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genes, erythrocyte membrane proteins, and ankyrin 1, appeared to be dramatically up-regulated in a *ZNF521*-silenced clone as compared with a control clone (supplemental Table 1). These results indicate that down-regulation of ZNF521 results in enhancement of erythroid differentiation in hematopoietic progenitors.

ZNF521 Inhibits GATA-1 Transcriptional Activity—Since GATA-1 is the key regulator of erythroid differentiation that plays a central role in erythroid lineage-associated gene expression (20), we speculated that ZNF521 might influence the transcriptional activity of GATA-1. To verify this possibility, we performed a luciferase assay with a reporter gene for GATA-1, pGL3-MαP-Luc, which contains three tandem repeats of the GATA-1-responsive element just upstream of the JunB promoter. We used NIH3T3 cells for reporter gene assays, because this cell line does not express endogenous GATA-1. Although transient transfection of the GATA-1 reporter with *GATA-1* cDNA alone increased the promoter activity, co-transfection of *ZNF521* cDNA with *GATA-1* cDNA exerted a significant repressive effect on GATA-1 activity (Fig. 2*A*). There was no significant effect of *ZNF521* cDNA on the basal activity of the JunB promoter, indicating that the inhibitory effect of ZNF521 was specific for the GATA-1-induced transcriptional activity. Next, we co-transfected the GATA-1 reporter with *GATA-1* cDNA and various amounts of *ZNF521* cDNA into NIH3T3 cells and examined the reporter activity. Consequently, *ZNF521* cDNA appeared to inhibit the GATA-1 activity in a dose-dependent manner (Fig. 2*B*). Next, we compared the *GATA-1* promoter activities in various K562 cell lines. The *GATA-1* promoter activities and GATA-1 protein levels were increased in the *ZNF521*-silenced clone and the *GATA-1*-overexpressing clone (Fig. 2*C*).

ZNF521 comprises an N-terminal repression motif and 30 ZFs (Fig. 3*A*). To determine which parts of ZNF521 inhibit GATA-1 transcriptional activity, we constructed deletion mutants of *ZNF521* cDNA and analyzed the effects of these products on GATA-1 transcriptional activity. Two kinds of mutant *ZNF521* cDNA, in which the N-terminal repression motif (ΔNT) or C-terminal ZF domains (Δ21–30) were deleted (Fig. 3*A*), were inserted into the FLAG-tagged expression vector. Production of wild-type and deleted proteins by these cDNAs was confirmed by Western blotting (Fig. 3*B*). The effects of these mutated ZNF521s on GATA-1 transcriptional activity were compared with that of wild-type ZNF521. As shown in Fig. 3*C*, the inhibitory effect of wild-type ZNF521 on GATA-1 promoter activity was partially abrogated by deletion of the N-terminal repression motif and C-terminal ZF domains, indicating that these parts are necessary for inhibition of GATA-1 activity. Because deletion mutants of the N-terminal repression motif and C-terminal ZF domains did not completely inhibit GATA-1 activity, another portion(s) of ZNF521 might also be involved in inhibiting GATA-1 transcriptional activity.

ZNF521 Does Not Affect the DNA Binding Activity of GATA-1—We performed an electrophoretic mobility shift assay using K562 clones. The DNA binding activity of GATA-1 was not decreased in *ZNF521*-silenced clones as compared with that in control clones. These data show that ZNF521 might not affect the DNA binding activity of GATA-1 (supplemental Fig. 1).

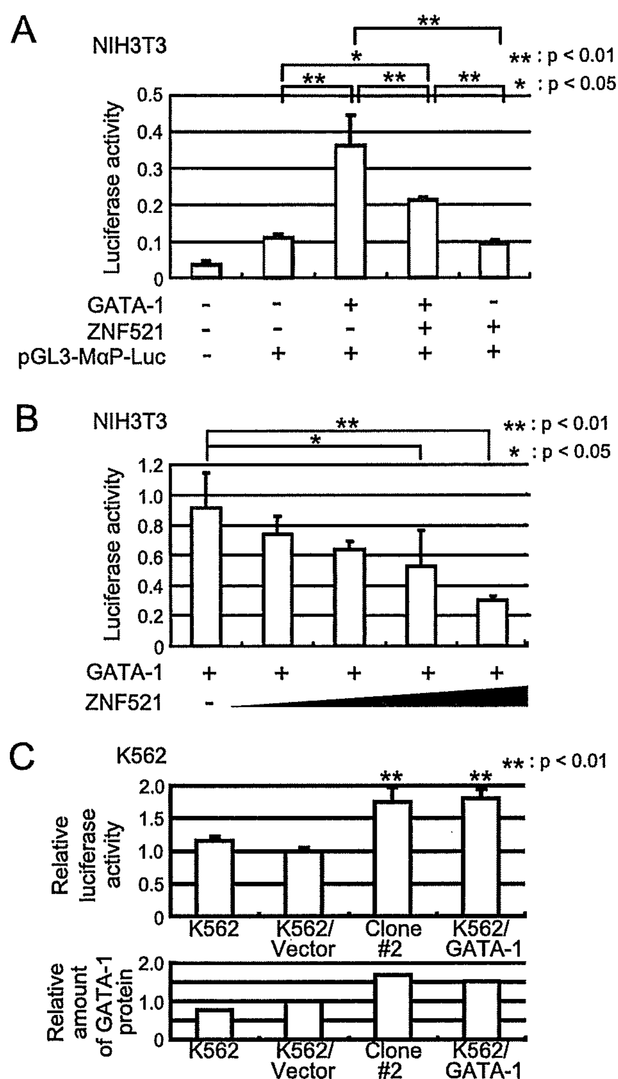

FIGURE 2. **Inhibitory effect of ZNF521 on transcriptional activity of GATA-1.** *A*, NIH3T3 cells were transduced with *GATA-1* reporter gene pGL3-MαP-Luc, sea pansy luciferase expression plasmid pRL-SV40, cDNA for *GATA-1*, and cDNA for *ZNF521*. After 16 h, cell lysates were measured for luciferase activity. The relative luciferase activity was calculated by normalizing the transfection efficiency relative to sea pansy luciferase activity. Results are mean ± S.D. for three independent experiments. *B*, NIH3T3 cells were transduced with pGL3-MαP-Luc, pRL-SV40, cDNA for *GATA-1*, and various amounts of *ZNF521*. After 16 h, cell lysates were measured for luciferase activity. *C*, plasmids (pGL3-MαP-Luc and pRL-SV40) were transduced into K562 cell lines. After 36 h, cell lysates were measured for luciferase activity. The GATA-1 protein level of K562 cell lines was analyzed by Western blot. As an internal control, β-actin protein level of K562 cell lines was analyzed. Western blot results were quantified by densitometry (Software Image J; National Institutes of Health). The expression level of GATA-1 protein was corrected by reference to that of β-actin protein, and the relative amount of GATA-1 protein in each sample was calculated.

ZNF521 Interacts Directly with GATA-1 in Vitro and in Vivo—Since ZNF521 inhibits GATA-1 transcriptional activity as shown above, we next examined the physical interaction between ZNF521 and GATA-1. To verify whether ZNF521 is capable of binding to GATA-1 *in vitro*, we performed a pull-down assay using a GST fusion protein comprising GATA-1 and three fragments of His_6-tagged ZNF521 protein, ZF1–8, ZF9–20, and ZF21–30 (Fig. 4*A*), which were synthesized *in*

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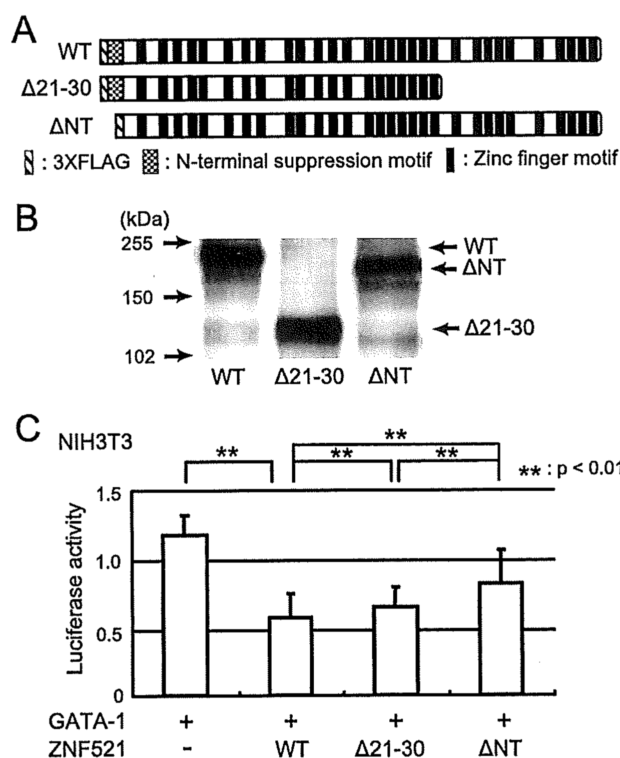

FIGURE 3. **Inhibitory effects of mutant ZNF521s on transcriptional activity of GATA-1.** *A*, the diagrams for ZNF521 and the deletion mutants (Δ21–30 and ΔNT) of ZNF521 tested for inhibition of the GATA-1 transcriptional activity. *B*, expression of the wild-type and mutant ZNF521 proteins was detected by Western blotting using anti-FLAG antibody. *C*, NIH3T3 cells were transfected with pGL3-MαP-Luc, cDNA for *GATA-1*, and cDNA for the indicated *ZNF521* mutant. After 16 h, cell lysates were measured for luciferase activity.

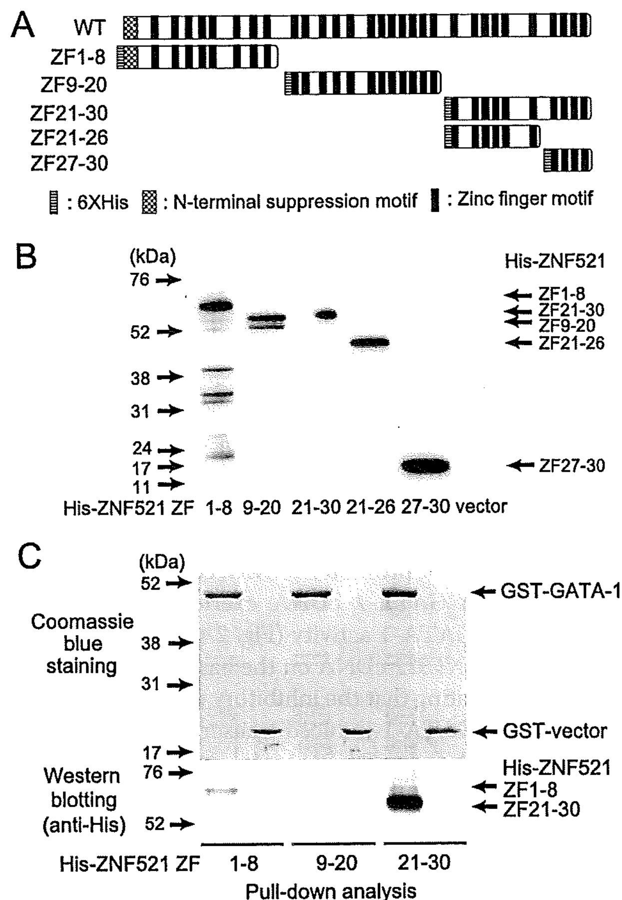

FIGURE 4. **Interaction between ZNF521 and GATA-1 *in vitro*.** *A*, diagrams of the His_6-tagged ZNF521 fragments. *B*, production of ZNF521 fragments was detected by Western blotting using anti-His antibody. *C*, purified GST-GATA-1 and GST proteins were mixed with purified ZNF521 fragment proteins. Bound proteins were fractionated by SDS-PAGE and detected by Western blotting using anti-His antibody. Loaded GST and GST-GATA-1 proteins were detected by Coomassie Blue staining.

vitro using a wheat cell-free protein synthesis system (Fig. 4*B*). GST-GATA-1 protein was immobilized onto glutathione-Sepharose 4B beads and incubated with His_6-tagged ZNF521 proteins. As shown in Fig. 4*C*, GST-GATA-1 bound to the C-terminal part of ZNF521 strongly and to the N-terminal part weakly but did not bind to the central portion of ZNF521. GST alone did not bind to any part of ZNF521. These data indicate that ZNF521 interacts directly with GATA-1 *in vitro*.

