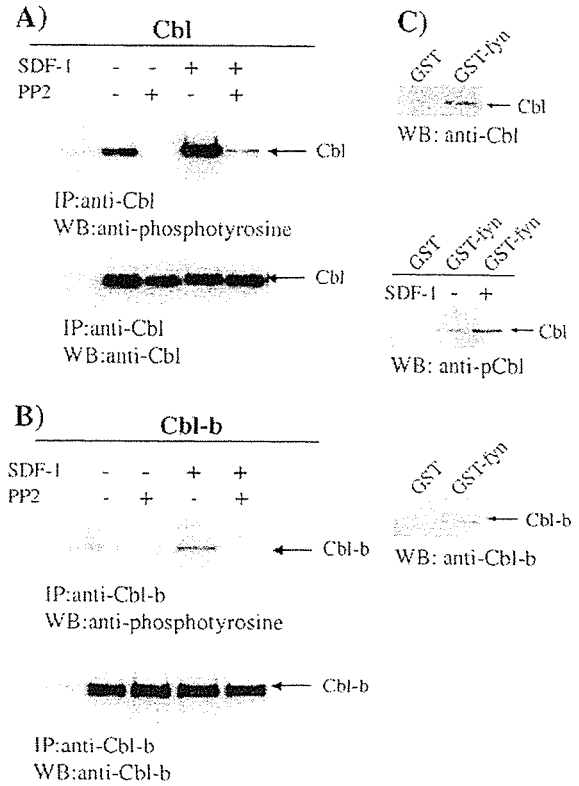

Fig. 2. Src kinase inhibitor PP2 blocks SDF-1/CXCL12 enhanced tyrosine phosphorylation of Cbl and Cbl-b. Jurkat cells were pretreated with src kinase inhibitor PP2 (10 μM) for 30 min and left unstimulated or stimulated with SDF-1α/CXCL12 at 100 ng/ml for 5 min. Cell lysates were immunoprecipitated with anti-Cbl Ab and immunoblotted with anti-phosphotyrosine or anti-Cbl Ab (A) or immunoprecipitated with anti-Cbl-b Ab and then immunoblotted with anti-phosphotyrosine or anti-Cbl-b Ab (B). Total cell lysates were incubated with GST or GST-fyn and then immunoblotted with anti-Cbl, anti-phospho-Cbl or anti-Cbl-b Ab (C). Results in panels A–C are one representative each of at least three similar experiments.

enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Bucks, UK) system with ECL film according to the manufacturer's specification.

Chemotaxis assay

Chemotaxis assays were performed using a Boyden chamber with a 5 μm pore size (Corning Incorporated, Corning NY) as described previously [18]. After incubation of the apparatus at 37°C for 2 h in humidified air with 5% CO_2, the filter was removed and the cells were counted by FACScan (Becton Dickinson, Mountain View) for 20 s.

Flow cytometric analysis

Cells were fixed with using Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA) or 1% paraformaldehyde PBS. Cells were incubated with anti-CXCR4 mAb (R&D systems, Minneapolis, MN) for 30 min at 4°C and then incubated with fluorescein-conjugated secondary antibody. The cells were monitored by flow cytometric analysis.

Electroporation of Jurkat cells

Jurkat cells were transfected by electroporation with siRNA for Cbl and Cbl-b. Cells were electroporated in 400 μl cultured medium at a density of 2×10^7/ml in electroporation cuvettes and mixed with 1 nmol siRNA. Cells were electroporated by using the Bio-Rad gene pulser II system at a setting of 0.2 kV

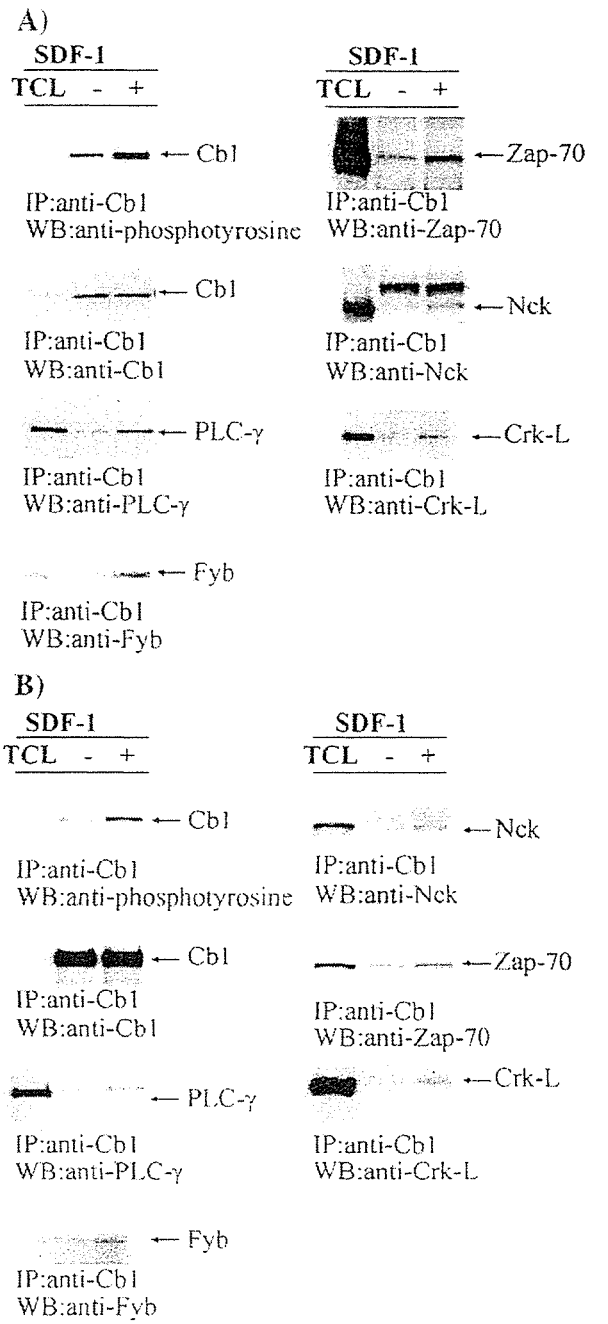

Fig. 3. SDF-1/CXCL12 enhances Cbl and Cbl-b association with Zap-70, Nck, PLC-γ, Crk-L and Fyb in Jurkat cells. Cbl (A) or Cbl-b (B) were immunoprecipitated from cell lysates of unstimulated or SDF-1α/CXCL12-stimulated cells and analyzed by Western blotting with anti-Zap-70, Nck, PLC-γ, Crk-L and Fyb Ab, or anti-Cbl Ab (A) or anti-Cbl-b Ab (B). Results in panels A and B are for 1 of at least 3 reproducible experiments.

and 950 μF capacitance. Fifteen minutes after electroporation, cells were diluted in culture medium and incubated at 37°C, 5% CO_2 and 92% humidity. After 48 h, cells were analyzed by chemotaxis assay and immunoblotting.

Results

SDF-1α/CXCL12 enhances tyrosine phosphorylation of Cbl and Cbl-b

Cbl is tyrosine-phosphorylated in response to stimulation via immune, growth factor, integrin and cytokine receptors [12] and SDF-1/CXCL12 [6]. In order to characterize signaling pathways activated by SDF-1/CXCL12, we used the Jurkat T cell line, which expresses the SDF-1α/CXCL12 receptor CXCR4. Jurkat T cells were serum-starved and stimulated with 100 ng/ml of SDF-1α/CXCL12 for the indicated times (Fig. 1). SDF-1α/CXCL12 enhanced tyrosine phosphorylation of Cbl (Fig. 1A), confirming the results of others [6]. Another Cbl family member Cbl-b was also tyrosine-phosphorylated in response to SDF-1/CXCL12 (Fig. 1B) extending information to a Cbl family member. Enhanced tyrosine phosphorylation of Cbl and Cbl-b was detected from 2 min to 30 min after treatment of cells with SDF-1/CXCL12. Determination of the loading of equal amounts of protein in all lanes was accomplished by stripping and blotting with Cbl and Cbl-b antibody.

