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
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 VLVEILDGL (HBZ19–27), GLLSEEL (HBZ26–34), AVLDDLSSL (HBZ42–50) and KLLQEKEDL (HBZ181–189), 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 51Cr-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 according to the manufacturer's instructions. Expression levels of HBZ 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 Ca2+-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=1), 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 HBZ26–34 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
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 tumor to tumor-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. 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 51Cr-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 51Cr-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.
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 by HBZ-1. 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/HTLV-1-infected T lymphocytes of 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...
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 (Azuza 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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