GATA-1 has two ZFs, an N-terminal finger (NF) and a C-terminal finger (CF). It has been reported that many kinds of protein can bind to GATA-1 via these two ZFs (21–28). To determine which ZF of GATA-1 interacts with which part of the ZFs of ZNF521, we constructed GST-GATA-1 NF, GST-GATA-1 CF, and two fragments of His_6-tagged ZNF521, ZF-(21–26) and ZF-(27–30) (Fig. 4*A* and 5*A*). GST-GATA-1 CF bound to ZNF521 ZF-(21–26) but not to ZNF521 ZF-(27–30), whereas GST-GATA-1 NF bound neither ZNF521 ZF-(21–26) nor ZF-(27–30) (Fig. 5*B*). These results indicate that ZNF521 interacts mainly with GATA-1 CF through the ZF-(21–26) portion.

Finally, we verified whether ZNF521 interacts with GATA-1 in viable cells. The vectors encoding FLAG-tagged *ZNF521* and Myc-tagged *GATA-1* were transfected into 293T cells, and the total cell lysates were isolated. As shown in Fig. 6*A*, immunoblotting with the anti-FLAG antibody on the anti-Myc immunoprecipitates revealed that ZNF521 was co-immunoprecipitated with Myc-tagged GATA-1. Immunoblotting with the anti-Myc antibody on the anti-FLAG immunoprecipitates revealed that GATA-1 was co-immunoprecipitated with ZNF521. In addition, we established K562 cell lines expressing 3×FLAG-ZNF521 to examine endogenous interaction between ZNF521 and GATA-1. Immunoblotting of the anti-FLAG immunoprecipitates with the anti-GATA-1 antibody revealed that ZNF521 was co-immunoprecipitated with endogenous GATA-1 (Fig. 6*B*). Furthermore, the data obtained from the ChIP assay using the K562/3×FLAG-ZNF521 cell line indicate that ZNF521 and GATA-1 form a transcription complex within the GATA-1-binding sites on the β-globin promoter, one of the GATA-1 target genes (Fig. 6*C*). These data confirm that ZNF521 interacts with GATA-1 *in vivo*.

DISCUSSION

In the present study, we found that shRNA-mediated silencing of the ZNF521-encoding gene in K562 and HEL cells resulted in erythroid differentiation. We also showed that ZNF521 inhibits the transcriptional activity of GATA-1. Deletion analysis of ZNF521 showed that the repressive effect of ZNF521 requires the N-terminal repression motif. Furthermore, direct interaction of ZNF521 with GATA-1 through

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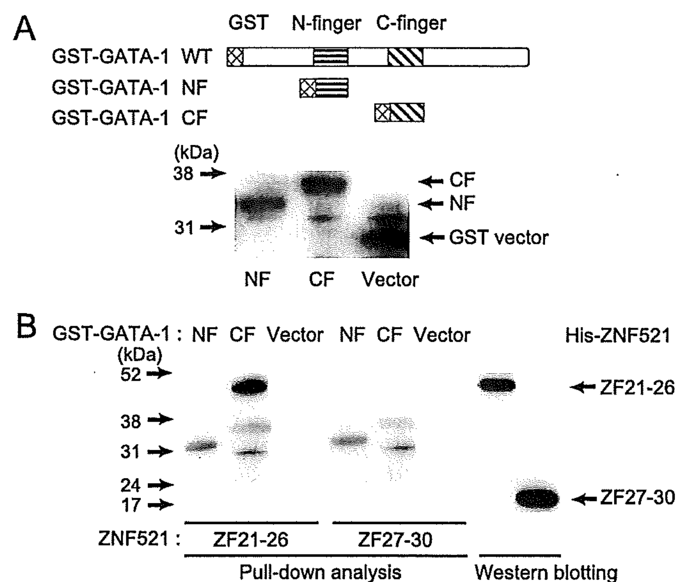

FIGURE 5. **GATA-1 CF interacts with ZNF521 via ZF-(21–26) *in vitro*.** *A*, diagrams of GST-GATA-1, GST-GATA-1 NF, and GST-GATA-1 CF. Production of GATA-1 proteins was detected by Western blotting using anti-GST antibody. *B*, purified GST, GST-GATA-1 NF, and GST-GATA-1 CF were mixed with either ZNF521 ZF-(21–26) or ZNF521 ZF-(27–30) protein. Produced ZNF521 fragment proteins and bound proteins fractionated by SDS-PAGE were detected by Western blotting using anti-His antibody. *WT*, wild type.

ZF-(21–26) of ZNF521 and CF of GATA-1 was demonstrated. These findings demonstrate a novel mechanism for differentiation of erythroid cells and maintenance of the "stemness" of hematopoietic progenitors.

GATA-1 is known to interact with a variety of proteins via ZF domains. Among them, GATA-1 NF interacts with FOG-1 (Friend of GATA-1) (21, 22) and c-Myb (23), whereas CF interacts with EKLF (24), Fli-1 (25), PU.1 (26), and EVI1 (27, 28). These interactions play an important role in hematopoiesis, since they lead to transcriptional activation or repression of GATA-1 target genes. On the other hand, ZNF521 contains an N-terminal suppression motif that is conserved among many transcriptional repressors, including FOG-1 and FOG-2 (9). Recently, this portion was shown to be the association site of the nucleosome remodeling and deacetylase corepressor complex (NuRD) (10, 11), which is involved in global transcriptional repression. In the present study, we demonstrated that deletion of the N-terminal motif of ZNF521 resulted in reduction of the inhibitory effect of ZNF521 against GATA-1 transcriptional activity. This finding suggests that ZNF521 inhibits GATA-1 transcriptional activity by recruiting NuRD.

Brand *et al.* (29) showed that binding of NuRD to the promoter of globin genes was increased in globin-nonproducing immature cells as compared with that in globin-producing erythroid-differentiated cells. This suggests that at an early stage of erythroid differentiation, expression of globlin genes is repressed by the NuRD complex, whereas at a more mature stage, this repression declines as a result of dissociation from NuRD. Taken together with our results, it seems likely that the complex of GATA-1 and ZNF521 inhibits globin gene expression at an early stage of erythroid differentiation through complex formation with NuRD.

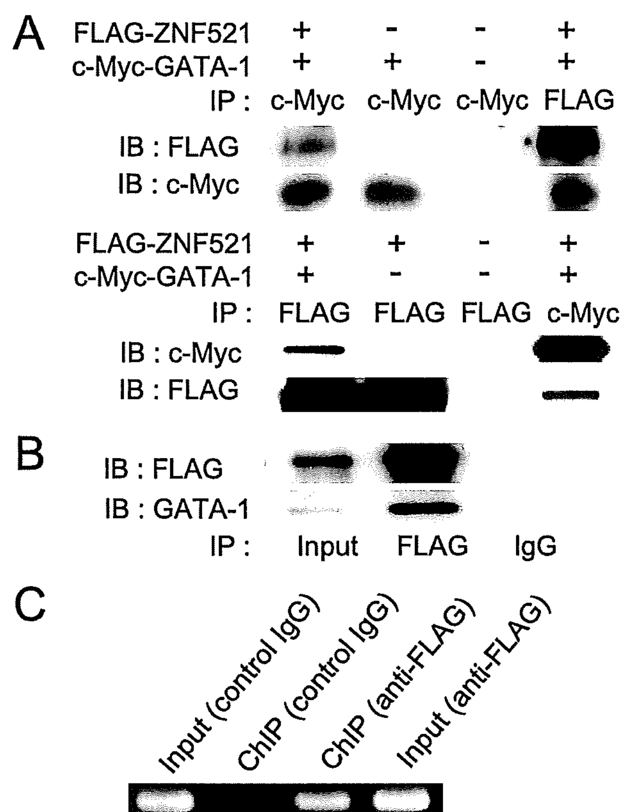

FIGURE 6. **ZNF521 binds to GATA-1 *in vivo*.** *A*, the 293T cells were transfected with or without cDNA for FLAG-tagged *ZNF521* and cDNA for Myc-tagged *GATA-1* as indicated. After 48 h, total cell lysates were isolated and subjected to immunoprecipitation analysis and Western blotting using the indicated antibodies. *B*, K562/3×FLAG-ZNF521 cells were lysed and subjected to immunoprecipitation analysis and Western blotting using the indicated antibodies. *C*, K562/3×FLAG-ZNF521 cells were sonicated, and a ChIP assay was performed using the indicated antibodies and primers for GATA-1-binding site on the β-globin promoter. *IP*, immunoprecipitation; *IB*, immunoblot.

In the present study, we demonstrated that ZNF521 interacts with GATA-1 through ZF-(21–30). We also demonstrated weak *in vitro* association of GATA-1 with ZNF521 ZF-(1–8). Since a ZNF521 mutant in which ZF-(21–30) was deleted inhibited GATA-1 activity, the association between GATA-1 and ZF-(1–8) of ZNF521 might also be involved in the repression of GATA-1 transcriptional activity. Fox *et al.* (30) showed that, in addition to a sixth ZF of FOG-1, ZF1, ZF5, and ZF9 of FOG-1 also interact with the NF of GATA-1 and that each finger contributes to the ability of FOG-1 to modulate GATA-1 activity. Furthermore, they showed that FOG-2 and U-shaped also contain multiple GATA-interacting fingers. These results indicate that GATA-binding proteins including ZNF521 use multiple ZFs to bind GATA-1.

Smad family proteins are essential for BMP and transforming growth factor-β signaling (31, 32). Recent reports have suggested that the association of Smads with other transcription factors is the key for recruitment of Smads to specific promoter elements. BMP4 can increase the proliferation of human hematopoietic progenitors (33, 34), whereas transforming growth factor-β acts as a negative regulator for hematopoietic progenitors *in vitro* (35, 36). BMP4 induces GATA-2 transcription (37, 38). Whereas GATA-1 regulates terminal differentiation of

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erythroid cells (39), GATA-2 is expressed in hematopoietic progenitors and controls early stages of hematopoiesis (40). ZNF521 has been demonstrated to interact with Smad1/4 in response to BMP signals and to activate BMP-responsive genes, resulting in proliferation of hematopoietic progenitors. Since GATA-2 was demonstrated to be repressed by GATA-1 during erythroid maturation (41), the inhibition of GATA-1 activity by ZNF521 might be important for constitutive expression of GATA-2 in order to maintain hematopoietic progenitor cells. Taken together, ZNF521 might play an important role in maintaining the "stemness" of hematopoietic progenitors.

