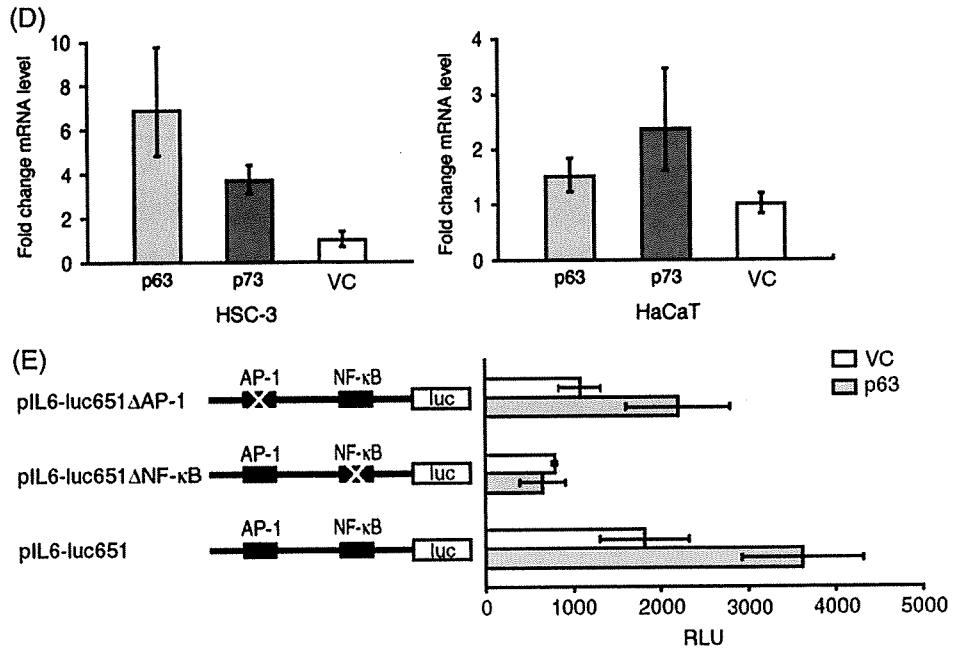

Figure 5. Continued

Numerous studies of the functional significance of p53-related factors have been mainly reported from the viewpoint of carcinogenesis, but the functions of p53-related factors in the immune system have not been fully investigated. In this study, we demonstrated the possible involvement of p53-related factors in the expression of IL-6 by TCECs. IL-6 of epithelial cells can be activated through the NF-κB axis by p63, which controls the delicate balance of proliferation and differentiation of keratinocytes as a determinant of epithelial stem cells [26]. Many NF-κB pathways can be affected by the p53 family, suggesting that other cytokines or biological factors that we did not investigate in this study might be related to the development of lymphoid follicles within CLGs of specialized LES of PPP tonsils. Furthermore, p63 itself may also be associated with the formation of LES of PPP tonsils, as suggested by the induction of epithelial cell hyperplasia when p63 is overexpressed [27]. It is known that p73-deficient mice exhibit generalized gastrointestinal mucositis without any clear dysfunction of leukocytes [28]. p73 is also suggested to have an important role in epithelial cell migration during wound healing and it might have a role in the natural mucosal defence mechanism [29]. Thus, p63 and p73 of TCECs in PPP tonsils may participate not only in the production of IL-6 to stimulate B cells, but also in cellular organogenesis of the TCEC meshwork of LES under inflammatory conditions.

Focal infection is a clinical entity characterized by a limited inflammatory process causing immune-related disorders. When tonsils are affected, various types of autoimmune diseases are elicited with particular symptoms and signs, including PPP, IgAN, and sternocostoclavicular hyperostosis [30,31]. Like the intestinal mucosa, tonsillar epithelia penetrate into the lymphoid stroma and form crypts as a mucosa-associated lymphoid tissue. Tonsil-related focal infection has been discussed as a disorder of mucosal immunity, usually based on its chronological sequence with initial and chronic phases [32]. In this regard, CLG may possibly be thought of as part of a chronic phase supporting the production of autoantibodies in patients with PPP. On the other hand, the initial event that is a prerequisite for the chronic phase in the PPP tonsil is still unknown. To address this issue, it is probably useful to refer to cases of molecular mimicry such as *Helicobacter pylori* infection of the stomach leading to chronic gastritis [33–35]. Possible linkage of a certain bacterial-cell moiety to a self-antigen specific to palmoplantar epithelium might be associated with the humoral immune response of PPP as in psoriasis vulgaris [36].

Our study indicates that dysregulated production of IL-6 by TCECs might stimulate B cells and induce abnormal secretion of the autoantibody in the PPP tonsil. Recently, an IL-6 receptor antagonist was reported to be effective for the treatment of autoimmune disorders such as rheumatoid arthritis [37]. Considering this, together with the pathophysiological similarity between PPP and rheumatoid arthritis as IL-6-mediated diseases, blockage of IL-6 signalling could become a new candidate for the treatment of PPP.

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Supplementary material

Supplementary material may be found at the web address http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2266.html

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Antigenic Peptide Vaccination: Provoking Immune Response and Clinical Benefit for Cancer

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Abstract: Recent immunotherapy depends largely on understanding of the molecular interactions between T cell receptors (TCR) on cytotoxic T lymphocytes (CTL) and peptide/MHC class I complexes on tumor cells. Many tumor antigens identified by cDNA library expression cloning methods, especially from malignant melanoma, have greatly contributed to clarifying such mechanisms and led to peptide vaccination trials, mainly for patients with melanoma. Although the objective tumor regression rate mediated by peptide vaccination is still low compared to adoptive cell transfer therapy, antigenic peptide vaccination can cause a constant objective response generally evaluated as stable disease or decreased serum levels of tumor markers. In addition, recent trials in the adjuvant setting showed some suppressive effects against recurrence. Therefore, peptide vaccination still has potential for clinical benefits in patients with various cancers. For further improvement of peptide vaccination, we considered that (i) novel antigenic peptides, (ii) effective adjuvants, (iii) more sensitive immunological monitoring and (iv) drugs up-regulating HLA class I molecules might be important.

Keywords: Peptide vaccination, tumor-associated antigen, CTL epitope, clinical trial.