SDF-1/CXCL12-enhanced phosphorylation of Cbl and Cbl-b is regulated by src family kinases

Tyrosine phosphorylation of Cbl appears to be mediated by several protein tyrosine kinases (PTKs), such as those of the src family PTKs [19,20]. Serum-starved Jurkat cells were pretreated with 10 μM of the specific src kinase inhibitor PP2 for 30 min and then stimulated with 100 ng/ml SDF-1α/CXCL12 for 5 min. Pretreatment of cells with PP2 completely or greatly blocked Cbl and Cbl-b phosphorylation (Figs. 2A and B). These results suggest that phosphorylation of Cbl and Cbl-b is regulated by src family kinases. We then assessed if Cbl and Cbl-b bound to the Src family member Fyn. SDF-1/CXCL12

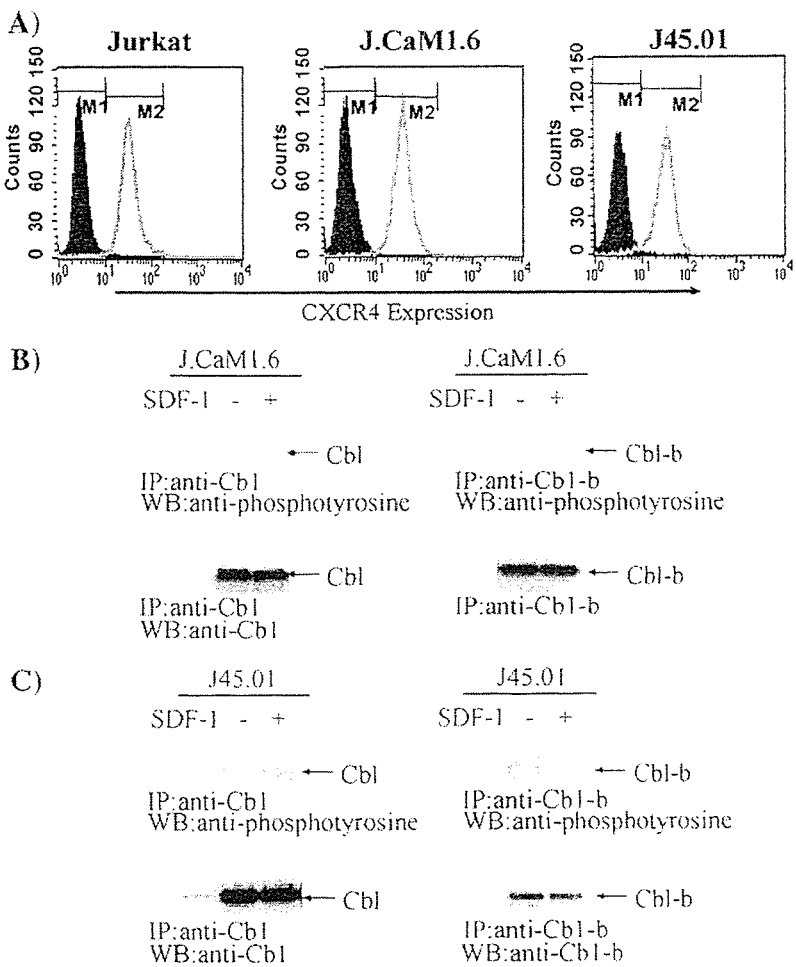

Fig. 4. SDF-1/CXCL12 does not enhance tyrosine phosphorylation of Cbl and Cbl-b in J.CaM1.6 and J45.01 cells. Cell surface expression of CXCR4. Jurkat, J.CaM1.6 and J45.01 cells were fixed by 1% paraformaldehyde PBS and stained with anti-CXCR4 Ab. After staining with a secondary Ab, surface CXCR4 levels were analyzed by FACScan (A). Tyrosine phosphorylation of Cbl or Cbl-b in J.CaM1.6 (B) or J45.01 (C). Cbl and Cbl-b were immunoprecipitates and analyzed by Western blotting with anti-phosphotyrosine or anti-Cbl or Cbl-b Ab. Results in panels A–C are for 1 of at least 3 reproducible experiments each.

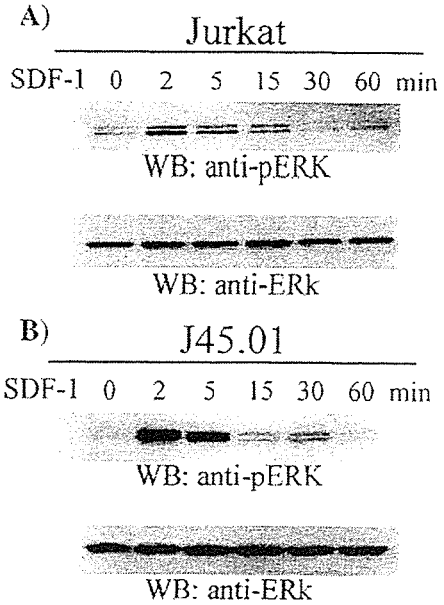

Fig. 5. SDF-1/CXCL12-enhanced MAPK activity is the same in J45.01 and parental Jurkat cells. Jurkat (A) and J45.01 (B) cells were left unstimulated (−) or stimulated with SDF-1α/CXCL12 (100 ng/ml) for the indicated times. Total cell lysates were immunoblotted with anti-phospho-ERK Ab or anti-ERK Ab. Results in panels A and B are representative of at least 3 complete experiments.

stimulation enhanced the binding of Cbl and Cbl-b to the Src family member Fyn as assessed by GST "pull-down" (Fig. 2C).

SDF-1/CXCL12 enhances the association of tyrosine-phosphorylated Cbl and Cbl-b with Crk-L, Zap-70, Nck, PLC-γ and Fyb

Cbl has been shown to form complexes with many proteins via its various functional domains. Tyrosine kinase binding domains of Cbl bind to several receptor PTKs, including Syk, Zap-70 and Src, and proline-rich regions of Cbl interact with SH3-containing proteins, such as Nck and Crk family adaptor proteins. Fyn binding protein (Fyb), also called SLAP-130 (SLP-76 associated phosphorylation of 130 kDa), includes several proline-rich regions and multiple tyrosine motifs [21]. SDF-1/CXCL12 enhanced the association of tyrosine-phosphorylated Cbl (Fig. 3A) and Cbl-b (Fig. 3B) with Crk-L, Zap-70, PLC-γ, Nck and Fyb.

Enhanced tyrosine phosphorylation of Cbl and Cbl-b in response to SDF-1/CXCL12 is not apparent in J.CaM1.6 and J45.01 cells

We showed above that tyrosine phosphorylation of Cbl and Cbl-b is regulated by src kinase family members. Lck is a member of the src kinase family and is important to T cell function. Lck regulates T cell surface receptors, such as CD2 and CD4 [22,23]. J.CaM1.6 cells are derived from Jurkat cells and are defective in expression of Lck. J45.01 cells are also derived from Jurkat cells but are defective in the phosphatase CD45. Surface expression of CXCR4 was similar between J. CaM1.6 and J45.01 cells and their parental cell line Jurkat (Fig. 4A). CD45 is a receptor-like protein tyrosine phosphatases (PTPs) expressed on all nucleated hematopoietic cells. CD45 serves as a positive regulator of src family kinases [24]. SDF-1/CXCL12 did not enhance tyrosine phosphorylation of Cbl and Cbl-b in J.CaM1.6 (Fig. 4B) or J45.01 (Fig. 4C) cells. These results demonstrate that Lck and CD45 are involved in regulation of tyrosine phosphorylation of Cbl and Cbl-b in response to SDF-1α/CXCL12.