Interestingly, EVI3, a murine homologue of ZNF521, has been demonstrated to be expressed in the brain, especially the cerebellum, as well as in hematopoietic progenitors (14). *In situ* hybridization analysis revealed striking enrichment of *Evi3* mRNA in the granule layer that hosts granule neural precursors in the postnatal cerebellum (14). These findings suggest that ZNF521 also plays an important role in development of the nervous system. To confirm this, we are now generating mice homozygous for an *Evi3* null allele.

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ORIGINAL ARTICLE

Clinical significance of co-expression of CD21 and LFA-1 in B-cell lymphoma

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Abstract We previously reported that the prognosis of CD21-positive diffuse large B-cell lymphoma (DLBCL) is significantly favorable to that of CD21-negative DLBCL (Otsuka et al. in Br J Haematol 127:416–424, 2004). In this study, we attempted to clarify the biological significance of CD21 expression in B-cell lymphoma (BCL) by performing in vitro experiments using *CD21* transfection into a CD21-negative lymphoma cell line and analyzing clinical data from lymphoma samples. Established clones of *CD21* transfectants showed homotypic aggregation in suspension culture. Analysis of integrin expression revealed that LFA-1 appeared to be expressed on *CD21* transfectants, and the cell aggregation was abrogated by anti-LFA-1 antibody. The *CD21* transfectants could adhere to plastic plates coated with ICAM-1. Moreover, flow cytometry and/or immunohistochemical analyses of clinical BCL samples ($n = 29$) revealed positive for CD21 in all cases; LFA-1 was also expressed without exception. All BCL cells isolated from cavity fluids ($n = 10$) failed to express both CD21 and LFA-1. These data suggest that CD21 is tightly related to LFA-1 expression in BCL and the absence of CD21/LFA-1 expression is associated with pleural/peritoneal fluid involvement by BCL, a potential indicator of disease progression of BCL.

Keywords CD21 · LFA-1 · B-cell lymphoma

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1 Introduction

Since non-Hodgkin lymphoma (NHL) includes a heterogeneous set of lymphoid malignancies, precise classification is essential for predicting the prognosis and determining the appropriate treatment strategy. Although recent studies using gene expression profile-based classification have successfully discriminated diffuse large B-cell lymphoma (DLBCL) into two subgroups reflecting the origin of the lymphoma cells and the disease prognosis [1–4], this method is complex and difficult to apply in routine clinical practice. Conversely, immunohistochemistry-based classification of NHL is readily applicable in clinical practice and has been widely used. Various surface markers are used for classification of NHL; however, associations between immunological

phenotype and clinical prognosis have not been clearly demonstrated. Since the anti-CD20 monoclonal antibody (mAb) rituximab has been successfully used for the treatment of B-cell lymphoma (BCL), CD20 expression has become one of the most significant prognostic factors for favorable outcome of BCL [5, 6]. Although the expression of antigens targeted by therapeutic antibodies, such as CD20, is certainly a good marker for predicting sensitivity to such immunotherapy, the biological significance for disease progression of surface molecules expressed on lymphoma cells has not been well clarified.

We previously reported the favorable prognostic value of CD21 for DLBCL. We also demonstrated that immunodeficient mice inoculated with a *CD21* gene-transfected BCL cell line survived significantly longer than those inoculated with the CD21-negative parent cell line [7]. The clinical significance of CD21 expression in predicting a good prognosis in DLBCL has also been reported by other investigators [8]. CD21 is a membrane protein that is normally expressed on B lymphocytes, follicular dendritic cells, early thymocytes, and a subset of mature T cells [9, 10]. CD21 appears later than CD19 and CD20 in B-cell ontogeny [11] and disappears from the cell surface during the early phase of B-cell activation [12]. It is well established that CD21 plays an important role in B-cell activation by forming, together with CD19 and CD81 [13], a co-receptor complex for the B-cell receptor (BCR). This gate-keeper complex is also utilized by Epstein-Barr virus to infect B lymphocytes [14, 15]. It is also well known that CD21 is physiologically essential for Ab production against T cell-dependent antigens [16–18]. In addition, CD21 has been reported to bind to CD23, resulting in cell aggregation [19].

These biological characteristics of CD21 indicate that it is widely involved in the B-lymphocyte immune response, including cell trafficking, migration, activation, and interaction with the T cell response. Although the function of CD21 in normal B lymphocytes has been widely studied, little is known about the biological significance of CD21 in progression of BCL. Here, we show in vitro and in vivo observations using *CD21* gene transfection and clinical samples and try to describe a new biological aspect of CD21 in BCL.

2 Materials and methods

2.1 Abs used in the experiments

The following mAbs were used for flow cytometric analysis and cell adhesion assays: anti-CD19 mAb (Beckman Coulter, Fullerton, CA; BD Biosciences, San Jose, CA), anti-CD21 mAbs (Immunotech, Westbrook, ME; Santa Cruz Biotechnology, Santa Cruz, CA; Beckman Coulter), anti-CD11a (LFA-1 αL subunit; αL integrin) mAbs (CosmoBio Co., Tokyo, Japan; Biolegend, San Diego, CA; Beckman Coulter), anti-CD18 (LFA-1β2 subunit; β2 integrin) mAbs (Beckman Coulter; Biolegend), anti-CD11b (MAC-1) mAb (Beckman Coulter), anti-CD11c (p150/95) mAb (Beckman Coulter), anti-CD49b (α2 integrin) mAb (CosmoBio Co.), anti-CD49c (α3 integrin) mAb (CosmoBio Co.), anti-CD49f (α6 integrin) mAb (CosmoBio Co.), anti-CD103 (αE integrin) mAb (CosmoBio Co.), anti-CD49d (α4 integrin) mAb (EMD Chemicals, San Diego, CA), anti-CD49e (α5 integrin) mAb (Southern Biotechnology Associates, Birmingham, AL), anti-CD51/CD61 (αv integrin and β3 integrin) mAb (Ancell, Bayport, MN), anti-CD54 (intracellular cell adhesion molecule-1; ICAM-1) mAb (Immunotech), and control IgG (mouse and rabbit IgG; Santa Cruz Biotechnology). These antibodies were used in accordance with the manufacturers' instructions.

2.2 NHL B-cell lines

NHL B-cell lines, Namalwa and Raji, were cultured in RPMI-1640 medium (GibcoBRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 50 U/ml of penicillin and 50 μg/ml of streptomycin (culture medium). Both Namalwa and Raji cells were positive for CD19, CD20, and CD54 (ICAM-1). On the other hand, Raji cells, but not Namalwa cells, were positive for CD21, CD11a, and CD18 [20, 21].

2.3 Flow cytometric analysis

The cells were labeled with the above-listed mAbs that had been conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and were then analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and Flowjo software (Tree Star, San Carlos, CA).

2.4 Generation of CD21 gene-transfected Namalwa cell line clones

CD21 gene-transfected Namalwa cell line clones were established as previously reported [7]. In brief, the full-length *CD21* cDNA (GeneBank accession number M26004) obtained from human mature B lymphocytes by reverse transcription polymerase chain reaction (RT-PCR) was cloned and inserted into pCMV-Script mammalian expression vector (Stratagene, La Jolla, CA). *CD21* gene-carrying vector and empty control vector were transfected into the parent Namalwa cells by electroporation with a Gene Pulser®II (Bio-Rad, Hercules, CA). After selection with G418, bulk cells were cloned by the limiting dilution method.

2.5 Quantitative real-time PCR (QRT-PCR)

Total RNA was extracted from samples using an Rneasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RT was performed using 1 μg of total RNA from each sample in a final volume of 100 μl with the TaqMan RT reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Gene expression levels of *CD11a*, *CD18*, and *CD21* were measured quantitatively with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). TaqMan Assays-on-demand probe and primer reagents (Applied Biosystems) for *CD21*, Hs01079099_m1, *CD11a*, Hs01035632_m1 and *CD18*, Hs01051753_m1 were utilized according to the manufacturer's instructions. QRT-PCR reactions of cDNA specimens and water as negative control were conducted in a total volume of 25 μl with TaqMan Master Mix (Applied Biosystems), 400 nM primers, and 200 nM probe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for QRT-PCR. Thermal cycler parameters included 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min (denature, annealing, and extension). All analyses were performed in duplicate. To normalize differences in RNA degradation between different samples and in RNA loading for the RT-PCR procedure, gene expression levels of both *CD11a* and *CD18* were defined relative to the expression level of the *GAPDH* gene in the sample. The *CD11a* and *CD18* gene expression levels in the Raji cell line, which strongly expresses both genes, were designated as 1.0 and expression levels in the experimental samples were calculated relative to those levels by the comparative threshold cycle method.

2.6 Cell aggregation and blocking experiment

The *CD21* gene-transfected cells and control vector-transfected cells suspended in culture medium were seeded in flat-bottomed plates at a concentration of 1×10^5 cells/ml/well. The cells were cultured with or without an optimal concentration of anti-LFA-1 mAb or a control Ab (mouse IgG) for 24 h. Then, cell aggregation was observed with an inverted microscope.

2.7 Cell adhesion assay

Flat-bottomed 96-well plates were coated with 5 μg/ml of goat anti-human IgG (Fc specific) (MP Biomedicals, Solon, OH) in 50 mM sodium bicarbonate buffer (pH 9.2) overnight at 4°C, with blocking by phosphate buffered saline (PBS) containing 0.5% of FCS. Then 50 μl of 1 μg/ml recombinant human ICAM-1/Fc chimeric Ab (R&D Systems, Minneapolis, MN) was added to each well and incubated for 1 h at room temperature. Plates coated with ICAM-1 were washed twice with washing buffer (PBS containing 0.1% FCS). Then, 5×10^4 cells of *CD21* gene-transfected, control vector-transfected cells, and parent Namalwa were seeded into plates with anti-LFA-1 mAbs or control Ab and incubated in culture medium containing 1 mM $MgCl_2$ for 1 h at 37°C in a 5% CO_2 incubator. Plates were then washed twice with washing buffer and treated with the lysis solution [50 mM sodium acetate (pH 5.0), *p*-nitrophenyl phosphate (6 mg/ml), and 1% Triton X-100] for 90 min at 37°C. Then, 1 M NaOH was added to each well. The rate of cell adhesion was determined by measuring total fluorescence at 405 nm wavelength with a plate reader (Nalge Nunc International, Tokyo, Japan).

2.8 Expression of CD21 and LFA-1 in clinical samples

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was provided by all patients.

Biopsied specimens and cells in pleural and peritoneal cavity fluids were collected from patients with BCL at Ehime University Hospital. Mononuclear cells were isolated by a centrifugation method with Ficoll-Hypaque. Clinical specimens which could not be analyzed immediately were kept in a liquid nitrogen freezer until use. Expressions of CD21 and LFA-1 (CD11a/CD18) were determined by flow cytometric analysis. When mAb-stained cells were detected in more than 30% of total cells, the specimen was defined to be positive, as described previously [22–24]. Expressions of CD21 and LFA-1 on lymphoma cells in cavity fluids were analyzed in the CD19-positive fraction to exclude non-lymphoma cells. All biopsied samples were examined immunohistochemically using biotinylated hen-eggwhite-lysozyme (HEL-biotin) and alkaline phosphatase streptavidin chemistry on fixed paraffin-embedded tissue sections. Some samples were also examined by flow cytometry using suspended viable cells. Staining intensity was scored at four levels [25]. The stained slides were reviewed twice by different pathologists with no prior knowledge of clinical outcomes, assigned randomly. Anti-CD21 mAbs (clone 1F8; Dako, Glostrup, Denmark) and anti-LFA-1 (anti-CD11a) mAb (clone EP1285Y; Epitomics, Burlingame, CA) were used for the immunohistochemical analysis.