INTRODUCTION

Recent immunotherapy depends largely on understanding of the molecular interactions between T cell receptors (TCR) on cytotoxic T lymphocytes (CTL) and peptide/MHC class I complexes on tumor cells. Many tumor antigens identified by cDNA library expression cloning, especially from malignant melanoma, have greatly contributed to clarifying such mechanisms and made feasible vaccinations, mainly for patients with melanoma. Various vaccination approaches, including those with antigenic peptides [1], recombinant viruses encoding antigenic genes [2], dendritic cells and antigenic proteins [3] were reported. Recent adoptive transfer of *ex vivo* expanded autologous tumor-infiltrating lymphocytes following chemotherapeutic lymphodepletion combined with total body irradiation [4] and adoptive transfer of T lymphocytes in which antigen-specific TCR is genetically engineered [5] resulted in strong clinical responses. Nevertheless, we are still focusing on peptide-based vaccination and have identified novel antigenic peptides by forward and reverse immunological approaches. In this review, we describe the recent status of the field of peptide-based vaccination immunotherapy and future perspectives on the basis of our work.

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IDENTIFICATION OF TUMOR ANTIGENS FOR PEPTIDE VACCINATION

Many tumor-associated antigenic genes and peptides recognized by CTLs have been identified since 1991 when the first CTL-defined tumor antigen, *MAGE*, was found [6]. Mainly in melanoma studies, tumor antigens were cloned by cDNA library expression cloning using CTL lines reacting with autologous tumor cells. This strategy is called the 'forward immunological approach.' The forward immunological approach can detect 'true' antigens naturally priming the cellular immune system of the patient. However, especially in non-melanocytic tumors, the establishment of autologous pairs of tumor cell-CTL lines is very difficult [7]. On the other hand, recent many antigenic tumor genes were screened by 'the reverse immunological approach', on the basis of the tumor-specific expression profiles obtained from cDNA microarrays and various bioinformatics databases, followed by *in vitro* stimulation of CTLs reacting with candidate antigen-derived peptides and natural tumor cells [8, 9]. This approach does not require a CTL line reacting with autologous tumor cells and makes feasible identification of tumor antigens associated with various cancers.

From melanoma studies, tumor antigens were categorized on the basis of their expression profiles in tumor tissues and normal organs into five groups: (i) cancer-testis antigens, (ii) melanoma-melanocyte differentiation antigens, (iii) mutated (unique) antigens, (iv) shared overexpression antigens and (v) ubiquitous antigens. This categorization is also adaptable

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for non-melanocytic tumors including antigens associated with epithelial cancer and sarcoma. The comprehensive database of CTL-defined tumor antigens and peptides in the context of HLA class I and class II is constantly updated by the Ludwig Institute for Cancer Research, Brussels Branch, Belgium (available at http://www.cancerimmunity.org/links/databases.htm). Considering the tumor-specific expression status of target antigens, CT antigens, differentiation antigens and overexpression antigens can be used as target molecules. We are still focusing on the identification of novel tumor antigens and antigenic peptides by forward and reverse immunological approaches [10]. Candidate tumor antigens and peptides we previously identified are shown in Table **1** [11-19].

CLINICAL STUDY: PEPTIDE VACCINATION AND ADOPTIVE CELL TRANSFER

Adoptive Cell Transfer: Strong Clinical Response

Since the first vaccination trial of a tumor-associated antigenic peptide in 1995 [20], much work on identification of CTL epitopes derived from tumor antigens has been conducted to promote clinical vaccination trials and immunomonitoring [1]. In the beginning, the immunological and clinical results suggested that peptide vaccination therapy was a promising modality against metastatic melanoma [21]. After one decade, Rosenberg *et al.* reviewed the past vaccination trials and concluded that this strategy could hardly mediate the objective response [22]. Although this pessimistic judgment of vaccination trials has been criticized [23, 24], general attention shifted from peptide vaccination to adoptive transfer. Adoptive tumor-infiltrating lymphocyte (TIL) transfer therapy, which started from 1996, reached an objective response of ≥50% in patients with metastatic melanoma in combination with lymphodepletion chemotherapy [25, 26]. This approach was augmented by total body irradiation for the further depletion of regulatory T cells and stimulation of innate immunity *via* Toll-like receptor (TLR) 4 [27, 28]. Moreover, adoptive transfer of T lymphocytes in which antigen-specific TCR was genetically engineered was performed in patients with metastatic melanoma [5]. This approach could be applicable for non-melanocytic cancer, for which there is limited availability of *ex vivo* expanded TIL [29]. Adoptive transfer of T lymphocytes activated *ex vivo* showed that adequate effector status of T cells is essential in addition to a sufficient number of T cells to kill the solid tumor mass. At present, adoptive effector cell transfer might be the most effective strategy mediating the objective regression of solid tumors graded by the RECIST criteria [30]. However, the adoptive cell transfer strategy still has the following limitations: (i) lymphodepletion chemotherapy can cause severe infectious disease and (ii) the requirement for special institutes meeting the criteria of the GMP grade for handling the T lymphocytes ex vivo limits the popularization of this approach.

Peptide Vaccination: Weak But Certain Clinical Response

On the other hand, peptide vaccination trials are still continuing and attempts have been made to trigger immunological responses and clinical responses. In addition to melanoma studies using the MAGE family and melanoma-melanocyte differentiation antigens (gp100, tyrosinase and Melan-A/MART-1), many tumor-associated antigens identified from non-melanocytic cancers were targeted to elicit T cell proliferation and activation (Table **2**) [31-47]. The target diseases for studies have also been expanded to non-melanocytic cancers. Although the precursor frequency of anti-vaccine CTLs in peripheral blood was still low for *in situ* detection using tetramers, cytokine ELISA and ELISPOT, anti-vaccine CTL responses *in vivo* were detected in many clinical studies including non-melanocytic cancers. The rate of objective tumor regression (CR or PR) was also estimated to be low, though antigenic vaccination could cause certain objective responses against disseminated cancers, including reduction of tumor masses, which was generally evaluated as SD, and reduction of serum tumor markers from the beginning of the vaccination trial [21]. Peptide vaccines have some advantages compared with adoptive T lymphocyte transfer therapy: (i) side effects more than grade 3

Table 1. Tumor-Associated Antigens and Candidate Peptides for Vaccination Trial

Antigen	Peptide	HLA	Disease	Vaccination Trial	Ref.
Forward Immunological Approach					
c98	YSWMDIITIC	A31	Gastric cancer		[11]
PBF	CTACRWKKACQR	B55	Osteosarcoma		[12]
	AYRPVSRNI	A24	Osteosarcoma	Planned	[13]
	ALPSFQIPV	A2	Osteosarcoma	Planned	[14]
Reverse Immunological Approach					
Survivin	AYACNTSTL	A24	Lung, gastric, colorectal, pancreatic and breast cancers	Ongoing	[15]
Livin	KWFPSCQFLL	A24	Lung cancer		[16]
Recoverin	QFQSIYAKFF	A24	Lung cancer		[17]
SYT-SSX	GYDQIMPKK	A24	Synovial sarcoma	Ongoing	[18]
	GYDQIMPKI*	A24	Synovial sarcoma	Ongoing	[19]

*Aggretope-substituted peptide.