MAPK activity is not decreased in J45.01 cells

MAPK is involved in signal transduction events mediating proliferation, differentiation and apoptosis in eukaryotic cells [25]. Extracellular-signal-regulated kinases 1/2 (ERK1/2) are typically stimulated by growth-related stimuli. We recently reported that MAPK activity is not different in J.CaM1.6 cells and parental Jurkat cells, suggesting that Lck is not involved in regulation of MAPK activity in response to SDF-1/CXCL12 [26]. We now evaluated whether or not the tyrosine phosphatase activity of CD45 was involved in SDF-1/CXCL12 effects on MAPK activity. MAPK activity was not decreased in J45.01 cells compared to Jurkat cells (Fig. 5), suggesting that CD45 does not regulate MAPK activity in response to SDF-1/CXCL12.

Chemotaxis response decreased in J45.01 cells

Jurkat cells respond chemotactically to SDF-1α/CXCL12 [6,18]. We chose to assess the chemotactic response of Jurkat (Parent), J.CaM1.6 (Lck-deficient) and J45.01 (CD45-deficient) cells to a concentration of SDF-1 (200 ng/ml) that is a plateau dose that induces maximum migration of Jurkat cells [18]. J. CaM1.6 cells were previously shown by us to be responsive to SDF-1/CXCL12-induced chemotaxis [26] and were used here as a control. As shown in Fig. 6, the chemotactic response to SDF-1α/CXCL12 was significantly decreased in J45.01 cells, but not in J.CaM1.6 cells, compared to Jurkat cells. This confirms the results of others [7] that CD45 is involved in cell migration in response to SDF-1α/CXCL12.

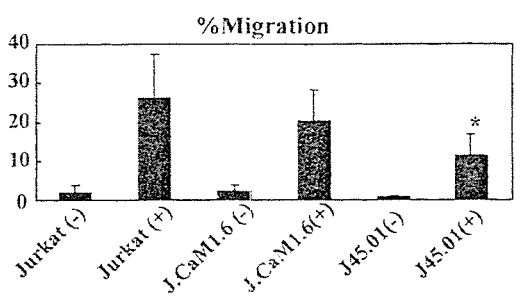

Fig. 6. J45.01 but not J.CaM1.6 cells are reduced in response to the chemotaxis activity of SDF-1/CXCL12 compared to parental Jurkat cells. Jurkat, J.CaM1.6 or J45.01 cells (1×10^5 each) were placed in the upper part of chemotaxis chambers. Cells that migrate to the lower chambers after 2 h were counted using FACScan. These experiments were done four times with similar results. $^*P < 0.05$, compared to Jurkat.

Chemotaxis response, but not MAPK activity, decreased after Cbl and Cbl-b siRNA transfection

To determine the effect of Cbl and Cbl-b in SDF-1α/CXCL12-induced migration, Cbl, Cbl-b or control siRNA was transfected into Jurkat cells by electroporation. Cbl and Cbl-b siRNAs reduced Cbl and Cbl-b protein levels 48 h after transfection (Fig. 7A). At this time, we examined the effect of Cbl and Cbl-b siRNA on chemotactic response to SDF-1α/CXCL12. The chemotactic response to SDF-1α/CXCL12 was significantly reduced in cells transfected with siRNA to Cbl or Cbl-b compared to that of control siRNA-transfected cells (Fig. 7B), but MAPK activity was not reduced (Fig. 7C). These results suggest that Cbl and Cbl-b are involved, at least in part, in SDF-1/CXCL12-induced chemotaxis of Jurkat cells, but MAPK activity is not influenced by this decrease in Cbl or Cbl-b.

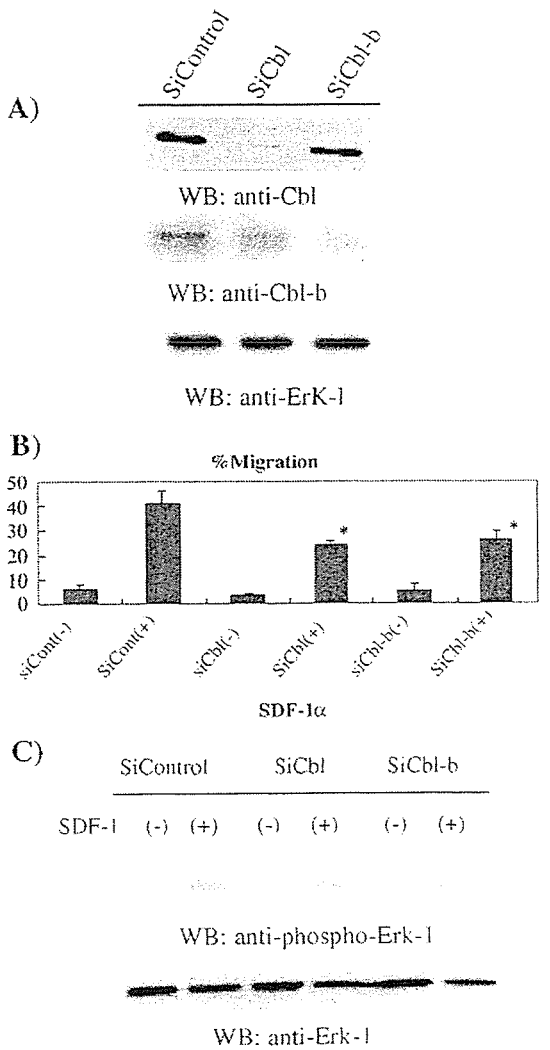

Fig. 7. Jurkat cells transfected with Cbl or Cbl-b siRNA are reduced in response to the chemotaxis induced by SDF-1/CXCL12 but are not reduced in MAPK activity. Jurkat cells were electroporated with 1 nmol siRNA for Cbl, Cbl-b or with control. (A) Cells were stimulated with SDF-1α/CXCL12 (100 ng/ml) and immunoblotted 48 h after electroporation. (B) Cells were placed in the upper part of chemotaxis chambers, and cells moving to the lower chamber were counted using FACScan. These experiments were done four times with similar results. $*P < 0.05$, compared to control. (C) Cells were stimulated by SDF-1/CXCL12 and evaluated for phosphorylation of Erk.

Discussion

SDF-1α/CXCL12 plays a key role in the regulation of migration and homing of hematopoietic cells. Cell migration is mediated by multiple signaling mechanisms. In the present study, we investigated the phosphorylation of Cbl and Cbl-b and the association of these adaptor molecules with other signaling molecules in T cell lines. We also assessed the functional effects of decreased Cbl and Cbl-b expression, after transfection of siRNA for Cbl and Cbl-b, on SDF-1/CXCL12-induced migration of the Jurkat T cell line. Our results both confirm and extend the findings of others [6] that Cbl family members function as adaptor proteins downstream of CXCR4 in response to SDF-1α/CXCL12.

Cbl and Cbl-b contain substrate recognition domains that interact with activated Src protein tyrosine kinases. Cbl interacts with the SH3 domains of src kinases, such as Lck, Fyn and Lyn [27]. We demonstrate here that Fyn associates with Cbl and Cbl-b and that Lck also regulates Cbl family phosphorylation in response to SDF-1α/CXCL12. Our observations that the Src kinase inhibitor PP2 blocks SDF-1α/CXCL12-induced tyrosine phosphorylation of Cbl and Cbl-b suggest that src kinases are upstream effectors of Cbl and Cbl-b in SDF-1α/CXCL12 actions.

Cbl is a negative regulator in the immune system of Syk/Zap-70 PTKs [28]. In Cbl-b knockout mice, Zap-70 is hyperphosphorylated in thymocytes and ERK is activated compared to wild type mice [15,16]. Cbl has no known catalytic function, but it contains several domains that are able to interact with a wide variety of substrates. We demonstrated that Cbl and Cbl-b interact with the Zap-70 tyrosine kinase and that phosphorylated Cbl and Cbl-b are enhanced in their capacity to associate with the adaptor proteins Crk-L, Nck, PLC-γ and Fyb in response to SDF-1/CXCL12. This extends the studies of others [6] on Cbl associations in response to SDF-1CXCL12 and demonstrates additional effects on Cbl-b and associated proteins.