3 Results

3.1 Transfection of CD21 gene into BCL cell line induces homotypic aggregation and LFA-1 expression at the transcription level

As we reported previously [7], the *CD21* gene was transfected into the CD21-negative Namalwa cell line and

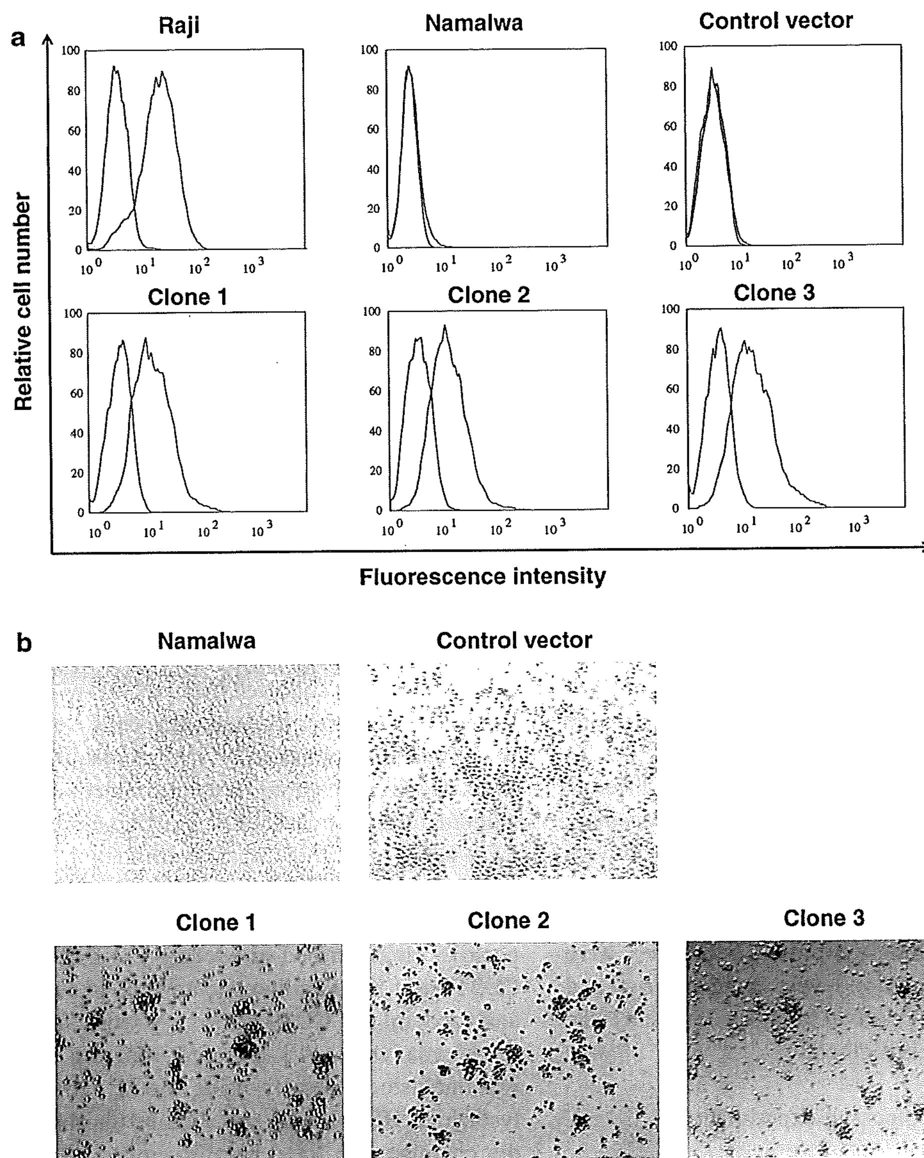

Fig. 1 Characteristic changes in BCL cell lines induced by CD21 gene transfection. **a** Expression of CD21 on *CD21* gene-transfected BCL cell lines. Expression of CD21 on the cell surface of the parent Namalwa cell line, the control vector-transfected cell line, and *CD21* gene-transfected clones was analyzed by flow cytometry. The CD21-positive cell line Raji was used as the positive control. **b** Cell aggregation of *CD21* gene-transfected BCL cells. Inverted microscopy shows that all *CD21* gene-transfected clones, but not parent or control vector-transfected cell lines, showed homotypic cell aggregation. **c** Expression of LFA-1 on *CD21* gene-transfected BCL cell lines. Expression of LFA-1 on the cell surface of the parent Namalwa cell line, the control vector-transfected cell line, and *CD21* gene-transfected clones was analyzed by flow cytometry. The LFA-1-positive cell line Raji was used as the positive control. **d** Expression of *LFA-1* (*CD11a*/*CD18*) mRNA. Expression levels of *CD11a* and *CD18* mRNAs in the parent Namalwa cell line, the control vector-transfected cell line, and *CD21* gene-transfected clones were determined by QRT-PCR as detailed in Sect. 2. The levels of *CD11a* and *CD18* mRNA expression in the Raji cell line, which expresses LFA-1, are shown as 1.0 and the expression levels in the samples were calculated relative to this value

CD21-expressing clones were established (Fig. 1a), and CD21-positive Namalwa cells showed homotypic aggregation (Fig. 1b). To clarify the mechanism of homotypic aggregation of CD21-positive lymphoma cells, we first examined the expression profiles of adhesion molecules on *CD21* gene-transfected cells and control cells, focusing on integrins. Among the various integrins screened, CD11a (α1 integrin) and CD18 (β2 integrin), which compose the heterodimer complex of the major adhesion molecule LFA-1, appeared to be expressed on CD21-positive clones but not on CD21-negative parent cells (Table 1). Flow cytometric analysis of CD11a and CD18 expression on

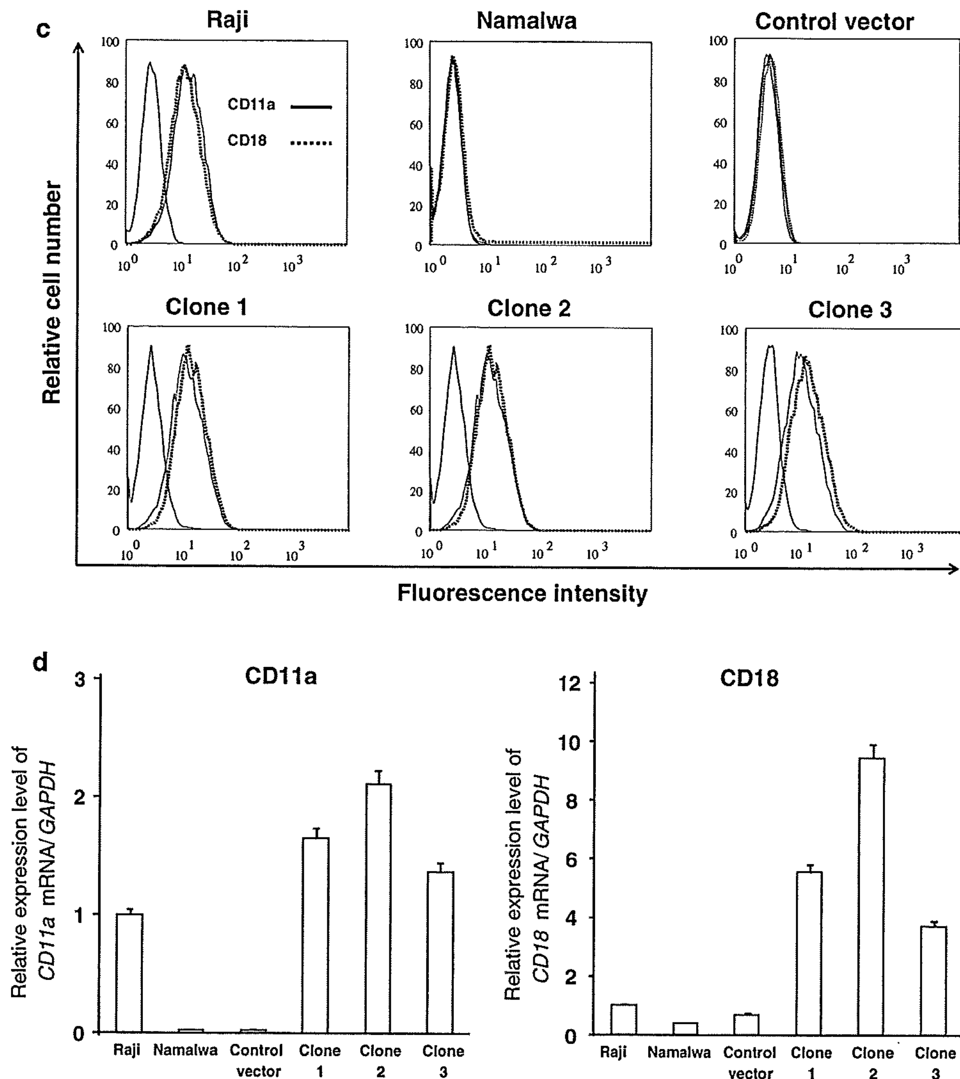

Fig. 1 continued

CD21-positive clones and CD21-negative parent cells is shown in Fig. 1c. The Raji cell line, which constitutively expresses high amounts of both CD21 and LFA-1 and spontaneously undergoes homotypic cell aggregation, was used as a positive control.

We next examined the mechanism of de novo expression of LFA-1 on *CD21* gene-transfected cells. As shown in Fig. 1d, QRT-PCR clearly revealed that mRNA expression of both *CD11a* and *CD18* was increased dramatically in *CD21* gene-transfected clones as compared with CD21-negative parent cells. Flow cytometric analysis failed to detect LFA-1 protein on the cell surface or in the cytoplasm of CD21-negative parent cells (data not shown), indicating that de novo expression of LFA-1 on *CD21* gene-transfected cells is generated at the transcription level rather than by intracytoplasmic transport.

3.2 The homotypic aggregation of CD21 gene-transfected BCL cells is mediated by the interaction between LFA-1 and ICAM-1

As shown in Fig. 2a, the aggregation of *CD21* gene-transfected BCL cells was abrogated by adding anti-LFA-1 mAbs (the combination of anti-CD11a mAb and anti-CD18 mAb), indicating that their homotypic aggregation was mediated by LFA-1 molecules which were newly expressed on their cell surface following transfection of the *CD21* gene. Anti-CD18 mAb alone completely abrogated the cell aggregation; however, the inhibitory effect of anti-CD11a mAb alone on homotypic aggregation appeared to be partial (data not shown).