Table 2. Phase I/II Clinial Trials of Antigenic Peptide Vaccination Since 2004

						Anti-Vaccine CTL Response		Clinical Response				
Target Antigen	Peptide Vaccine	HLA Restriction	Adjuvant	Disease	n	Method	Response	Criteria	Response	Adverse Effect ‡	Correlation†	Ref.
NY-ESO-1	SLLMWITQV* WITQCFLPVFLA QPPSGQRA	A2 DP4	IL-2	Melanoma	37	ELISA	100%	RECIST	PR; 3%	2%	ND	[31]
gp100	GRAMLGTHT MEVTV	A2, (DR53, DQ6)	IFA GM-CSF	Melanoma	28	Tetramer	57%	RECIST	SD; 4%	None	No	[32]
gp100	IMDQVPFSV	A2	IL-2	Melanoma	26	ELISPOT Tetramer	65% 31%	RECIST	SD; 31%	27%	No	[33]
hTERT	YLFFYRKSV * RLFFYRKSV	A2 A2	IFA	NSCLC	22	ELISPOT Pentamer	88% 90%	RECIST	SD; 36%	None	Yes	[34]
hTERT	YLFFYRKSV * RLFFYRKSV	A2 A2	IFA	Various	19	Tetramer	93%	WHO	SD; 21%	None	ND	[35]
WT1	CMTWNQMNL CYTWNQMNL*	A24	IFA	Various	26	Tetramer Intracellular FACS	50%	Tumor marker Number of blast cells	Reduction; 76%	12%	Yes	[36]
Survivin	AYACNTSTL	A24	None	Colorectal cancer	15	Tetramer	50%	RECIST Tumor marker	MR; 7% Reduction; 40%	None	ND	[37]
Survivin	AYACNTSTL	A24	IFA	Breast cancer	14	Tetramer ELISPOT	50%	RECIST	SD; 14%	None	ND	[38]
SYT-SSX	GYDQIMPKK	A24	None	Synovial sarcoma	6	Tetramer	50%	RECIST	SD; 17%	None	No	[39]
CA9	EYRALQLHL AYEQLLSRL RYFQYEGSL	A24	IFA	RCC	23	ELISA	76%	WHO	PR; 13% SD; 26%	None	Yes	[40]
Multiple (12 antigens)	Multiple (48 peptides)	A2, A24	IFA	RCC	10	ELISA	5%	RECIST	SD; 60%	None	ND	[41]
Multiple (9 antigens)	Multiple (16 peptides)	A24	IFA	Prostate cancer	16	ELISA	57%	Serum PSA level	Reduction; 100%	None	ND	[42]
Multiple (7 antigens)	Multiple (14 peptides)	A24	IFA	Prostate cancer	10	ELISA	50%	Serum PSA level	Reduction; 20%	None	No	[43]
Multiple (8 antigens)	Multiple (16 peptides)	A2	IFA	Prostate cancer	10	ELISA	40%	Serum PSA level	Reduction; 30%	None	No	[44]
Adjuvant setting												
NY-ESO-1	SLLMWITQC	A2	IFA	Ovarian cancer	9	Tetramer ELISPOT	78%	Recurrence-free rate at 22 months	33%	None	No	[45]
HER2/neu	KIFGSLAFL	A2, A3	GM-CSF	Breast cancer	186	Immuno-globlin dimer assay	ND	Recurrence-free rate at 20 months	Vaccinated group; 94.4% Non-vaccinated group; 85.8%	2%	ND	[46]
Multiple (6 antigens)	Multiple (4 peptides)	A1, A2, A3	IFA tetanus helper peptide GM-CSF	Melanoma	52	ELISPOT	87%	Overall survival at 24 months	89%	37%	Yes	[47]

*Aggretope-substituted peptide.
†Correlation between immunological response and clinical response.
‡The proportion of reactions scaled as more than grade 3, according to the National Cancer Institute Common Toxicity Criteria.

are merely observed and generally tolerable, (ii) there is no requirement for special institutes, and (iii) costs for manufacturing and vaccination are relatively low. Recent studies of adjuvant vaccination with MAGE3 protein increased the 5-year survival rate in patients with non-small-cell lung cancer (NSCLC) [48, 49]. In addition, peptide vaccination trials in the adjuvant setting were also performed [45-47]. These results have encouraged many researchers.

Peptide Vaccination: Current Problems

(i) Status of Circulating Anti-Vaccine CTLs: Function and Frequency

Discrepancies between immunological responses and clinical responses remain unsolved. With regard to the immunological aspect, we support the idea that thorough monitoring is still required to detect the immunological status provoked by vaccination and to improve the current vaccination strategy for the next generation [23, 24]. In cases in which anti-vaccine CTLs positively detected by tetramers, cytokine ELISPOT or ELISA could not mediate tumor regression, the functional status of CTLs *in vivo* was altered from effector-memory or memory to effector by manipulation with *in vitro* stimulation. Adoptive T lymphocyte transfer studies also supported the idea that adequate *ex-vivo* activated T cells could reject large tumor masses. Speiser *et al.* reported that *ex-vivo* five-cell PCR of sorted tetramer-positive cells from peripheral blood showed that cytokine profiles affecting the natural status were provoked by vaccination [50, 51].

On the other hand, immunosuppressive cells might affect the effector function of CTLs. Regulatory T cells (Treg) have been reported and reviewed in detail as the critical suppressive factor in peripheral blood and the tumor microenvironment in patients bearing cancer [52]. Several drugs depleting Treg, including denileukin diftitox (ONTAK), the anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibody and anti-folate receptor 4 antibody have been shown to have potential for the enhancement of anti-vaccine CTLs in *in vivo* studies [53-55]. Recently, myeloid-derived suppressor cells (MDSC) were focused on with regard to immune escape. In the peripheral lymphoid organs, MDSC present antigens to antigen-specific T lymphocytes and induce nitration of TCR and CD8 molecules on the T-lymphocyte surface. This results in conformational changes in these molecules and induces loss of their ability to bind to the peptide-MHC complex on tumor cells [56]. The blockade of peroxynitrite generation, which could induce nitration, might have the possibility to enhance the anti-tumor immunity.