A functional role for both Cbl and Cbl-b in SDF-1/CXCL12-induced chemotaxis was identified in our studies in which Jurkat T cells, which were transfected with siRNA for either Cbl or Cbl-b and was decreased in expression of both these molecules, were also decreased in response to SDF-1/CXCL12-induced chemotaxis. Of interest was that MAPK activity in response to SDF-1/CXCL12 was not influenced by the decreased Cbl and Cbl-b expression in the Cbl- and Cbl-b-transfected cells.

CD45 is an important regulator of src family kinases [24]. The phosphatase activity of CD45 appears to be required for activation of src family kinase members. In contrast, in some reports, CD45 inhibits activation of src family kinases in macrophages and some lymphocytes. Thus, CD45 function while important is still somewhat controversial. In our studies, we found using a CD45-deficient cell line that SDF-1α/CXCL12 did not enhance tyrosine phosphorylation of Cbl or

Cbl-b, demonstrating that CD45 is required for the enhanced phosphorylation of both these intracellular signals. Moreover, CD45 was, but MAPK was not, required for the chemotactic response of cells to SDF-1α/CXCL12, in part adding valuable confirmation and extension to the work of others [7] in this still somewhat controversial area. Thus, our results implicate Cbl and Cbl-b as intracellular and functional mediators in cell migration induced by SDF-1α/CXCL12, effects that likely involve interactions of Cbl and Cbl-b with Crk-L, Zap70, Nck, PLC-γ, Fyb and Fyn and regulation by CD45 and Src kinases.

Acknowledgments

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Clonally Expanded T-Cells in the Peripheral Blood of Patients with Idiopathic Thrombocytopenic Purpura and *Helicobacter pylori* Infection

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Abstract

Eradication of *Helicobacter pylori* leads to platelet recovery in some patients with idiopathic thrombocytopenic purpura (ITP). Therefore, the pathogenesis of a subgroup of ITP is probably associated with *H pylori* infection (*H pylori*–related ITP). If *H pylori*–related ITP is a definite subgroup of ITP, specific oligoclonal T-cells might accumulate in the peripheral blood (PB). To address this issue, we performed single-strand conformation polymorphism analysis of complementarity-determining region 3 (CDR3) of the T-cell receptor β-chain genes of PB T-cells. Fourteen ITP patients with *H pylori* infection and 12 age-adjusted healthy volunteers were studied. Of the 14 patients, 8 patients (responders) exhibited a platelet response after successful *H pylori* eradication therapy, but 6 patients (nonresponders) did not. Vβ5.2, Vβ15, and Vβ19 gene usage by clonally expanded T-cells in PB obtained before *H pylori* eradication therapy was significantly higher in responders than in nonresponders or healthy volunteers (Vβ5.2, $P = .023$; Vβ15, $P = .004$; Vβ19, $P = .036$). Furthermore, an abrogation of clonally expanded T-cells was observed after therapy in some responders. These findings suggest that specific T-cell clones accumulate in *H pylori*–related ITP and that such clones may be associated with immune-mediated destruction of platelets.
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Key words: Idiopathic thrombocytopenic purpura (ITP); *Helicobacter pylori*; T-cell clonality; T-cell receptor; Single-strand conformation polymorphism (SSCP)

1. Introduction

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder caused by autoantibodies against platelets. Platelet membrane glycoproteins (GPs) such as GP IIb-IIIa and GP Ib have been identified as target autoantigens. Recently, many investigators reported that eradication of *Helicobacter pylori* leads to platelet recovery in patients with ITP (response rate, 33%-100%) [1-11]. However, a few investigators have shown the opposite result [12-14]. Therefore, *H pylori* infection may play an important pathophysiological role in a subgroup of ITP (*H pylori*–related ITP).

Oligoclonal T-cells that respond to antigenic stimulation accumulate in the peripheral blood (PB) in autoimmune diseases. Single-strand conformation polymorphism (SSCP) analysis of complementarity-determining region 3 (CDR3) of the T-cell receptor (TCR) β chain has demonstrated that oligoclonal T-cells accumulate in the PB of ITP patients [15]. This result suggests that specific oligoclonally expanded T-cells that drive B-cells to produce autoantibodies against platelets may be present in the PB of patients with ITP.

H pylori infection is prevalent among healthy people. In Japan, the frequency of *H pylori* infection is greater than 50% in healthy adults and 70% to 80% among elderly people [16]. Therefore, the occurrence of complicating *H pylori* infection in ITP patients does not necessarily mean that these patients have *H pylori*–related ITP. The diagnosis of *H pylori*–related ITP is made retrospectively according to the platelet response following *H pylori* eradication therapy. If a patient with *H pylori* infection recovers from thrombocytopenia after successful eradication therapy (ie, a responder), a diagnosis of *H pylori*–related ITP can be made. On the other hand, if a patient fails to recover from thrombocytopenia even after *H pylori* has successfully been eradicated (ie, a nonresponder), a diagnosis of *H pylori*–unrelated ITP can be made. If specific clonally expanded T-cells that are different from those of nonresponders and healthy volunteers can be demonstrated to be present in the PB of responders, it may

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Table 1.
Clinical and Laboratory Characteristics of 14 Patients with Idiopathic Thrombocytopenic Purpura*

Patient No.	Age, y	Sex	Disease Duration, mo	Previous Treatment	^{13}C-Urea Breath Test, ‰		*H pylori* Eradication	Platelets, $\times 10^9$/L		Response
					Before *H pylori* Eradication	After *H pylori* Eradication		Before *H pylori* Eradication	After *H pylori* Eradication	
1	50	M	38	PSL	38	0.9	Yes	14	138	+
2	52	M	6	—	16	2.1	Yes	14	239	+
3	56	M	18	PSL	4	1.3	Yes	54	95	+
4	56	M	10	PSL	14.9	1.8	Yes	18	105	+
5	63	F	23	PSL, Sp	10.2	1.2	Yes	20	92	+
6	74	F	58	Vitamin C	20	1.1	Yes	49	182	+
7	74	F	6	—	19.8	1.4	Yes	35	125	+
8	76	M	11	—	3	1.2	Yes	23	95	+
9	34	F	9	PSL	43.8	0.2	Yes	52	67	−
10	43	F	133	mPSL, Dan	4.3	1	Yes	19	11	−
11	50	F	78	—	49.3	0.6	Yes	73	66	−
12	53	F	16	—	10.3	1.2	Yes	70	92	−
13	58	F	60	PSL	20.1	1.3	Yes	31	18	−
14	61	F	63	Vitamin C	39	0.8	Yes	38	43	−

*Eight patients (nos. 1-8) showed a platelet response after *Helicobacter pylori* eradication therapy, and 6 patients (nos. 9-14) did not show a platelet response after eradication therapy. PSL indicates prednisolone; Sp, splenectomy; mPSL, methylprednisolone; Dan, danazol.

be possible to show that *H pylori*–related ITP is indeed a definite subgroup of ITP. To address this issue, we performed SSCP analysis of TCR Vβ-chain genes of PB T-cells in ITP patients with *H pylori* infection and investigated T-cell repertoire usage by the clonally expanded T-cells.

2. Materials and Methods

2.1. Patients

Fourteen patients with chronic ITP complicated by *H pylori* infection were studied. The patients comprised 5 men and 9 women with a median age of 56 years (range, 35-72 years). Chronic ITP was defined as thrombocytopenia (platelets $<100 \times 10^9$/L) lasting for at least 6 months, normal or increased numbers of megakaryocytes in the bone marrow, and absence of other apparent causes of thrombocytopenia. *H pylori* infection was diagnosed by a positive value of greater than 2.5% in the breath test using carbon 13 (^{13}C)-labeled urea [17].