We next attempted to identify the molecule(s) that can bind to LFA-1 expressed on *CD21* gene-transfected BCL

Table 1 Cell surface expression of adhesion molecules on cell lines

CD	Namalwa	Control vector	Clone 1	Clone 2	Clone 3
CD21	−	−	+	+	+
CD18	−	−	+	+	+
CD11a	−	−	+	+	+
CD11b	−	−	−	−	−
CD11c	−	−	−	−	−
CD49b	−	−	−	−	−
CD49c	−	−	−	−	−
CD49d	+	+	+	+	+
CD49e	−	−	−	−	−
CD49f	+	+	+	+	+
CD103	−	−	−	−	−
CD51	−	−	−	−	−
CD61	−	−	−	−	−
CD54	+	+	+	+	+

Expression of various cell surface molecules on the parent Namalwa cell line, the control vector-transfected cell line, and the *CD21* gene-transfected clones was analyzed by flow cytometry

cells. As shown in Table 1, CD54 (intercellular adhesion molecule-1; ICAM-1), which is well established as the ligand for LFA-1, was abundantly expressed on all cell lines including the parent Namalwa cells, control vector-transfected cells, and *CD21* gene-transfected cells. On the basis of this finding, we investigated whether interaction between LFA-1 and ICAM-1 on CD21-positive lymphoma cells is responsible for their homotypic aggregation. As shown in Fig. 2b, *CD21* gene-transfected cells appeared to adhere strongly to ICAM-1-coated plastic plates, and their adhesion was almost completely inhibited by adding anti-LFA-1 mAbs to the culture medium ($p < 0.05$ by Wilcoxon-signed rank test). These data indicate that homotypic aggregation of CD21-positive lymphoma cells is mediated by the interaction between LFA-1 and ICAM-1 expressed on their cell surface. Several examinations have reported that ICAM-1, -2, and -3 were expressed on the leukocytes and blood vessels [26], and that ICAM-4 and -5 are dominantly expressed on red blood cells and neurological tissues [27–30]. ICAM-1 has been well documented to be involved in homing and activation of tumor lymphocytes [25, 31, 32]. Our blocking experiments that use anti-LFA-1 antibodies to *CD21* gene transfectants indicate cell aggregation and adhesion resulting from the interaction between LFA-1 and ICAM family, especially ICAM-1.

3.3 Close correlation between expression of CD21 and LFA-1 in clinical samples of BCL

On the basis of the data obtained from the series of experiments described above, we addressed the question whether the correlation between CD21 and LFA-1 can also be detected in clinical BCL samples. We examined a series of 39 clinical samples (including 27 DLBCLs in solid organs, 1 DLBCL transformed from follicular lymphoma, 1 primary mediastinal large BCL, and 10 DLBCLs disseminated in cavity fluids) obtained from patients newly diagnosed with BCL in our hospital. On the basis of expression of CD21 and LFA-1, BCL cases could be divided into four subgroups: CD21$^+$/LFA-1$^+$, CD21$^-$/LFA-1$^+$, CD21$^+$/LFA-1$^-$, and CD21$^-$/LFA-1$^-$. As shown in Table 2, without exception all CD21$^+$ cases appeared to co-express LFA-1; this correlation was statistically significant ($p < 0.05$ by Fisher's exact probability test). This result suggests the close correlation between expression of CD21 and LFA-1 observed in the in vitro experiments using *CD21* gene-transfected lymphoma cell lines.

3.4 Lymphoma cells isolated from pleural and peritoneal effusions lack both CD21 and LFA-1

The expression of CD21 and LFA-1 on lymphoma cells isolated from cavity fluid (pleural and peritoneal effusions) is shown in Table 2. Interestingly, all 10 samples isolated from effusion involvement appeared to be negative for CD21 and LFA-1. The detailed data for these cases are shown in Table 3. Among the 10 cases with cavity fluid lymphoma, the primary lesions, including lymph nodes, intestine, mediastinal mass, and oral mucosa, could also be examined in 7 cases. Although all cases with lymphoma in cavity fluids were CD21$^-$/LFA-1$^-$, 2 of the 7 cases appeared to be CD21$^-$/LFA-1$^+$ in their primary lesions. These findings suggest that disappearance of LFA-1 can occur during disease progression, and this may result in cell migration from the primary lesions and dissemination in cavity fluid.

4 Discussion

We previously reported on the basis of analysis of clinical data that CD21 expression is associated with favorable prognosis of DLBCL. We also reported in the same paper that nude mice transplanted with *CD21* gene-transfected human lymphoma cells exhibited significantly reduced tumor growth and longer survival as compared with those transplanted with CD21-negative parent lymphoma cells. Interestingly, all established *CD21* gene-transfected cell lines showed homotypic aggregation in the culture medium and anti-CD21 Abs did not inhibit this aggregation, suggesting that CD21 does not by itself play an important role in homotypic aggregation. In the current study, we focused on expression of adhesion molecules, and the data obtained from the present series of experiments are as follows. Comparative analysis of integrin expression on *CD21*

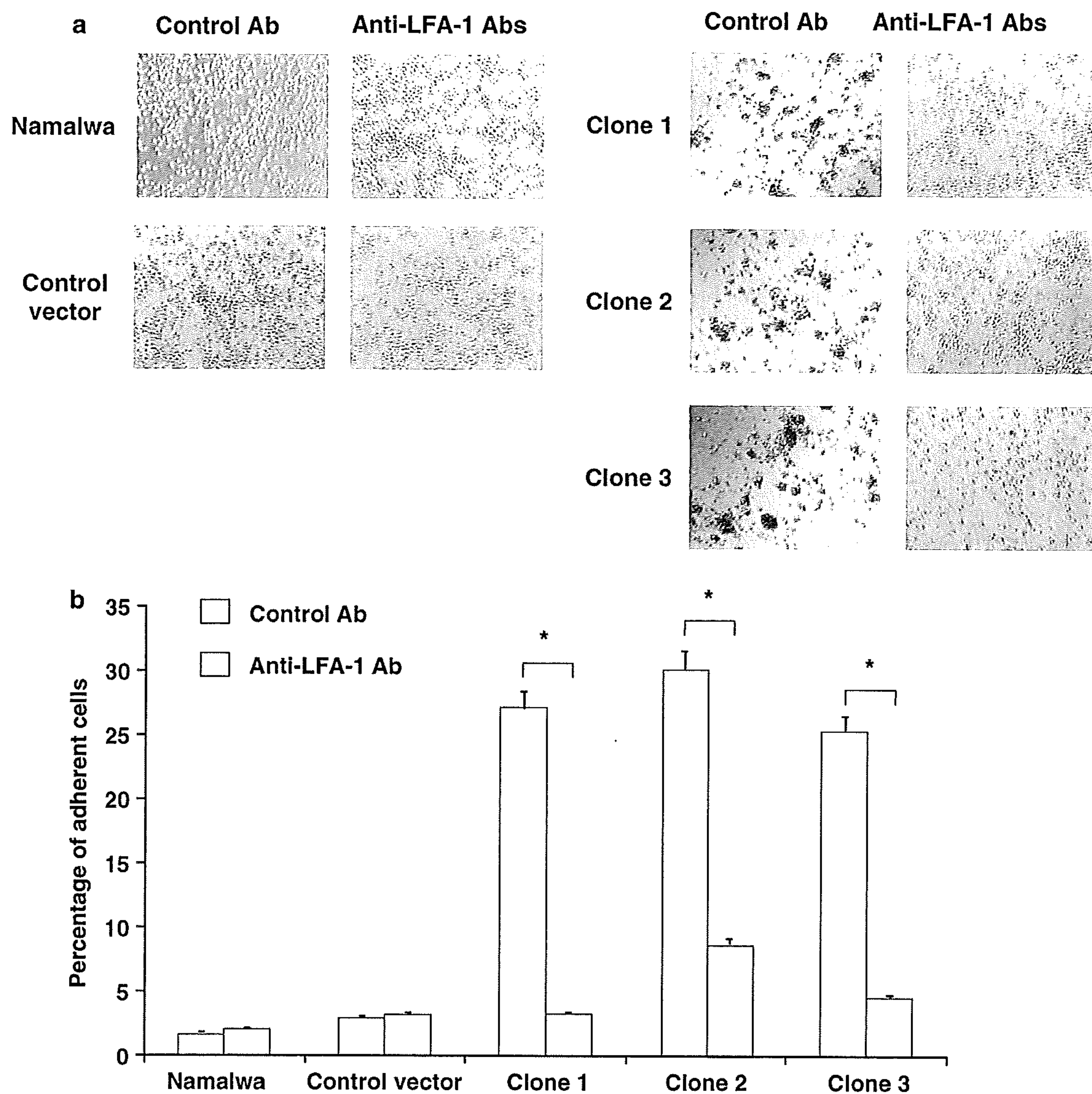

Fig. 2 Inhibition of cell aggregation and cell adhesion by anti-LFA-1 mAbs. **a** Inhibition of homotypic aggregation by anti-LFA-1 mAbs. *CD21* gene-transfected clone cells were cultured with anti-LFA-1 (the combination of anti-CD11a and anti-CD18) mAbs or control Ab (mouse IgG) at a concentration of 10 μg/ml for 24 h. Homotypic cell aggregation of *CD21* gene-transfected cells was inhibited by anti-LFA-1 mAbs but not by control Ab. **b** Inhibition of cell adhesion to ICAM-1-coated plates. The parent Namalwa cell line, the control vector-transfected cell line, and *CD21* gene-transfected clones were cultured on ICAM-1-coated plates with anti-LFA-1 (the combination of anti-CD11a and anti-CD18) mAbs or control Ab (mouse IgG) at a concentration of 10 μg/ml for 1 h. *CD21* gene-transfected clone cells but not the parent Namalwa cells or control vector-transfected cells adhered to ICAM-1-coated plates, and their adhesion was inhibited by anti-LFA-1 mAbs but not by control Ab. Asterisks indicate significant differences ($p < 0.05$ by Wilcoxon-signed rank test)

gene-transfected and control vector-transfected BCL cell lines revealed that transcription of LFA-1 appeared to be up-regulated by *CD21* gene transfection. The *CD21* gene-transfected BCL cells could bind to plastic plates coated with ICAM-1, and this binding and their homotypic aggregation were completely abrogated by adding anti-LFA-1 mAbs. We also analyzed co-expression of CD21 and LFA-1 in clinical lymphoma samples. All cases positive for CD21 also co-expressed LFA-1. In addition, all lymphoma cells isolated from cavity fluids appeared to express neither CD21 nor LFA-1. In previous papers, the lack of LFA-1 on the cell surface of intravascular malignant lymphomatosis and extra-nodal lymphoma has been reported [33–35]. These clinical observations suggest that the LFA-1-negative malignant B lymphocytes can easily migrate out of the lymphoid organs. Our data suggest a close correlation between expression of CD21 and of LFA-1 in BCL cells, and that this interaction might at least in part underlie the favorable prognosis in CD21-positive DLBCL.