Considering that antigenic peptides are derived from self-antigens, most anti-vaccine CTLs might have low- or moderate-affinity TCR because of clonal deletion of T lymphocytes reacting to self-antigens with high affinity TCR in the thymus, which is called central tolerance. Recently, Janicki *et al.* reported that CTLs having high affinity TCR could form tumor-infiltrating lymphocytes, although they lose effector function. Meanwhile, T lymphocytes recognizing self-antigens could become tolerant as a result of the conformational change of TCR modified by addition of inhibitory or removal of activating molecules [57]. This suggests that expansion of the anti-vaccine CTLs having adequate characteristics of TCR by active peptide vaccination is still difficult. However, we think that novel tumor antigens, epitopes and vaccination have some possibility to induce effective CTLs having such TCR. Adjuvants also might be able to alter the clonal diversity of TCR repertoire [58].

If anti-vaccine CTLs cannot be detected by standard monitoring procedures in spite of positive clinical responses, more sensitive procedures are required to detect them at extremely low frequencies. Limiting dilution (LD)/mixed lymphocyte peptide culture (MLPC) followed by tetramer-based frequency analysis is the most sensitive method now available [59-62]. Collected peripheral blood mononuclear cells (PBMCs) are stimulated with antigenic peptides *in vitro* under limiting dilution conditions (200,000 cells/well of 96-well microculture plates), followed by detection of tetramer-positive anti-vaccine CTLs. With many internal negative pools, the positive pools including tetramer-positive cells are carefully identified. This procedure could provide the sensitivity to detect anti-vaccine CTLs under the 10^{-7} level in non-vaccinated patients and healthy donors. Moreover, the sensitivity might be increased by increasing the amount of PBMCs. We analyzed the precursor frequency of CTLs against osteosarcoma antigen papillomavirus binding factor (PBF)-derived peptide in the context of HLA-A24 and A2 by LD/MLPC/tetramer analysis [13, 14]. Among non-vaccinated patients with osteosarcoma, the peripheral frequency of anti-PBF CTLs was detected at between $5x10^{-7}$-$7x10^{-6}$ and $2x10^{-7}$-$5x10^{-6}$ in HLA-A*2402-positive patients and HLA-A*0201-positive patients, respectively. In addition, the frequency of anti-PBF CTLs was detected at between $8x10^{-7}$-$5x10^{-6}$ and $1x10^{-7}$-$5x10^{-7}$ in HLA-A*2402-positive and HLA-A*0201-positive healthy donors, respectively (Tsukahara *et al.* unpublished observation 2008). However, this procedure requires intensive laboratory work [63, 64].

(ii) Status of Tumor Cells: The Loss of Antigens and HLA Class I Molecules

With regard to tumor biology, the problem of tumor escape after vaccination remains. Tumor escape results from the loss of antigens and the loss of antigen-presenting HLA class I molecules. The loss of antigens is easy to resolve by using multiple peptides or targeting molecules essential for tumor cell survival. We performed vaccination trials targeting the inhibitor of apoptosis protein survivin, which plays a key role in resistance to various apoptotic stimuli [15, 37, 38]. As described above, we consider that intensive laboratory work to identify novel tumor-associated antigens and related peptides is still required. The loss or down-regulation of HLA class I molecules is another classic but important problem. It is well known that tumor cells can lose HLA class I molecules on the cell surface and escape from immune pressure [65-67]. We observed that the loss or down-regulation of HLA class I molecules occurred in 100% and 45% of non-responders and responders to survivin-derived peptide vaccination, respectively (Torigoe *et al.* unpublished observation 2007). Although the sample size was very small, the expression of HLA class I was negative in 3 of 3 synovial sarcoma specimens. The propensity of synovial sarcoma cells to lose HLA class I may also serve as an obstacle for immunotherapeutic trials such as one we undertook using SYT-SSX fusion gene-derived peptide vaccine [39]. We also observed that epigenetic silencing of beta2-microglobulin was the key point to explain the loss or down-regulation of

HLA class I. Moreover, oral administration of the histone deacetylase inhibitor valproic acid caused retrieval of the HLA class I expression on xenograft tumors in mouse models (Torigoe *et al.* unpublished observation 2007). In addition, the correlation between the loss or down-regulation of HLA class I molecules and poor prognosis in renal cell cancer [68], NSCLC [69] and osteosarcoma [70] also supports the important role of HLA class I expression in the immune escape of various tumors.

Peptide Vaccination in the Future: Augmentation with TLR Agonists

To strengthen the vaccine-mediated immunological response, novel adjuvant drugs are highly desirable. Some candidates were already described above. On the basis of studies regarding TLR signaling in innate immunity, TLR agonists were introduced as adjuvants for the activation of antigen-presenting dendritic cells by vaccination. Many drugs, including TLR agonists, were reviewed and scored in the NCI Immunotherapy Workshop Proceedings (available at the NCI-Frederick web site; http://web.ncifcrf.gov/research/brb/workshops.asp). In addition to CpG (a TLR9 agonist) and poly I:C (a TLR3 agonist), monophosphoryl lipid-A (MPLA; a TLR4 agonist) was introduced as a novel adjuvant candidate. MPLA is a low-toxicity derivative of lipopolysaccharide (LPS; a component of the bacterial wall) and could trigger production of type I interferon (interferon-alpha and -beta) and T cell proliferation equal to LPS [71]. We used interferon-alpha as an adjuvant in peptide vaccination trials and found a strong immune response and clinical response (PR graded by RECIST) in one patient with recurrent pancreatic cancer (Iwayama *et al.* unpublished observation, 2007). Although it is still unclear what adjuvant is optimal to activate and expand anti-vaccine T lymphocytes, the finding of additional novel TLR agonists as adjuvants is anticipated.

OUR FUTURE PERSPECTIVES

Our further projects are composed of (i) a PBF-derived peptide vaccination trial for patients with osteosarcoma, and (ii) peptide vaccination with heat-shock protein as a novel adjuvant. As described above, without these further approaches, it seems to be difficult to enhance anti-vaccine CTLs having adequate TCR avidity and effector function. The adjuvant effects of TLR ligands, drugs depleting Treg and cytokines should be clinically assessed. Nevertheless, in the future, we believe that antigenic peptide vaccination with strong adjuvants will provoke immune responses and objective responses against cancer.