H pylori infection was initially eradicated by treatment with amoxicillin (750 mg twice daily), clarithromycin (200 mg twice daily), and lansoprazole (30 mg twice daily) for 7 days. After a minimum of 2 months, the ^{13}C-urea breath test was again performed to evaluate the effect of *H pylori* eradication therapy. *H pylori* was eradicated in 12 of the 14 patients. The 2 patients (patients 13 and 14) who failed to respond were then successfully treated with metronidazole (250 mg twice daily), amoxicillin (750 mg twice daily), and lansoprazole (30 mg twice daily). Therefore, *H pylori* infection was eventually eradicated in all 14 patients.

The platelet response due to *H pylori* eradication was evaluated 6 months after treatment. A response was defined as an absolute increase in the platelet count of $>30 \times 10^9$/L from the baseline. The response criteria are based on the previous report by Vianelli et al [18]. As shown in Table 1, eradication of *H pylori* led to a platelet response in 8 patients (patients 1-8) but no response in 6 patients (patients 9-14). All of the responders are now in remission with a platelet count of $>100 \times 10^9$/L for at least 34 months after treatment.

2.2. SSCP Analysis of TCR β-Chain Genes

Samples of heparinized PB were obtained from all patients before eradication therapy. Samples were also obtained 3 to 6 months after eradication therapy for some patients. As a control, PB samples were also obtained from 12 age-adjusted healthy volunteers (5 men and 7 women with a median age of 57.5 years [range, 35-72 years]) with no history of recent infection. All patients gave informed consent.

SSCP analysis of TCR β-chain genes was performed as described elsewhere [19]. In brief, mononuclear cells were separated from the PB samples by density-gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden), and total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method [20]. RNA was converted to complementary DNA in a solution containing 200 U reverse transcriptase (SuperScript; Gibco BRL, Gaithersburg, MD, USA) and 100 pmol random hexamer oligonucleotide primer (Gibco BRL). For amplification of each TCR Vβ gene family, a biotinylated primer for the constant region of the β chain and a Vβ-specific primer were used. The sequences of the Vβ-specific primers were as described elsewhere [21]. The polymerase chain reaction was performed with deoxynucleoside triphosphates and *Taq* DNA polymerase (TaKaRa Bio, Shiga, Japan) for 35 cycles in a thermocycler (PerkinElmer, Norwalk, CT, USA). Following dilution and heat denaturation, amplified DNA fragments were separated on the basis of differences in their single-strand conformation by electrophoresis on nondenaturing 4% polyacrylamide gels containing 10% glycerol. The electrophoresed DNA fragments were transferred to membranes (GeneScreen; NEN Life Science Products, Boston, MA, USA) and visualized by subsequent incubations with strep-

tavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Phototope-Star Detection kit; New England BioLabs, Beverly, MA, USA). We counted distinct bands as corresponding to clonal T-cell expansions. Although bands usually were easily recognized visually, a densitometer (ACD-25DX; ATTO Technology, Tokyo, Japan) was also used to confirm the presence of the bands. If one or more distinct bands were present in a certain Vβ gene family, the existence of clonally expanded T-cells was demonstrated. The presence of clonally expanded T-cells in each TCR Vβ gene family was individually assessed, and then the proportion of patients (or healthy volunteers) with clonally expanded T-cells was calculated for each TCR Vβ gene.

2.3. DNA Sequencing

TCR Vβ gene transcripts obtained before and after eradication therapy from one patient who had recovered from thrombocytopenia after *H pylori* eradication were extracted from the SSCP gel and cloned with a TA cloning kit (Invitrogen, Carlsbad, CA, USA). Approximately 20 plaques were randomly chosen and subjected to dideoxy direct sequencing.

2.4. Statistical Analysis

Differences in proportions among the groups were evaluated by the Kruskal-Wallis test, the Student *t* test, or the chi-square test. A P value <.05 was considered statistically significant.

3. Results

3.1. Analysis of the Clonally Expanded T-Cells in ITP Patients with H pylori Infection

We performed SSCP analyses of 20 major TCR Vβ gene families in patients with *H pylori* infection to investigate whether clonally expanded T-cells were present in PB. The median number of TCR Vβ gene families with clonally expanded T-cells in responders, nonresponders, and healthy subjects was 7 (range, 1-21), 3.5 (range, 2-11), and 4 (range, 1-6), respectively. The number of Vβ gene families with clonally expanded T-cells was significantly greater in responders (P = .032).

TCR Vβ subfamilies that frequently (>50% of cases) revealed clonally expanded T-cells were observed in 7 families (Vβ1, Vβ2, Vβ5.2, Vβ7, Vβ11, Vβ15, and Vβ19) in responders, 2 families (Vβ1 and Vβ5.1) in nonresponders, and 3 families (Vβ7, Vβ10, and Vβ11) in healthy volunteers (Figure 1). We analyzed the differences in Vβ usage of clonally expanded T-cells among responders, nonresponders, and healthy volunteers and found that the usage of Vβ5.2, Vβ15, and Vβ19 genes of clonally expanded T-cells was significantly higher in responders than in nonresponders or healthy volunteers (Vβ5.2, P = .023; Vβ15, P = .004; Vβ19, P = .036). We investigated whether the distinct bands in the SSCP analysis of Vβ5.2 (patients 1, 2, 3, and 6), Vβ15 (patients 3, 4, and 8), or Vβ19 (patients 3, 6, and 8) genes that were found in some responders before therapy disappeared after therapy. Disappearance of distinct bands from Vβ5.2, Vβ15, and Vβ19 genes was observed in none of 4 responders, 1 (patient 3) of 3 responders, and 1 (patient 3) of 3 responders, respectively (data not shown).

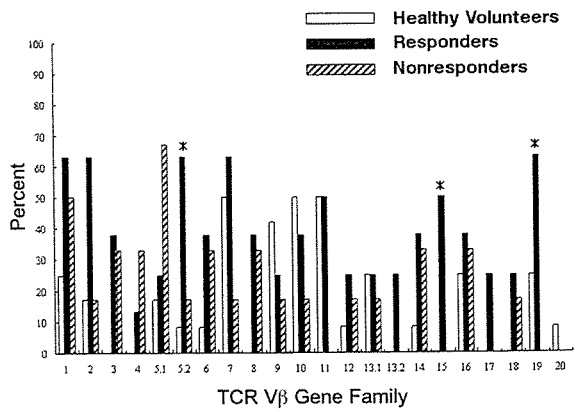

Figure 1. T-cell receptor (TCR) Vβ subfamily usage by clonally accumulated T-cells for responders, nonresponders, and healthy volunteers. Peripheral blood samples from patients with idiopathic thrombocytopenic purpura were obtained before eradication therapy and analyzed. If one or more distinct bands for a given Vβ gene were present in the single-strand conformation polymorphism analysis, the existence of clonally expanded T-cells was confirmed. The presence of clonally expanded T-cells for each TCR Vβ gene was individually assessed, and then the proportion of patients (or healthy volunteers) with clonal T-cell expansion was calculated for each TCR Vβ gene. Responders indicates patients who recovered from thrombocytopenia after successful *Helicobacter pylori* eradication; nonresponders, patients who failed to recover from thrombocytopenia even after *H pylori* was successfully eradicated. *Vβ5.2, Vβ15, and Vβ19 gene usage was significantly higher in responders than in nonresponders or healthy volunteers (P = .023, .004, and .036, respectively).

We compared nonresponders and healthy volunteers with respect to Vβ usage by clonally expanded T-cells and found Vβ3, Vβ4, Vβ5.1, and Vβ8 gene usage to be significantly higher in nonresponders (P = .034, .034, .034, and .034, respectively).

3.2. DNA Sequencing of TCR CDR3

In a patient (patient 1) who recovered from thrombocytopenia after *H pylori* eradication, the distinct band that was seen in the SSCP analysis of the Vβ8 gene before eradication therapy disappeared after eradication therapy (Figure 2). To confirm the disappearance of clonally expanded T-cells in Vβ8 following *H pylori* eradication, we determined the CDR3 DNA sequences of the TCR Vβ genes. In the sample obtained before *H pylori* eradication, all 18 subcloned genes showed the same sequence. However, in the sample obtained after *H pylori* eradication, all 19 subcloned genes showed different sequences, and none of these sequences were identical to the sequence seen before *H pylori* eradication (Table 2).