Table 2 Expression of CD21 and LFA-1 in clinical samples

Diagnosed sites (*n*)	CD21$^+$/LFA-1$^+$	CD21$^+$/LFA-1$^-$	CD21$^-$/LFA-1$^+$	CD21$^-$/LFA-1$^-$
Lymph node (12)	6	0	3	3
Stomach/intestine (6)	0	0	3	3
Bone marrow (4)	0	0	1	3
Nasal cavity (1)	0	0	0	1
Mediastinum (1)	0	0	0	1
Extradural (1)	0	0	1	0
Liver (1)	1	0	0	0
Adrenal gland (1)	0	0	0	1
Oral mucosa (1)	0	0	0	1
Bone (1)	0	0	1	0
Body cavity (10)	0	0	0	10
$n = 39$	7	0	9	23

Table 3 Expression of CD21 and LFA-1 in pleural and peritoneal effusion lymphoma cells

Case	Pleural and peritoneal effusion lymphoma cells (expression of CD21/CD11a)	Disease	Primary site	Lymphoma cells at primary lesions (expression of CD21/CD11a)
#1	–/–	DLBCL	Lymph node	–/+
#2	–/–	DLBCL	Lymph node	–/–
#3	–/–	DLBCL	Intestine	–/–
#4	–/–	DLBCL	Bone marrow	–/+
#5	–/–	DLBCL	Bone marrow	–/–
#6	–/–	Mediastinal B	Mediastinum	–/–
#7	–/–	DLBCL	Oral mucosa	–/–
#8	–/–	DLBCL	Lymph node	ND
#9	–/–	DLBCL	Lymph node	ND
#10	–/–	DLBCL	Lymph node	ND

DLBCL diffuse large B-cell lymphoma, *mediastinal B* mediastinal large B-cell lymphoma, *ND* not done, because of unfavorable physical condition

One of the interesting findings in this study is that de novo expression of LFA-1 is induced by *CD21* gene transfection in lymphoma cells. It has been reported that the *CD11a* gene promoter directs LFA-1 (CD11a/CD18) integrin expression, a process that depends on two overlapping sequences within the MS7 element, RUNX-110 and CEBP-100 [36], which are recognized by the RUNX and C/EBP transcription factor families, respectively. Recognition of MS7 differs in lymphoid (RUNX) and myeloid (C/EBP and RUNX) cells [37]. The functional relevance of these elements is demonstrated by the fact that RUNX3 overexpression leads to enhanced LFA-1 levels, whereas RUNX1-ETO-expressing cells exhibit weak or absent expression of LFA-1. RUNX3, which can induce immortalization of B lymphocytes, is known to be overexpressed in Burkitt lymphoma cells [38]. CD21 together with CD19 and CD81 forms a complex that acts as a coreceptor, enhancing signaling and antigen processing by the BCR in response to stimulation. Taken together with the evidence that LFA-1 is expressed physiologically in activated B lymphocytes, we suggest that activation of B-lymphoma cells induced by signaling through the complex formed by CD21 might induce RUNX3 activation, resulting in LFA-1 expression.

The other interesting finding in this study is the coexpression pattern of CD21 and LFA-1 in clinical lymphoma samples. All CD21-positive cases appeared to be positive for LFA-1. In additional, all lymphoma cells isolated from pleural or peritoneal fluid were negative for both CD21 and LFA-1. In our current analysis using clinical samples, all six patients with BCL expressing both CD21 and LFA-1 (double-positive) are in remission and are still alive following standard chemotherapy (R-CHOP). In contrast, two out of three patients with BCL expressing neither CD21 nor LFA-1 (double-negative) died due to lymphoma within ten months. There was no statistical

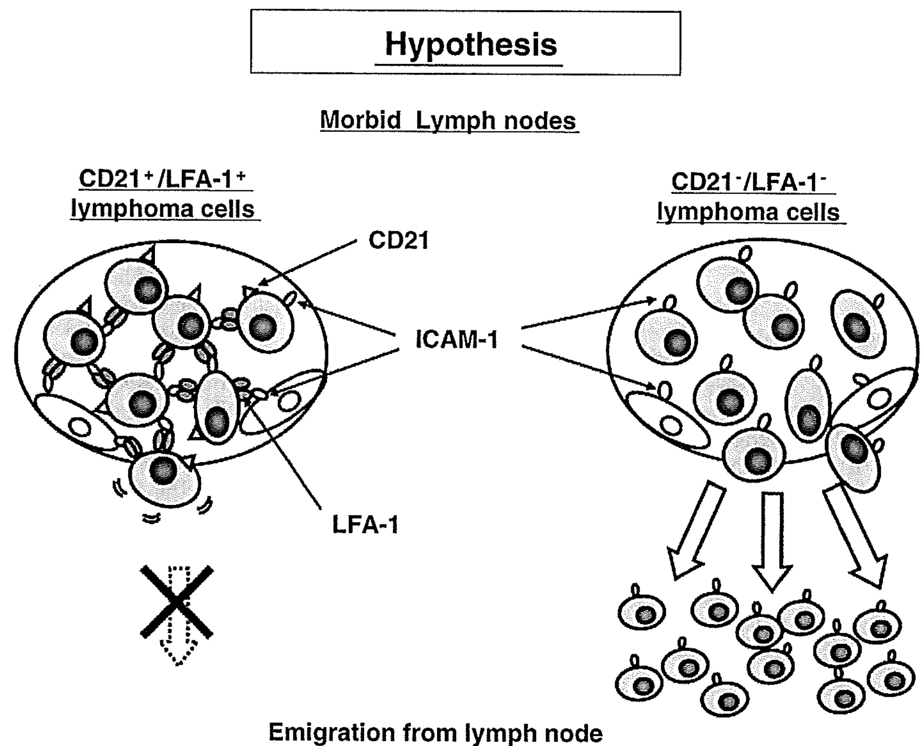

Fig. 3 Postulated negative contribution of CD21 related LFA-1 expression on lymphoma cells to the disease progression in the morbid lymph nodes. In lymphoma, interaction between LFA-1 and ICAM-1 in CD21$^+$LFA-1$^+$ lymphoma cells and stromal cells causes the mutual cell adhesion of lymphoma cells. In CD21$^-$LFA-1$^-$ lymphoma, the adhesion is abrogated, resulting in their emigration from lymph nodes

difference, however, the patients with tumors expressing both CD21 and LFA-1 (double-positive) had a greater instance of remission.

Lymphocyte homing is regulated by multistep processes. Among the various adhesion molecules, LFA-1 mediates interaction of B lymphocytes with follicular dendritic cells in germinal centers [39]. This integrin-mediated interaction inhibits apoptosis of germinal B lymphocytes and may be crucial for affinity maturation [40, 41]. Patients with NHL expressing LFA-1 were reported to present more frequently with advanced stage and bone marrow infiltration [25]. In contrast to these unfavorable aspects of LFA-1 expression in malignancies, the present study provides a new insight into the role of LFA-1 expression in the progression of lymphoma cells. The endothelial interactions and migration of normal lymphocytes are regulated by various kinds of adhesion molecule, including LFA-1/ICAM-1. In lymphoma, interaction between LFA-1 and ICAM-1 on lymphoma cells causes homotypic aggregation, and this may limit proliferation in the affected lesions. Lack of this interaction may allow localized proliferation of lymphoma cells, resulting in their disease progression (Fig. 3). Taken together, these considerations suggest that homotypic aggregation of lymphoma cells mediated by interaction between CD21-related LFA-1 and ICAM-1 may be one of the mechanisms underlying the favorable prognostic value of CD21 in DLBCL as we reported before [7].

Another question is whether CD21$^+$ DLBCL epigenetically loses CD21 expression during disease progression or whether CD21$^+$ and CD21$^-$ DLBCLs arise from originally distinct B-cell lineages. In the literature, almost 30% of primary DLBCL cases are reported to be CD21-positive [42–45]. Recently, Miyazaki et al. [46] reported a comparative analysis of gene expression profiles in CD21$^+$ and CD21$^-$ DLBCLs. They described that survival of patients with CD21$^+$ DLBCL was significantly better than that of patients with CD21$^-$ DLBCL, as reported previously. Their gene expression profiling analysis showed that genes associated with immune response, cell cycle regulation, and apoptosis are differentially expressed in CD21$^+$ and CD21$^-$ DLBCLs. These data suggest that CD21$^+$ and CD21$^-$ DLBCLs are distinct in their origin. On the other hand, our present data demonstrate that disappearance of CD21 and LFA-1 can occur during disease progression in some cases, and that this phenomenon may result in involvement of lymphoma cells in cavity fluid. Together, the previous findings and our present data suggest that the mechanisms underlying the favorable prognosis of CD21$^+$ DLBCL may be multi-factorial, and further studies are required to clarify this issue.

In summary, the present study demonstrates for the first time the biological significance of co-expression of CD21 and LFA-1 in lymphoma cells. Our data may shed light on the mechanisms responsible for progression of lymphoma cells, and further studies focusing on the association of

CD21 and adhesion molecules may be expected to lead to novel strategies for treatment of lymphoma.

Conflict of interest statement No potential conflict of interest was disclosed.

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Short Communication

HBZ is an immunogenic protein, but not a target antigen for human T-cell leukemia virus type 1-specific cytotoxic T lymphocytes

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Recently, HBZ has been reported to play an important role in the proliferation of adult T-cell leukaemia (ATL) cells and might be a target of novel therapy for ATL. To develop a novel immunotherapy for ATL, we verified the feasibility of cellular immunotherapy targeting HBZ. We established an HBZ-specific and HLA-A*0201-restricted cytotoxic T lymphocyte (CTL) clone. Detailed study using this CTL clone clearly showed that HBZ is certainly an immunogenic protein recognizable by human CTLs; however, HBZ-specific CTLs could not lyse ATL cells. Failure of HBZ-specific CTLs to recognize human T-cell leukemia virus type 1 (HTLV-1)-infected cells might be due to a low level of HBZ protein expression in ATL cells and resistance of HTLV-1-infected cells to CTL-mediated cytotoxicity. Although HBZ plays an important role in the proliferation of HTLV-1-infected cells, it may also provide a novel mechanism that allows them to evade immune recognition.

Adult T-cell leukaemia (ATL) is a neoplasm of peripheral T lymphocytes generated by a human retrovirus, human T-cell leukemia virus type 1 (HTLV-1) (Satou & Matsuoka, 2007). The prognosis of ATL is very poor despite intensive chemotherapy, and the current mean survival time of patients with aggressive ATL is less than 1 year. Recently, however, longer survival than that achieved solely by chemotherapy has been achieved in ATL patients after allogeneic haematopoietic stem cell transplantation (HSCT) (Utsunomiya *et al.*, 2001; Fujiwara *et al.*, 2008). The clinical effect of allogeneic HSCT is thought to be mediated mainly by the anti-HTLV-1 immune response. Because Tax-specific cytotoxic T lymphocytes (CTLs) are frequently detected in peripheral blood of patients with ATL who have undergone allogeneic HSCT (Harashima *et al.*, 2004), immunotherapy targeting Tax might be a promising strategy for treatment of ATL. However, universal clinical application of Tax-targeted immunotherapy seems unlikely because *tax* mRNA is detected in only about 40% of ATL cases (Taylor& Matsuoka, 2005). Therefore, identification of a novel target antigen recognized by CTLs and directed against ATL cells is desirable.

Recently, mRNA encoding an open reading frame in the minus strand of the HTLV-1 provirus has been identified (Gaudray *et al.*, 2002). This mRNA encodes HBZ (HTLV-1 bZIP factor), a protein that contains an N-terminal transcriptional activation domain and a leucine zipper motif at its C terminus. HBZ was found to inhibit Tax-mediated transactivation of viral transcription from the 5′LTR by interaction with cellular factors of the JUN and ATF/CREB families (Basbous *et al.*, 2003; Lemasson *et al.*, 2007). The *HBZ* transcript is reportedly detectable in all ATL cases without exception (Taylor& Matsuoka, 2005). Importantly, it has been reported that downregulation of HBZ results in inhibition of ATL cell growth, and that conversely, the expression of HBZ in human T-cell lines promotes their proliferation (Satou *et al.*, 2006). These data strongly suggest that HBZ plays an important role in the proliferation of ATL cells, and might be a universal target of novel therapy for ATL. On the basis of this concept, we attempted to verify the feasibility of cellular immunotherapy for ATL targeting HBZ.