New Target: Osteosarcoma Antigen PBF

Osteosarcoma is a high-grade malignancy originating from mesenchymal cells. Before 1970, the 5-year survival rate of patients with osteosarcoma was less than 10%. To develop new treatment modalities, vaccination trials for osteosarcomas were initially conducted for patients with osteosarcoma during 1970s [72]. Surprisingly, autologous tumor lysate vaccination showed some effect to increase the survival rate [73]. However, during the same period, multidrug adjuvant chemotherapy including high dose methotrexate was demonstrated to raise the 5-year survival to 60-70% [74, 75]. Although vaccination could not outperform chemotherapy, its potential to trigger the host immune system and reject tumor cells conferring metastasis, especially in the adjuvant setting, is certainly present. As the first step, we identified osteosarcoma-associated antigen PBF using an autologous pair comprised of an osteosarcoma cell line and a CTL clone [12, 76]. PBF is a nuclear-cytoplasmic shuttling transcription factor that regulates apoptosis [77]. PBF protein was expressed in 92% of primary osteosarcoma tissues. Moreover, PBF-positive osteosarcomas conferred a poorer prognosis than those with negative expression of PBF [13]. Therefore, PBF might be a candidate target for peptide vaccination clinical trials. As the next step, we analyzed the frequency and function of anti-PBF CTLs in peripheral blood of patients with osteosarcoma [13, 14]. Among non-vaccinated patients with osteosarcoma, the peripheral frequency of anti-PBF CTLs was between $5x10^{-7}$-$7x10^{-6}$ and $2x10^{-7}$-$5x10^{-6}$ in HLA-A*2402-positive patients and HLA-A*0201-positive patients, respectively. The low frequency of anti-PBF CTLs might support the evidence that spontaneous regression of osteosarcoma is extremely rare [78, 79]. Now we are planning a phase I study of PBF-derived peptide vaccination with IFA or interferon-alpha in end-stage patients with osteosarcoma. Although strong objective clinical responses in many peptide vaccination trials for various cancers could hardly be observed, vaccination targeting a novel tumor-associated antigen PBF for osteosarcoma might have a certain possibility to induce some objective responses in addition to immunological responses. Considering the early study of vaccination with autologous tumor lysates [73], PBF-derived peptide vaccination trials in adjuvant or neoadjuvant settings seem attractive.

New Adjuvant: Heat-Shock Protein

As mentioned above, new adjuvants are expected to elicit strong immune responses. Activation of innate immunity in addition to acquired immunity against a vaccine might be essential to further increase efficacy. We focused on molecular chaperone heat-shock protein 90 (hsp90), which could elicit anti-tumor CTL responses in mouse models [80]. Our preclinical study demonstrated that DCs could take up the exogenous hsp90-peptide vaccine complex and present the peptide on DCs in the context of HLA class I molecules *via* a cross-presentation pathway. As a result, the hsp90-antigenic peptide complex could elicit anti-vaccine CTLs [81]. Moreover, hsp90 could induce the production of inflammatory cytokines (TNF-alpha, IL-1, IL-6 and IL-12) *via* TLR-2 and -4 signaling pathways [82]. Therefore, hsp90 might be promising for an adjuvant effect in the peptide vaccination strategy.

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Prognostic impact and immunogenicity of a novel osteosarcoma antigen, papillomavirus binding factor, in patients with osteosarcoma

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To develop peptide-based immunotherapy for osteosarcoma, we previously identified papillomavirus binding factor (PBF) as a cytotoxic T lymphocytes (CTL)-defined osteosarcoma antigen in the context of human leukocyte antigen (HLA)-B55. In the present study, we analyzed the distribution profile of PBF in 83 biopsy specimens of osteosarcomas and also the prognostic impact of PBF expression in 78 patients with osteosarcoma who had completed the standard treatment protocols. Next, we determined the antigenic peptides from PBF that react with peripheral T lymphocytes of HLA-A24$^+$ patients with osteosarcoma. Immunohistochemical analysis revealed that 92% of biopsy specimens of osteosarcoma expressed PBF. PBF-positive osteosarcoma conferred significantly poorer prognosis than those with negative expression of PBF ($P = 0.025$). In accordance with the Bioinformatics and Molecular Analysis Section score, we synthesized 10 peptides from the PBF sequence. Subsequent screening with an HLA class I stabilization assay revealed that peptide PBF A24.2 had the highest affinity to HLA-A24. CD8$^+$ T cells reacting with a PBF A24.2 peptide were detected in eight of nine HLA-A24-positive patients with osteosarcoma at the frequency from 5×10^{-7} to 7×10^{-6} using limiting dilution/mixed lymphocyte peptide culture followed by tetramer-based frequency analysis. PBF A24.2 peptide induced CTL lines from an HLA-A24-positive patient, which specifically killed an osteosarcoma cell line that expresses both PBF and HLA-A24. These findings suggested prognostic significance and immunodominancy of PBF in patients with osteosarcoma. PBF is the candidate target for immunotherapy in patients with osteosarcoma. (*Cancer Sci* 2008; 99: 368–375)

Osteosarcoma is the most common primary malignant tumor of bone. The past three decades have witnessed remarkable advances in the treatment of osteosarcoma. These include the introduction of adjuvant chemotherapy, establishment of guidelines for adequate surgical margins, and the development of postexcision reconstruction.[1,2] There have also been advances in the field of immunotherapy for osteosarcoma that, unfortunately, have received less attention.[3,4] However, the current stagnation in chemotherapy-based treatments for osteosarcoma has reignited interest in immunotherapeutic approaches.[5,6]

Recent immunotherapy depends largely on understanding of the molecular interactions between T cell receptors (TCR) on cytotoxic T lymphocytes (CTL) and antigenic peptides on tumor cells. This has led to a variety of vaccination approaches, including those with antigenic peptides[7] recombinant viruses encoding antigenic genes[8] dendritic cells[9] and T lymphocytes, in which the TCR recognizing an antigenic peptide is genetically engineered.[10] Nevertheless, such immunotherapeutic approaches were hampered in osteosarcoma by a lack of defined antigens until we recently identified papillomavirus binding factor (PBF) using an osteosarcoma cell line and an autologous CTL clone restricted by human leukocyte antigen (HLA)-B*5502.[11,12] PBF is a DNA-binding transcription factor with unknown function.[13,14] The oncogenic role of PBF in osteosarcoma and its immunogenicity in patients with common HLA alleles such as HLA-A2 and HLA-A24 needs to be disclosed before development of clinically applicable PBF-based immunotherapy.