4. Discussion

We performed an SSCP analysis of TCR Vβ-chain genes of PB T-cells from ITP patients with *H pylori* infection and

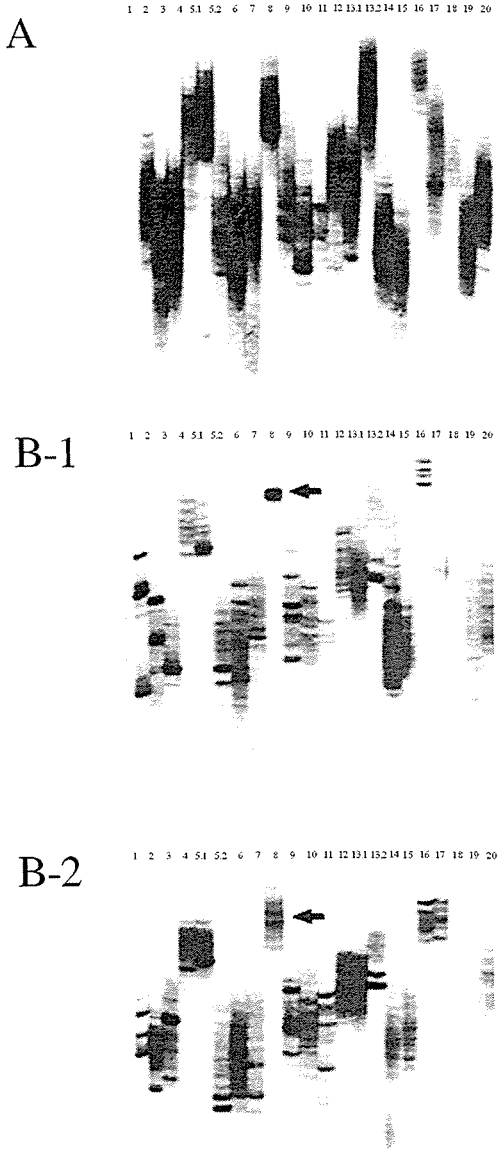

Figure 2. Single-strand conformation polymorphism analysis of T-cell receptor (TCR) Vβ genes in peripheral blood T-cells. The number at the top of each lane indicates the TCR Vβ gene subfamily. Results obtained from a typical healthy control subject (A) and patient 1 (responder) (B) are shown. In patient 1, a distinct band for the Vβ8 gene segment present before *Helicobacter pylori* eradication (B-1) disappeared after *H pylori* eradication (B-2) (arrows).

investigated T-cell repertoire usage by clonally expanded T-cells. The number of TCR Vβ gene families with clonally expanded T-cells was significantly higher in the patients with *H pylori* infection who subsequently recovered from thrombocytopenia after successful eradication therapy (responders) than in the patients who failed to recover from thrombocytopenia after successful eradication therapy (nonresponders). In addition, the usage of Vβ5.2, Vβ15, and

Table 2.
Deduced Amino Acid Sequences of the T-Cell Receptor (TCR) β Chains Carrying the Vβ Gene Segment Derived from the Peripheral Blood of Patient 1

Vβ8	nDn	Jβ (Gene Segment)	TCR β Chains, n
Before *Helicobacter pylori* eradication			
CASS	FSYCSA	NYGYT (J1S2)	18 (100%)
After *Helicobacter pylori* eradication			
CASSL	AWSGRY	TGELF (J2S2)	1 (5.3%)
CAS	RTTGG	SYEQY (J2S7)	1 (5.3%)
CASS	FSGGR	ETQYF (J2S5)	1 (5.3%)
CASS	KTGYE	QYFGP (J2S3)	1 (5.3%)
CAS	SRLAGGHPPT	QYFGP (J2S7)	1 (5.3%)
CAS	TRPEGGT	YNEQFF (J2S1)	1 (5.3%)
CAS	EEG	NTEAF (J1S1)	1 (5.3%)
CAS	SRFPAGA	YEQYF (J2S7)	1 (5.3%)
CA	SRPLAP	QETQYF (J2S5)	1 (5.3%)
CASS	SATV	SYEQY (J2S7)	1 (5.3%)
CASS	PRLDG	SYEQY (J2S7)	1 (5.3%)
CASS	RDFRA	NYGYT (J1S2)	1 (5.3%)
CASS	FGGTAR	QETQYF (J2S5)	1 (5.3%)
CASS	GTGTTSD	EQFFGPG (J2S1)	1 (5.3%)
CASSL	RPY	QPQHFG (J1S5)	1 (5.3%)
CAS	QGQH	NSPLHF (J1S6)	1 (5.3%)
CAS	NRLAGGHP	DTQYFGP (J2S3)	1 (5.3%)
CASSL	ELQDGYA	FGSGTRL (J1S2)	1 (5.3%)
CAS	RL	SGANVLT (J2S6)	1 (5.3%)

Vβ19 genes by clonally expanded T-cells was significantly higher in responders than in nonresponders. This difference notably does not derive from the presence or absence of *H pylori* infection, because all of the patients had been infected with *H pylori*. These results suggest that some clonally expanded T-cells with specific TCR Vβ subfamily usage are present in patients with *H pylori*–related ITP. Distinct bands seen in the SSCP analysis of Vβ genes before *H pylori* eradication therapy in some of the responders disappeared after *H pylori* eradication therapy. Furthermore, we confirmed the disappearance of clonally expanded T-cells after *H pylori* eradication therapy in a patient with *H pylori*–related ITP by analyzing the DNA sequences of CDR3 of Vβ genes. Our results indicate that clonally expanded T-cells were abrogated by *H pylori* eradication and suggest that the disappearance of clonally expanded T-cells was responsible for platelet recovery.

H pylori infection is associated with various autoimmune diseases, including rheumatoid arthritis, Sjögren syndrome, and autoimmune hypothyroidism [22-25]. Clinical data from patients with these disorders raise the possibility that immune reactions against *H pylori* have pivotal roles in the onset of autoimmune diseases. As for *H pylori*–related ITP, why *H pylori* eradication is able to induce platelet recovery is unknown. However, one possible explanation is that anti–*H pylori* antibodies bind to platelets in the presence of cross-mimicry between platelet surface antigens and *H pylori* antigens, resulting in platelet destruction. This speculation is supported by recent work demonstrating that platelet-associated immunoglobulin possesses cross-reactivity to

H pylori cytotoxin-associated gene A (CagA) [26]. If this mechanism operates in *H pylori*–related ITP, it strongly suggests that *H pylori* eradication induces a reduction in the T-cell clones against CagA that drive B-cells to produce cross-reactive antibodies, resulting in the reduction of cross-reactive antibodies. Our data demonstrating the subsequent disappearance of clonally expanded T-cells after *H pylori* eradication support this speculation.

Cytotoxic T-cell–mediated lysis of autologous platelets has recently been demonstrated in active ITP, and T-cell–mediated cytotoxicity has been suggested to be an alternative mechanism of platelet destruction in ITP [27]. Therefore, it is also possible that clonally expanded T-cells observed in *H pylori*–related ITP are cytotoxic T-cells against *H pylori* with cross-reactivity to platelets.

In conclusion, our findings suggest that specific T-cell clones accumulate in *H pylori*–related ITP and that these clones may be associated with immune-mediated platelet destruction. Further studies are needed to elucidate the role of the clonally expanded T-cells observed in *H pylori*–related ITP.