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was provided by all patients. HTLV-1-infected cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and with or without 10 U IL-2 ml^{-1}. Four 9 aa peptides derived from the HBZ

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sequence, which were predicted to bind with high affinity to the HLA-A*0201 molecule, were designed by computer algorithms available at the BioInformatics & Molecular Analysis Section (BIMAS) website (http://www-bimas.cit.-nih.gov/molbio/hla_bind/) and SYFPEITHI website (http://www.syfpeithi.de/home.htm), as described previously (Suemori *et al.*, 2008). The amino acid sequences of the synthetic peptides were LVEELVDGL (HBZ_{19-27}), GLLSLEEEL (HBZ_{26-34}), AVLDGLLSL (HBZ_{42-50}) and KLLQEKEDL ($HBZ_{181-189}$), and their binding affinities for the HLA-A*0201 molecule were evaluated by an HLA stabilization assay, as reported previously (Kuzushima *et al.*, 2001). We attempted to generate HBZ peptide-specific CTLs from HLA-*A0201-positive individuals by stimulating $CD8^+$ T lymphocytes with peptide-loaded autologous dendritic cells, as reported previously (Ohminami *et al.*, 2000). The epitope specificity, HLA restriction, and cytotoxic activity of the induced CTLs were determined by standard ^{51}Cr-release assays, as reported previously (Yasukawa *et al.*, 1999; Suemori *et al.*, 2008).

Using TaqMan assay reagent target kits (Applied Biosystems), quantitative real-time PCR (QRT-PCR) for *HBZ* mRNA and *tax* mRNA was performed in accordance with the manufacturer's instructions. Expression levels of HBZ protein were determined by Western blotting using anti-HBZ serum, which was produced by immunizing rabbits with purified six-His-tagged HBZ polypeptide corresponding to the bZIP domain of HBZ, as reported previously (Gaudray *et al.*, 2002). Tetramer assays were performed by flow cytometry with a tetramer comprising HBZ_{26-34} peptide and HLA-A*0201 molecule with genetically altered $\alpha3$ domain, and Tax_{11-19} (LLFGYPVYV) peptide and HLA-A*0201 molecules (Medical & Biological Laboratories), as reported previously (Azuma *et al.*, 2004).

Among the four synthetic HBZ peptides, HBZ_{26-34}, HBZ_{42-50}, and $HBZ_{181-189}$ appeared to bind strongly to the HLA-A*0201 molecule (data not shown). We tried to generate HBZ-specific CTLs by stimulating peripheral blood lymphocytes with these peptides. Consequently, an HBZ_{26-34} peptide-specific CTL line, designated HBZ-1, was established from an HLA-A*0201-positive individual. HBZ-1 was able to lyse an autologous B-lymphoblastoid cell line (LCL) loaded with a low concentration (10^{-4} μmol l^{-1}) of HBZ peptide (Fig. 1a), demonstrating that HBZ-1 might express the high-affinity T-cell receptor (TCR). Moreover, direct sequencing of the HBZ-1 *TCR β-chain* gene revealed that the HBZ-1 cell line appeared to carry Vβ10-3*01/D1*01/J1-5*01. In addition, all HBZ-1 cells were brightly stained with HLA-A*0201/HBZ_{26-34} tetramer (Fig. 1b), indicating that this cell line is a CTL clone derived from a single HBZ_{26-34}-specific T cell. As shown in Fig. 1(c), HBZ-1 exhibited cytotoxicity against an HBZ_{26-34} peptide-loaded but not peptide-unloaded autologous B-LCL, HLA-A*0201-positive allogeneic LCL, and an *HLA-A*0201* gene-transduced C1R cell line (C1R-A*0201: kindly provided by Dr A. John Barrett). HBZ-1 did not show any cytotoxicity against HBZ_{26-34} peptide-loaded HLA-A2-negative allogeneic LCLs. Autologous LCLs loaded with other HLA-A*0201-binding peptides were not lysed by HBZ-1 (data not shown). The CD107a assay demonstrated that HBZ-1 showed granule exocytosis upon recognition of HBZ peptide-loaded but not HIV-gag peptide-loaded HLA-A*0201-positive B-LCL (Fig. 1d). In addition, the cytotoxicity of HBZ-1 against HBZ peptide-loaded B-LCL appeared to be significantly abrogated by the Ca^{2+}-chelating agent EGTA and an inhibitor of vacuolar-type H^+-ATPase, concanamycin A (data not shown). These data strongly suggest that the cytotoxicity of HBZ-1 is mediated through the granule exocytosis pathway.

Mean expression levels of *HBZ* mRNA and *tax* mRNA in HTLV-1 infected cell lines (n=8), freshly isolated ATL cells (n=4), and peripheral blood mononuclear cells (PBMCs) of HTLV-1 carriers (n=4) were measured by QRT-PCR. As reported previously (Satou *et al.*, 2006), *HBZ* mRNA was detected in all HTLV-1-infected cell lines and freshly isolated ATL cells examined, and *tax* mRNA was detected in all HTLV-1-infected cell lines and half of the ATL cases (data not shown).

Next, we examined the cytotoxicity of HBZ-1 against HTLV-1-infected cells. Unexpectedly, HBZ-1 failed to lyse HLA-A*0201-positive HTLV-1-infected cell lines or freshly isolated ATL cells (Fig. 2a). We further examined whether HBZ is an immunogenic protein that can be processed within cells, and whether HBZ-derived peptides can be presented on the cell surface in context with the HLA class I molecule. To address this issue, the *HBZ* gene was transfected into *HLA-A*0201* gene-transfected K562 (K562-A*0201: kindly provided by Dr Marieke Griffioen) and C1R (C1R-A*0201) cell lines and their susceptibility to HBZ-1-mediated cytotoxicity was examined. As shown in Fig. 2(a), HBZ-1 exerted cytotoxicity against the *HBZ* gene-transfected K562-A*0201 and C1R-A*0201 cell lines. HBZ-1 did not show any cytotoxicity against empty vector-transfected cell lines. These data indicate that HBZ_{26-34} peptide can be produced by processing HBZ protein in the cells, and can be expressed on the cell surface in context with HLA-A*0201 molecules.

We further addressed the issue of why HTLV-1-infected HLA-A*0201-positive cells cannot be recognized by HBZ-specific CTLs even though *HBZ* mRNA is expressed in HTLV-1-infected cells. Western blotting for HBZ protein expression in various cells is shown in Fig. 2(b). As expected, HBZ protein was detected in abundance in *HBZ* gene-transfected K562-A*0201 and C1R-A*0201 cell lines. In contrast, HBZ protein was scarcely detectable in HTLV-1-infected cell lines and freshly isolated ATL cells. These findings strongly suggest that the amount of HBZ protein produced in HTLV-1-infected T lymphocytes is insufficient for recognition by HBZ-specific CTLs. Interestingly, we found that there was no correlation between the levels of expression of *HBZ* mRNA and HBZ protein in the samples. These data suggest that the efficiency of *HBZ* mRNA translation into HBZ protein depends on cell type, and that

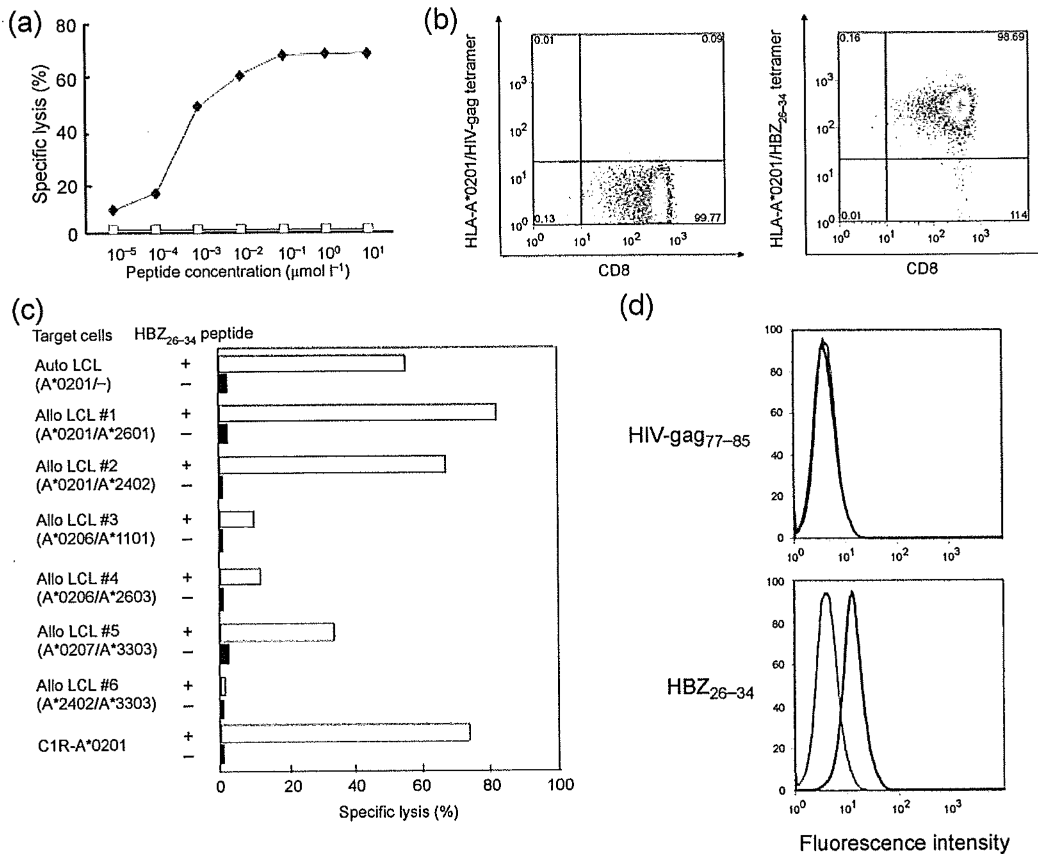

Fig. 1. Establishment of an HLA-A*0201-restricted and HBZ_{26-34} peptide-specific CTL line, HBZ-1. (a) HBZ peptide-specific and concentration-dependent cytotoxicity of HBZ-1. HBZ_{26-34}-specific CTLs were generated and their cytotoxicity against HBZ_{26-34} peptide-loaded autologous B-LCL (◆) and HBZ_{26-34} peptide-loaded HLA-A*0201-negative allogeneic B-LCL (□) was determined by ^{51}Cr-release assays at an effector : target cell (E : T) ratio of 5 : 1. Target cells were preincubated with and without HBZ_{26-34} peptide at various concentrations for 1 h. (b) Tetramer assay for HBZ-1 cell line. HBZ-1 cells were stained with HLA-A*0201/HBZ_{26-34} tetramer, but not with HLA-A*0201/HIV-gag_{77-85} tetramer. (c) Cytotoxicity of HBZ-1 against various cells. The cytotoxicity of the CTL line designated HBZ-1 against various LCLs and *HLA-A*0201* gene-transfected cells (C1R-A*0201), which were loaded or unloaded with HBZ_{26-34} peptide, was determined by 4 h ^{51}Cr-release assays at an E : T ratio of 5 : 1. Experiments were performed three times and representative data are shown. (d) Granular exocytosis of HBZ-1 upon antigen recognition. Granular exocytosis of HBZ-1 upon target antigen recognition was determined by CD107a assay. Granular exocytosis of HBZ-1 cells was detected when they were stimulated with HBZ_{26-34} peptide-loaded, but not irrelevant HLA-A*0201-binding HIV-gag_{77-85} peptide-loaded autologous B-LCL. Grey zones show negative control without peptide.