In the present study, we analyzed the distribution profile, prognostic impact, and immunogenicity of PBF in patients with osteosarcoma. Immunogenicity analysis focused on frequency and cytotoxicity of T cells in patients with HLA-A24 allele by using limiting dilution (LD)/mixed lymphocyte peptide culture (MLPC)/tetramer assays.

Materials and Methods

This study was approved under institutional guidelines for the use of human subjects in research. The patients and their families as well as healthy donors gave informed consent for the use of blood samples and tissue specimens in our research.

Generation of anti-PBF antibody. A polyclonal antibody against PBF was generated by immunizing rabbits with 100 μg of a 15-mer peptide, CGDTVDSDQFKREED, once per week for six weeks (SigmaGenosys, Sapporo, Japan). The serum was collected seven days after the last immunization and purified using Protein A column. The specificity of the anti-PBF antibody was confirmed previously by Western blotting and immunostaining.[12]

Immunohistochemistry. Formalin-fixed paraffin-embedded sections were obtained from 83 biopsy specimens of the primary lesion of osteosarcoma (Table 1). The sections were deparaffinized, boiled in a microwave oven, and blocked with 1% non-fat dry milk before staining with streptavidin-biotin-complex (Nichirei, Tokyo, Japan) as previously described.[15] Hematoxylin was used for counter staining. The reactivity of the anti-PBF antibody was determined by staining of the nuclei of tumor cells.[12] The expression status of PBF was graded semiquantitatively according to the modified classification described by Al-Batran *et al.*[16,17] negative (positive cells <5%), low (≤5% positive cells ≤50%), and high (positive cells >50%) (Fig. 1). Diffuse expression and heterogeneous expression were regarded as high grade and low grade, respectively. Focal expression was graded as low or negative according to the percentage of positive cells.

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Table 1. The grade of PBF expressioni and the progonosis in 83 patients with osteosarcoma

Patients	Age	Gender	Location	Surgical stage	Histological type	Chemotherapy	Histological response grade	Operation	PBF expression	EFS (months)	OS (months)	Prognosis
1	15	M	Femur	IIB	Osteoblastic	T-12	2	Amputation	High	39	46	DOD
2	20	M	Humerus	IIB	Osteoblastic	T-12	0	Amputation	Low	12	24	DOD
3	9	F	Femur	IIB	Chondroblastic	T-12	0	WE+FVFG	High	14	22	DOD
4	12	M	Femur	IIB	Osteoblastic	T-12	0	Amputation	Low	3	9	DOD
5	10	F	Humerus	IIB	Teleangiectatic	T-12	1	WE+FVFG	High	103	103	CDF
6	17	F	Humerus	IIB	Osteoblastic	T-12	2	WE+FVFG	Low	97	97	CDF
7	14	F	Femur	IIB	Fibroblastic	NSH-7	2	WE+FVFG	Negative	119	119	CDF
8	14	M	Fibula	IIB	Chondroblastic	NSH-7	2	WE	High	34	102	NED
9	11	F	Tibia	IIB	Osteoblastic	NSH-7	2	WE+FVFG	Negative	117	117	CDF
10	42	F	Tibia	IIB	Osteoblastic	NSH-7	2	WE+Prosthesis	High	72	72	CDF
11	14	M	Tibia	IIB	Osteoblastic	NSH-7	3	WE+FVFG	High	32	96	NED
12	18	M	2nd rib	IIB	Osteoblastic	NSH-7	0	WE	High	106	106	CDF
13	33	M	Pelvis	IIB	Chondroblastic	NECO93J	1	WE+FVFG	High	8	13	DOD
14	20	M	Femur	IIB	Fibroblastic	NECO93J	2	WE+FVFG	High	72	72	CDF
15	15	M	Femur	IIB	Osteoblastic	NECO93J	2	WE+FVFG	High	106	106	CDF
16	15	M	Femur	IIB	Osteoblastic	NECO93J	2	WE+FVFG	High	20	33	DOD
17	20	F	Femur	IIB	Osteoblastic	Not done†	(–)†	Not done†	Negative	0	7	DOD
18	16	M	Tibia	IIB	Chondroblastic	NECO93J	2	WE+FVFG	High	6	6	DOC†
19	14	F	Femur	IIB	Osteoblastic	NECO93J	2	WE	Negative	5	5	DOC†
20	15	M	Fibula	IIB	Osteoblastic	NECO93J	0	Amputation	High	13	74	DOD
21	13	M	Humerus	IIB	Chondroblastic	NECO93J	0	Amputation	High	7	9	DOD
22	7	F	Femur	IIB	Osteoblastic	NECO95J	1	WE+FVFG	High	85	85	CDF
23	10	F	Tibia	IIB	Osteoblastic	NECO95J	2	Amputation	Negative	72	72	CDF
24	13	M	Femur	IIB	Osteoblastic	NECO95J	0	WE+RP	Low	6	11	DOD
25	27	M	Tibia	IIB	Osteoblastic	NECO95J	0	WE+FVFG	High	8	18	DOD
26	18	F	Femur	IIIB	Fibroblastic	NECO95J	1	WE+Prosthesis	High	0	16	DOD
27	20	F	Humerus	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	Negative	67	67	CDF
28	69	F	Femur	IIB	Fibroblastic	NECO95J	0	WE+Prosthesis	Low	62	63	NED
29	15	F	Femur	IIB	Osteoblastic	NECO95J	3	WE+FVFG	High	32	32	CDF
30	46	M	Pelvis	IIB	Fibroblastic	NECO95J	0	WE+Fillet	Low	47	60	NED
31	40	F	Femur	IIB	Fibroblastic	NECO95J	2	Amputation	Low	12	20	DOD
32	19	M	Tibia	IIB	Osteoblastic	NECO95J	2	Amputation	High	41	43	DOD
33	15	F	Femur	IIB	Fibroblastic	NECO95J	3	WE+FVFG	High	52	52	CDF
34	48	M	Femur	IIB	Osteoblastic	NECO95J	2	Amputation	High	17	37	DOD
35	15	F	Femur	IIIB	Chondroblastic	NECO95J	1	WE+FVFG	High	0	17	NED
36	42	F	Sacrum	IIB	Osteoblastic	NECO95J	(–)§	Not done§	High	0	18	NED
37	7	M	Tibia	IIB	Osteoblastic	CCCH2	1	WE+RP	Low	13	86	DOD
38	12	F	Femur	IIB	Chondroblastic	CCCH2	0	Amputation	Low	5	84	DOD
39	17	M	Tibia	IIB	Chondroblastic	CCCH2	1	WE+RP	High	180	180	CDF
40	14	F	Femur	IIB	Fibroblastic	CCCH2	0	WE+Prosthesis	High	13	167	NED
41	19	M	Tibia	IIB	Fibroblastic	CCCH2	0	WE+RP	Low	20	36	DOD
42	14	F	Tibia	IIB	Osteoblastic	CCCH2	0	WE+Prosthesis	Low	19	44	DOD
43	12	M	Tibia	IIB	Osteoblastic	CCCH2	2	WE+Prosthesis	High	167	167	CDF
44	22	M	Femur	IIB	Fibroblastic	NECO93J	3	WE+RP	Low	164	164	CDF
45	21	M	Femur	IIB	Chondroblastic	NECO93J	1	WE+RP	High	34	80	DOD
46	20	M	Tibia	IIB	Osteoblastic	NECO93J	1	WE+RP	High	37	131	NED
47	17	M	Femur	IIB	Osteoblastic	NECO93J	3	WE+FVFG	High	127	127	CDF
48	8	F	Humerus	IIB	Osteoblastic	NECO93J	3	WE+FVFG	Low	126	126	CDF
49	20	F	Humerus	IIB	Fibroblastic	NECO93J	1	WE+FVFG	High	126	126	CDF
50	16	F	Femur	IIB	Osteoblastic	NECO93J	2	WE+RP	High	124	124	CDF
51	18	M	Tibia	IIB	Osteoblastic	NECO93J	0	WE+RP	Low	10	15	DOD
52	13	F	Femur	IIB	Osteoblastic	NECO95J	1	WE+Prosthesis	Low	100	100	CDF
53	11	M	Tibia	IIB	Chondroblastic	NECO93J	2	WE+RP	High	121	121	CDF
54	12	F	Tibia	IIB	Osteoblastic	NECO95J	1	WE+Prosthesis	High	114	114	CDF
55	24	M	Femur	IIB	Osteoblastic	NECO95J	1	WE+Prosthesis	Negative	79	79	CDF
56	14	F	Humerus	IIB	Chondroblastic	NECO95J	1	WE+FVFG	High	29	96	NED
57	26	M	Tibia	IIB	Osteoblastic	NECO95J	0	WE+Prosthesis	High	89	89	CDF
58	17	M	Radius	IIB	Osteoblastic	NECO95J	1	WE+FVFG	Low	84	84	CDF
59	18	F	Femur	IIB	Chondroblastic	NECO95J	1	WE+Prosthesis	High	17	69	DOD
60	15	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	Low	75	75	CDF
61	17	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	Low	74	74	CDF
62	13	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	Low	72	72	CDF
63	11	F	Femur	IIB	Osteoblastic	NECO95J	3	WE+Prosthesis	Low	66	66	CDF