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ORIGINAL ARTICLE

Hyperactivation of the RAS signaling pathway in myelodysplastic syndrome with *AML1/RUNX1* point mutations

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AML1/RUNX1 mutations have been reported frequently in myelodysplastic syndrome (MDS) patients, especially those diagnosed with refractory anemia with excess blast (RAEB), RAEB in transformation (RAEBt), or AML following MDS (these categories are defined as MDS/AML). Although AML1 mutations are suspected to play a pivotal role in the development of MDS/AML, acquisition of additional genetic alterations is also necessary. We analyzed gene alterations in MDS/AML patients with AML1 mutations, comparing them to alterations in those without an AML1 mutation. AML1 mutations were significantly associated with −7/7q-, whereas MDS/AML patients without AML1 mutations showed a high frequency of −5/5q- and a complex karyotype. Patients with AML1 mutations showed more mutations of their *FLT3*, *N-RAS*, *PTPN11*, and *NF1* genes, resulting in a significantly higher mutation frequency for receptor tyrosine kinase (RTK)–RAS signaling pathways in AML1-mutated MDS/AML patients compared to AML1-wild-type MDS/AML patients (38% versus 6.3%, $P<0.0001$). Conversely, *p53* mutations were detected only in patients without AML1 mutations. Furthermore, blast cells of the AML1-mutated patients expressing surface c-KIT, and SHP-2 mutants contributed to prolonged and enhanced extracellular signal-regulated kinase activation following stem cell factor stimulation. Our results suggest that MDS/AML arising from AML1/RUNX1 mutations has a significant association with −7/7q- alteration, and frequently involves RTK–RAS signaling pathway activation.

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Introduction

Somatically acquired point mutations of critical genes have been demonstrated to contribute to the development of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Genes encoding key regulatory factors for cell division, differentiation, or cell survival of hematopoietic progenitors, as well as transcription factors, are frequent mutation targets. The *AML1/RUNX1* gene was also found to be altered by point mutations in AML and MDS, and unique features associated with these mutations have been revealed by several studies. First, although the frequency of AML1 mutations in *de novo* AML is low, they have been detected with a substantially higher frequency in poorly differentiated AML M0,[1–3] and in radiation-associated and therapy-related AML.[4,5] Second, germ-line mutations of *AML1* have been shown to occur in a rare autosomal dominant disorder, familial platelet disorder with predisposition to AML (FPD/AML).[6,7] Third, we previously reported a high frequency of AML1 mutations in MDS patients, especially those with refractory anemia with excess blast (RAEB), RAEB in transformation (RAEBt), and AML following MDS (these categories are defined as MDS/AML).[8] This suggests that AML1 point mutations are strongly associated with these specific types of hematopoietic malignancy. In the molecular pathogenesis, *de novo* AML is often associated with oncogenic chimeras such as AML1–ETO (MTG8), which are considered to be the major cause of malignant transformation of hematopoietic progenitors, while point mutations are likely a complement for chimeras. However, AML1 point mutations should be considered one of the major decisive factors of the development of MDS/AML, similar to chimeras in *de novo* AML.

All of the AML1 mutants detected so far, with a few exceptions, showed a lowering or loss of their *trans*-activation potential, regardless of their DNA-binding potential. This suggests that various AML1 mutants act through a loss-of-function mechanism, and contribute to development of the same type of myeloid malignancy, MDS/AML. However, loss of AML1 function caused by AML1 mutations in hematopoietic stem cells is not sufficient for an individual to develop MDS/AML, and this explains the long latency period before development of AML among people having FPD/AML pedigrees with congenital AML1 mutations. Moreover, AML1$^{+/-}$ mice, conditional AML1$^{-/-}$ mice or conditional heterozygous AML1–ETO knockin mice do not develop leukemia without mutagenic reagents, in spite of the increase in number and the self-renewal capacity of their hematopoietic stem cells.[9,10] This in turn suggests that the acquisition of some additional genetic alterations that cooperate with the AML1 mutations is needed for development of MDS/AML. Recently, c-kit activating mutations were identified in 20–30% of CBF leukemia patients who had blasts containing either AML1–ETO or CBFβ–MYH11 fusion proteins.[11] This was not observed in any of the non-CBF leukemias, suggesting that the expression of CBF fusion proteins provides a selective advantage for acquisition of a c-kit mutation, and that these events function together in the development of leukemia. This theory supports a 'two-hit' model for leukemogenesis.[12] The basis for the hypothesis is that AML is the consequence of a collaboration between at least two broad classes of mutation: class I (proliferative) mutations that

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confer a proliferative and/or survival advantage to cells, including gene alterations of *RAS, c-KIT, FLT3, PTPN11* and *NF1*, and class II (blocking) mutations that primarily impair hematopoietic differentiation and subsequent cellular apoptosis, including *AML1–ETO* and *CBFβ–MYH11* fusion genes. *AML1* point mutations are classified as class II mutations, and *FLT3* mutations were frequently observed in AML1-mutated AML M0 patients.[13]

It is hard to explain the molecular mechanisms in MDS that contribute to the transformation of hematopoietic progenitors by a simple 'two-hit' model. Many cases of MDS are generally considered to develop as a result of accumulated gene deletions and point mutations. Genetic alterations associated with MDS have been identified, including receptors for hematopoietic growth factors, RAS signaling molecules, cell cycle regulators and transcriptional factors.[14,15] However, these are all relatively rare (<10%) and none are specific to MDS. Chromosomal abnormalities are also associated with MDS, and they vary from single numerical or structural changes to complex genomic lesions.[14,15] Unbalanced numeric chromosomal alterations, including partial and complete chromosome loss (especially 5q-, −7, −Y and 20q-) or chromosome gain (most frequently +8), predominate in MDS. A model of stepwise genetic progression has emerged, based on genetic and cytogenetic findings, to explain the development and evolution of MDS.[16] In this model, a primary genetic event incites the initial DNA damage and subsequently increases the susceptibility to further damage. Secondary genetic events promote acquisition of molecular-genetic or cytogenetic abnormalities common to MDS and precipitate additional abnormalities.

Myelodysplastic syndrome is a quite heterogeneous disease category. We have been trying to re-classify MDS based on the molecular pathogenesis, and have proposed a new disease entity, MDS/AML with AML1 point mutations. In this subgroup of MDS, an AML1 mutation is considered to be the primary genetic event. Similar to CBF leukemia, patients with MDS/AML with AML1 mutations probably also have some selective secondary genetic events. Here, we report a high frequency of mutations affecting the class III receptor tyrosine kinase (RTK)–RAS signaling pathway in AML1-mutated MDS/AML patients. Our data suggest that AML1 point mutations affect activation of the RTK–RAS signaling pathway, and that this may be one of the molecular mechanisms to develop MDS/AML.

Materials and methods

Patients

We examined 625 patients with hematologic diseases, including MDS, AML, acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML) and myeloproliferative disorder (MPD), all of whom were diagnosed at Hiroshima University Hospital and its affiliated hospitals between 1990 and 2004. Diagnosis was based on morphologic and immunophenotypic studies according to the French–American–British (FAB) classification. Three disease categories corresponding to subgroups of MDS and AML were identified (i.e., RAEB, RAEBt and AML following MDS) as MDS/AML.[8] Cytogenetic analyses using standard procedures were performed according to the International System of Human Cytogenetic Nomenclature (1995).[17] The majority of the patients in this study were treated at Hiroshima University Hospital or Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital using a protocol involving intensive chemotherapy and bone marrow transplantation.

Patient samples were taken after obtaining informed consent and approval from the institutional review board at Hiroshima University. Mononuclear cells were isolated from bone marrow or peripheral blood samples by Ficoll-Conray density gradient centrifugation. Genomic DNA was extracted with a Puregene Kit (Gentra, Minneapolis, MN, USA) and total RNA was extracted using a TRIzol Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturers' instructions.

Identification of AML1 mutations

Mutation analysis of *AML1* exons 3–8 was performed by polymerase chain reaction–single–strand conformation polymorphism (PCR–SSCP) of all 625 patients as described previously.[8] Briefly, 100 ng of genomic DNA was amplified by PCR using the flanking intronic, forward/reverse primers (Supplementary Table). To identify *AML1* mutations, SSCP analysis was performed on a GenePhor system (Amersham Pharmacia Biotech, Buckinghamshire, UK). PCR products that showed abnormal bands were sequenced in both directions. To confirm the mutations, PCR products from cDNA were also sequenced.