HBZ mRNA might be inefficiently translated in T lymphocytes.

Previous reports have shown that mature T lymphocytes are relatively resistant to CTL-mediated cytotoxicity in comparison with other cell types (Jiang *et al.*, 1990; Muller & Tschopp, 1994). We have also reported that the sensitivity of various kinds of tumour to tumour-associated antigen-specific CTLs differs (Azuma *et al.*, 2004). These findings led us to investigate whether HTLV-1-infected T lymphocytes are resistant to cytotoxicity mediated by HBZ-specific CTLs. To address this question, we compared the cytotoxic activities of HBZ-1 against HBZ peptide-loaded B-LCL and HTLV-1-transformed T-cell lines. As shown in Fig. 3, HBZ-1 was not cytotoxic to HTLV-1-transformed T-cell lines loaded with HBZ peptide at low concentrations; however, at these low concentrations, autologous B-LCL was lysed in a dose-dependent manner. At high HBZ peptide concentrations, HLA-A*0201-positive HTLV-1-transformed T-cell lines were lysed by HBZ-1, although cytotoxic activity against HBZ peptide-loaded B-LCL was higher than that against HBZ peptide-loaded T-cell lines.

In this study, we succeeded for the first time in establishing an HBZ peptide-specific CTL clone, and a detailed study using this CTL clone and *HBZ* gene-transfected cells clearly revealed that HBZ_{26-34} is an immunogenic epitope

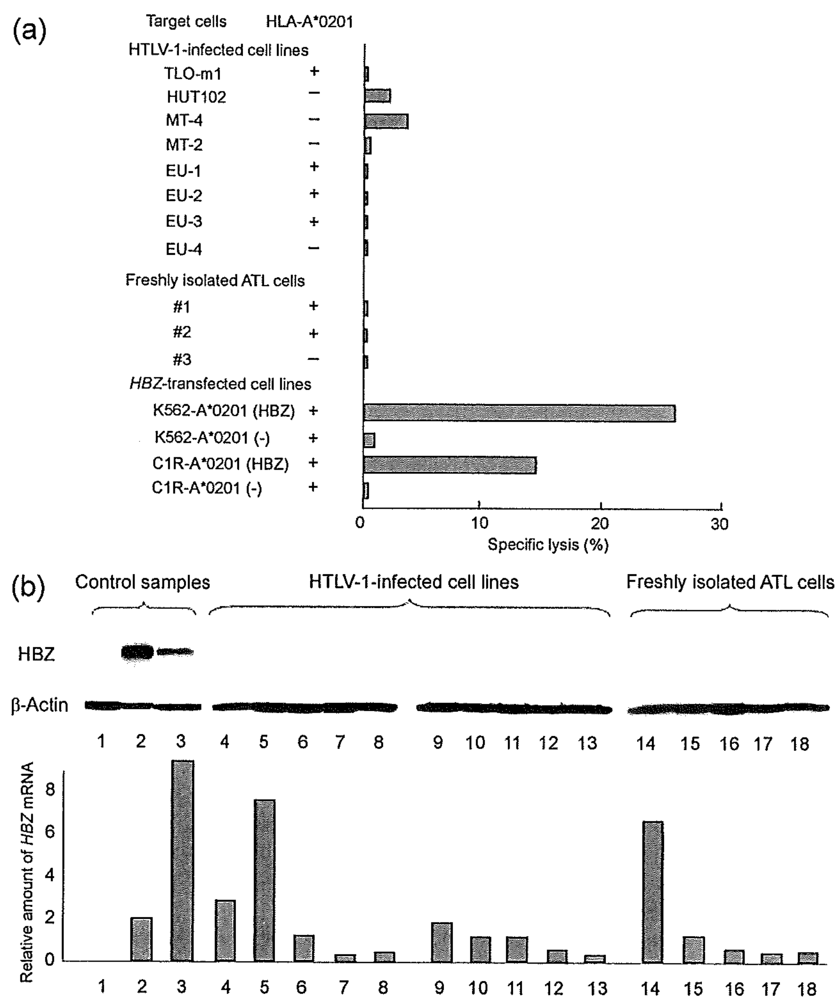

Fig. 2. Susceptibility of ATL cells and HTLV-1-infected cells to HBZ-1-mediated cytotoxicity and expression of *HBZ* mRNA and protein in various cells. (a) Cytotoxicity of the HBZ_{26-34} peptide-specific CTL line HBZ-1 against various cells. The cytotoxicity of HBZ-1 to HLA-A*0201-positive and HLA-A*0201-negative HTLV-1-infected cell lines, freshly isolated ATL cells and *HBZ* gene-transfected and -untransfected K562-A*0201 and C1R-A*0201 cell lines was determined by 4 h ^{51}Cr-release assays at an E : T ratio of 10 : 1. Experiments were performed three times and representative data are shown. (b) Expression of *HBZ* mRNA and protein in leukaemia cell lines, freshly isolated ATL cells, and normal PBMCs. HBZ protein expression was examined by Western blotting using anti-HBZ antibody and anti-β-actin antibody as the control. Expression levels of *HBZ* mRNA in the cells were determined by QRT-PCR. The expression level of *HBZ* mRNA in MT-4 is shown as 1.0 and the expression levels in samples were calculated relative to this value. 1, PBMCs; 2, *HBZ*-transfected K562-A*0201; 3, *HBZ*-transfected C1R-A*0201; 4, MT-1; 5, MT-2; 6, MT-4; 7, TL-MAT; 8, TLO-m1; 9, EU-1; 10, EU-2; 11, EU-3; 12, EU-4; 13, EU-5; 14, ATL#1; 15, ATL#2; 16, ATL#3; 17, ATL#4; 18, ATL#5.

recognizable by HLA-A*0201-restricted CTLs. However, HBZ-specific CTLs could not lyse HTLV-1-infected cells. In addition, cytokine production by HBZ-specific CTLs in response to stimulation with HTLV-1-infected cells in an HLA-restricted manner could not be detected (data not shown). The hypothesis that downregulation of HLA class I molecules on HTLV-1-infected cells is the cause of unsuccessful recognition of HTLV-1-infected cells by HBZ-1 seems unlikely, because flow cytometry showed strong expression of HLA class I molecules on HTLV-1-infected cells, and HBZ_{26-34} peptide-loaded HLA-A*0201-positive HTLV-1-infected cells were efficiently lysed by HBZ-1 (data not shown).

Because *HBZ* gene-transfected cells abundantly expressing HBZ protein were lysed by HBZ-specific CTLs in an HLA-A*0201-restricted manner, and Western blotting revealed a very low level of HBZ protein expression in HTLV-1-infected cells, we concluded that HBZ protein can certainly be processed in the cells and presented in context with the HLA-A*0201 molecule; however, HBZ-specific CTLs cannot discriminate HTLV-1-infected from HTLV-1-uninfected cells due to the small amount of HBZ protein in the former. Furthermore, HLA-A*0201/HBZ_{26-34} tetramer analysis of freshly isolated PBMCs from HLA-A*0201-positive ATL patients ($n=5$) and a HLA-A*0201-positive HTLV-1 carrier ($n=1$) revealed that HBZ-specific CTLs were scarcely detectable in HTLV-1-infected individuals (data not shown). Because PBMCs from HLA-A*0201-positive HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients were not available, tetramer assays for HBZ-specific T-cell immune response in HAM/TSP could not be performed. Although the possibility that HBZ protein could be presented by HTLV-1-infected T lymphocytes of HAM/TSP patients cannot be excluded, these data strongly support our interpretation that HBZ protein cannot be presented by T lymphocytes naturally infected with HTLV-1. Although *HBZ* mRNA is expressed in all ATL cases, and previous studies using overexpression and gene silencing methods have clearly demonstrated the important role of *HBZ* mRNA in proliferation of ATL cells (Satou *et al.*, 2006), the detailed characteristics and functional role of HBZ protein in leukaemogenesis and progression of HTLV-1-infected cells

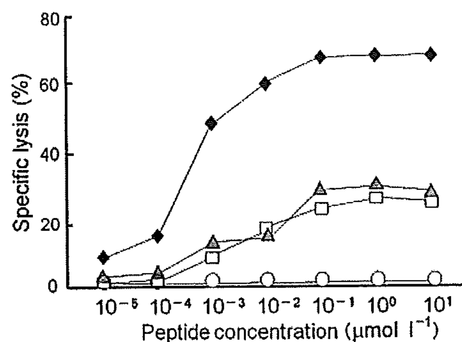

Fig. 3. Effect of HBZ peptide concentration on cytotoxicity of HBZ-1 against B-LCL and HTLV-1-transformed T-cell lines. The cytotoxicity of HBZ-1 against autologous B-LCL (◆), autologous HTLV-1-transformed $CD4^+$ T-cell line (□), HLA-A*0201-positive allogeneic HTLV-1-transformed $CD4^+$ T-cell line (△), and HLA-A*0201-negative allogeneic HTLV-1-transformed $CD4^+$ T-cell line (○), loaded with various concentrations of HBZ peptide for 1 h was determined by 4 h ^{51}Cr release assays at an E : T ratio of 5 : 1. Experiments were performed three times and representative data are shown. The cytotoxic activities of HBZ-1 against HBZ peptide-loaded autologous and HLA-A*0201-positive allogeneic HTLV-1-transformed $CD4^+$ T-cell lines are significantly lower than that against HBZ peptide-loaded autologous B-LCL ($P<0.01$ by paired sample *t*-test).

are still obscure. Notably, we also found that the expression levels of *HBZ* mRNA and HBZ protein in HTLV-1-infected cells were not parallel. This suggests that the machinery for translation of *HBZ* mRNA and/or the degradation pathway of HBZ protein may differ, and that the degree of this difference may be determined by cell type.

Another interesting finding of this study was that HTLV-1-infected T lymphocytes were relatively resistant to CTL-mediated cytotoxicity, compared with B-LCLs. We have previously reported that myeloma cells are more sensitive to the perforin-mediated granule exocytosis pathway of CTLs than lymphoma cells, and that susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity (Azuma *et al.*, 2004). Resistance to perforin-mediated cytotoxicity, possibly induced by membrane-stabilizing mechanisms, has been demonstrated in human cytomegalovirus-infected fibroblasts (Odeberg *et al.*, 2003). In addition, it has been reported that the human leukaemia cell line ML-2 can be recognized by natural killer (NK) cells but is resistant to NK cell-mediated cytotoxicity because of a defect in perforin binding (Lehmann *et al.*, 2000). Some molecules, including cathepsin B (Balaji *et al.*, 2002) and PI-9 (Bird *et al.*, 1998), have been proposed to play an important role in protection of target cells from CTL-mediated cytotoxicity. Although there has been no obvious evidence in the relationship between the resistance of HTLV-1-infected T lymphocytes to CTL-mediated cytotoxicity and cathepsin B or PI-9, further studies focusing on these molecules seem to be needed to clarify the mechanism underlying the resistance of HTLV-1-infected T lymphocytes to CTL-mediated cytotoxicity.

In summary, we conclude that HBZ is certainly immunogenic for CTLs, but that ATL cells cannot be lysed by HBZ-specific CTLs. Although further clarification of the mechanism underlying the resistance of HTLV-1-infected T lymphocytes to CTLs is needed, our present data strongly suggest the presence of a novel mechanism that allows HTLV-1 to evade immune recognition.

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