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Table 1. *Continued*

Patients	Age	Gender	Location	Surgical stage	Histological type	Chemotherapy	Histological response grade	Operation	PBF expression	EFS (months)	OS (months)	Prognosis
64	13	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	High	65	65	CDF
65	13	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	Negative	57	57	CDF
66	19	F	Femur	IIB	Osteoblastic	NECO95J	1	WE+Prosthesis	High	54	54	CDF
67	24	M	Tibia	IIB	Fibroblastic	NECO95J	0	WE+Prosthesis	Low	45	45	CDF
68	14	F	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	High	48	48	CDF
69	15	F	Femur	IIB	Osteoblastic	NECO95J	1	WE+Prosthesis	Low	7	18	DOD
70	19	M	Radius	IIB	Osteoblastic	NECO95J	1	WE+FVFG	Low	27	35	NED
71	16	F	Fibula	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	Low	33	33	CDF
72	10	M	Tibia	IIB	Osteoblastic	NECO95J	2	Amputation	High	12	32	AWD
73	11	M	Femur	IIB	Osteoblastic	NECO95J	1	WE+FVFG	Low	31	31	CDF
74	29	F	Fibula	IIB	Osteoblastic	NECO95J	1	WE	High	25	25	CDF
75	10	M	Femur	IIB	Chondroblastic	NECO95J	0	WE+Prosthesis	High	9	20	DOD
76	8	M	Femur	IIB	Osteoblastic	NECO95J	1	WE+RP	High	6	19	AWD
77	20	F	Tibia	IIB	Osteoblastic	NECO95J	2	Amputation	High	16	16	CDF
78	12	M	Femur	IIB	Osteoblastic	NECO95J	3	WE+Prosthesis	Low	15	15	CDF
79	65	M	Tibia	IIB	Osteoblastic	NECO95J[¶]	(–)[§]	WE+Prosthesis	High	2	13	NED
80	20	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	High	12	12	CDF
81	16	M	Ilium	IIB	Osteoblastic	NECO95J	1	WE	High	13	13	CDF
82	12	M	Femur	IIB	Osteoblastic	NECO95J	2	Amputation	High	8	13	DOD
83	20	M	Femur	IIB	Osteoblastic	NECO95J	2	WE+Prosthesis	High	12	12	CDF

[†]Chemotherapy and operation were refused by the patient and her family. [‡]Died of acute hepatitis B during postoperative chemotherapy. [§]Carbon ion radiotherapy was chosen instead of operation. [¶]Chemotherapy was instituted only postoperatively. AWD, alive with disease; CDF, continuously disease free; DOC, death of other cause; DOD, death of disease; EFS, event-free survival; F, female, FVFG, free vascularized fibula graft; M, male; NED, no evidence of disease; OS, overall survival; RP, rotational plasty; PBF, papillomavirus binding factor; WE, wide excision.