We selected patients for further studies according to their diagnosis and their *AML1* mutations, and they were divided into three disease categories as follows: (1) 34 cases of MDS/AML with an AML1 mutation (10 RAEB, 16 RAEBt and 8 AML following MDS); (2) 80 cases of MDS/AML without an AML1 mutation (32 RAEB, 21 RAEBt and 27 AML following MDS) and (3) 25 cases of CBF leukemia (19 with 8; 21 translocation and 6 with inversion 16).

Polymerase chain reaction of N-RAS, K-RAS, c-KIT, PTPN11, NF1 *and* p53 *fragments*

The PCR amplified the DNA sequence of interest: exons 1 and 2 of *N-RAS* and *K-RAS*;[18] exons 8 and 17 of *c-KIT*;[19] exons 3 and 13 of *PTPN11*;[20] exons 1–49 of *NF1*[21] and exons 5–8 of *p53*.[22] Genomic DNA(100 ng) was amplified by PCR in a total volume of 25 μl containing 1 × PCR buffer II (Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$ (Applied Biosystems), 0.2 mM dNTP (deoxynucleotide triphosphate; Roche, Mannheim, Germany), 0.2 μM of each primer, and 0.5 unit of AmpliTaq Gold (Applied Biosystems). PCR was performed on genes of interest using the flanking intronic, forward/reverse primers listed in the Supplementary Table. Amplicons were checked by agarose gel electrophoresis to make sure that only the specific product was amplified.

Denaturing high performance liquid chromatography analysis

PCR-amplified samples were warmed to 95°C, then cooled to 25°C over approximately 45 min to promote heteroduplex formation. Denaturing high performance liquid chromatography (DHPLC) was performed on a WAVE DNA fragment analysis system (Transgenomic, Omaha, NE, USA). Briefly, 5 μl of each DNA sample was injected into a high-throughput DNASep column and eluted through a 260 nM photodetector with concentrations of buffer A and B (Transgenomic) adjusted automatically as calculated by the Navigator software package (Transgenomic). All samples were run at the oven temperature listed in the Supplementary Table. The Navigator software predicted that each segment of the exonic component of the amplicon would be under partially denaturing conditions at these temperatures. For each abnormal elution profile, genomic

DNA was re-amplified and the PCR products were directly sequenced.

Direct sequencing

The PCR-amplified product was electrophoresed through a 2% agarose gel. The bands of interest were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified DNA fragments were sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and were analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). To confirm the mutations, a second independent PCR amplification and subsequent bidirectional sequencing were performed.

Screening for the internal tandem duplication and the D835 mutation of the FLT3 *gene*

The FLT3 internal tandem duplication (ITD) was examined by amplification of the juxtamembrane (JM) domain using forward/reverse primers (Supplementary Table), followed by electrophoresis in an agarose gel, as reported previously.[23] Additional bands, indicative of ITD, were cut out for direct sequencing. Screening for the D835 mutation was carried out by amplification of genomic DNA using forward/reverse primers (Supplementary Table). PCR products were digested with *EcoRV* and were then resolved on an agarose gel, as described previously.[24] Direct sequencing was carried out on samples with an undigested band.

Plasmid constructions

PCR-generated fragments of *PTPN11* cDNA encoding SHP-2 or SHP-2 mutants with the C-terminus c-myc epitope tag were subcloned into the pcDNA3.1 expression vector (Invitrogen). The wild-type *PTPN11* cDNA was mutated at nucleotides 181 (G>T) or 226 (G>A) using PCR-based mutagenesis to yield amino-acid changes D61Y or E76K, respectively. The integrity of the amplified sequence was confirmed by DNA sequencing.

Cell culture and transfection

HEL cells were maintained in RPMI1640 (Invitrogen) with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37°C in a humidified atmosphere with 5% CO_2. The plasmids were transfected into HEL cells by electroporation (300 V, 1050 μF) using a Gene Pulser (Bio-Rad, Hercules, CA, USA). Transfected cells were selected with 1 mg/ml G418 (Invitrogen), cloned by limiting dilution, and analyzed for SHP-2 expression by Western blot. Cells were washed with RPMI1640 containing 10% FCS three times, resuspended in the same medium alone or supplemented with 100 ng/ml of stem cell factor (SCF) (PeproTech, Rocky Hill, NJ, USA).

Immunoblotting

For immunoblot analysis, cells were solubilized in NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP-40) containing a protease inhibitor mixture (Complete; Roche). In order to detect phosphorylation, a phosphatase inhibitor mixture (50 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate) was added to the lysis buffer. Protein concentrations were determined with Bradford reagents (Bio-Rad). For Western blot, the lysates were boiled in Laemmli buffer and then separated by 15% SDS-PAGE gel and transferred to Hybond enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech). The membrane was blocked in 5% non-fat milk in PBS containing 0.1% Tween-20 and incubated sequentially with primary antibodies and a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). The primary antibodies used in this study were anti-Myc antibody, anti-c-kit polyclonal antibody, anti-SHP-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p44/42 MAP kinase polyclonal antibody and anti-phospho-p44/42 MAP kinase polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA). Bound antibodies were detected by ECL using a Western blotting kit (Amersham Pharmacia Biotech).

Results

The difference of cytogenetic findings in MDS/AML patients with or without an AML1 point mutation

We previously reported 32 cases of hematological disease associated with somatic mutations of the *AML1* gene.[8] We then extended the mutation analysis by PCR–SSCP assay using genomic DNA, and thereby found 13 new cases. The clinical findings of 10 patients with MDS/AML are summarized in Table 1 (cases 33 – 42). Two patients with secondary AML had frame shift and nonsense mutations, and one patient with reactive leukocytosis had the same silent mutation we previously reported in an MDS RA patient (case 1).[8]

We selected the patients according to their diagnosis and *AML1* mutations, and divided them into three disease categories: (A) MDS/AML patients with an AML1 mutation; (B) MDS/AML patients without an AML1 mutation; and (C) CBF leukemia patients (8;21 translocation and inversion 16). Two patients (cases 2 and 15) who had silent mutations were excluded from the group of patients with AML1 mutations because these mutations represent a simple polymorphism, and indeed we found one silent mutation in a reactive leukocytosis patient as well. We analyzed 34 MDS/AML patients with AML1 mutations summarized in Table 1, including the previously published 24 cases designated as nos. 3–9 and 16–32,[8] 80 MDS/AML patients without an AML1 mutation and 25 CBF leukemia patients. This analysis included 28 cases of secondary MDS/AML: 13 with AML1 mutations (six atomic-bomb survivors, five patients who received previous chemotherapy and/or radiotherapy, one atomic-bomb survivor who also received previous chemotherapy and one case of exposure to mustard gas), and 15 patients without AML1 mutations (seven atomic-bomb survivors and eight therapy-related patients).

The cytogenetic findings of AML1-mutated patients were compared with those of AML1-wild-type patients to see whether the AML1-mutated patients had distinct cytogenetic characteristics (Table 2). Five previously reported patients who showed a normal karyotype (cases 9, 19, 25, 26 and 29) had minor but clonal karyotypic abnormalities; case 9: 47, XX, +1, der(1;7)(q10;p10), i(21)(q10), case 19: 47, XY, del(7)(q22q32), +8, case 25: 47, XY, dup(1)(q21q44), +8, case 26: 45, XY, −7 and case 29: 44, XX, −7, −17. Clonal cytogenetic abnormalities could be detected in more than half of the patients with MDS/AML, 19 (56%) of the 34 patients with AML1 mutations and 47 (59%) of the 80 patients without AML1 mutations. A complex karyotype (five or more aberrations) was seen in 20 (25%) of the 80 patients without an AML1 mutation, whereas it was not seen in the patients with an AML1 mutation ($P=0.0007$).