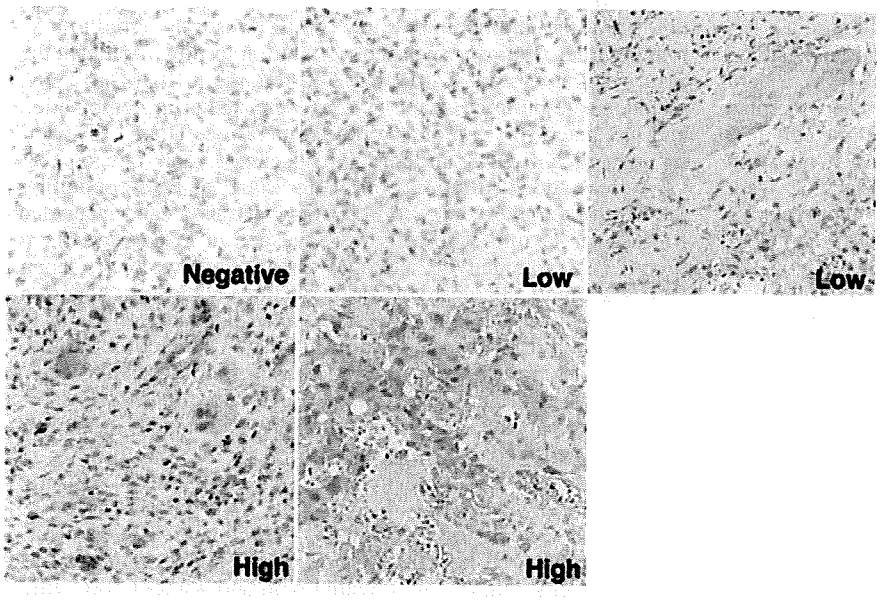

Fig. 1. Immunohistochemical grading of tumor specimens. Representative sections of osteosarcoma specimens stained with an anti-papillomavirus binding factor antibody are shown (original magnification ×200). Negative indicates that less than 5% of tumor cells were stained positively. Low indicates a positive tumor cell number from 5% to 50%. High indicates a positive tumor cell number of over 50%.

Survivorship analysis. Survivorship analysis was performed for 78 patients with osteosarcoma who had completed the protocols consisting of pre- and postoperative chemotherapy and underwent resection of the primary tumor with wide margin or amputation (Table 1). Five patients excluded were due to refusal (Patient 17) or incompletion of the chemotherapy (Patients 18, 19, and 79) and choice of non-surgical treatment (Patient 36). There were 43 males and 35 females with an average age of 17.9 years. Primary tumors were located in the femur (41 patients), tibia (19 patients), humerus (8 patients), fibula (4 patients), pelvis (3 patients), radius (2 patients) and rib (1 patient). According to Enneking's surgical staging system,[18] 76 patients were stage IIB and 2 patients were stage IIIB. There were 53 osteoblastic, 12 chondroblastic, 12 fibroblastic and 1 teleangiectatic osteosarcomas. Adjuvant chemotherapy protocols comprised of high-dose methotrexate-based multidrug regimen, including T-12,[19] NSH-7,[20] CCCH2,[21] NECO93J,[22] and NECO95J.[23,24] The responses of the tumors to preoperative chemotherapy were histologically graded according to the classification of the Japanese Orthopaedic Association:[25] Grade 0 (tumor necrosis <50%), 1 (≤50% tumor necrosis <90%), 2 (tumor necrosis ≥90%), 3 (No viable tumor cells in the histological sections). These 78 patients were followed up for an average of 65.0 months (range from 9 to 180 months).

The prognostic significance of the following variables overall and in the event-free survival of patients with osteosarcoma was

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Table 2. Sequences and binding affinities of PBF-derived peptides with HLA-A*2402 binding motif

Peptide	Position	Sequence	Binding score[†]	HLA-peptide binding affinity[‡] (% MFI increase ± SD)
A24.1	84–93	WYGGQECTGL	200	−3.0 ± 3.2
A24.2	145–153	AYRPVSRNI	84	119.2 ± 7.3
A24.3	409–418	AYQALPSFQI	75	25.3 ± 6.0
A24.4	254–263	GFETDPDPFL	30	2.6 ± 10.3
A24.5	320–328	DFYYTEVQL	20	2.3 ± 9.6
A24.6	118–127	RVEEVWLAEL	15.8	22.5 ± 9.6
A24.7	254–262	GFETDPDPF	15	−1.2 ± 5.9
A24.8	12–20	RSLLGARVL	12	14.2 ± 5.8
A24.9	415–424	SFQIPVSPHI	10.5	11.7 ± 7.9
A24.10	104–113	VTVWILEQKL	9.5	7.1 ± 1.2
HIV		RYLRDQQLLGI		41.2 ± 9.3

[†]Binding score was determined by BIMAS HLA Peptide Binding Predictions. [‡]The affinity of each peptide was evaluated by a HLA class I stabilization assay. BIMAS, bioinformatics and molecular analysis section; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; PBF, papillomavirus binding factor; SD, standard deviation.

determined by univariate analysis using the generalized Wilcoxon test: age (≥ 15), gender (male or female), stage (IIB or IIIB), histological type (osteoblastic, chondroblastic or fibroblastic), response to chemotherapy (Grades 0 and 1 or Grades 2 and 3), and PBF expression status (negative, low or high). The relationship between each variable and PBF expression status was determined by the chi-squared test. A probability of less than 0.05 was considered statistically significant.

Cell lines. An osteosarcoma cell line, OS2000, and an Epstein–Barr virus-transformed B cell line, LCL-OS2000, were established previously from a 17-year-old patient.[11] Osteosarcoma cell lines HOS and U2OS, and the erythroleukemia cell line K562 were purchased from American Type Culture Collection (Manassas, VA, USA). OS2000, HOS, U2OS and K562 were PBF^+ and LCL-OS2000 was PBF^-.[12] The HLA genotypes of osteosarcoma cell lines were as follows: OS2000, A*2402, B*5502, B*4002, Cw*0102; HOS, A*0211, B*5201, Cw*1202; U2OS, A*0201, A*3201, B*4402, Cw*0501, Cw*0704.

Design and synthesis of PBF-derived peptides. Based on the entire amino acid sequence of PBF, peptides with the ability to bind to HLA-A24 class I molecules were searched through the Internet site, Bioinformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (http://bimas.cit.nih.gov/).[26] Based on the binding scores, 10 peptides were selected and synthesized (Table 2).

HLA class I stabilization assay. The affinity of peptides for HLA-A24 molecules was evaluated by cell surface HLA class-I stabilization assay as described previously.[27,28] An HLA-A*2401-binding HIV peptide (RYLRDQQLLGI) was used for positive control. Assays were performed in triplicate. The affinity of each peptide for HLA-A*2402 molecules was evaluated by the percent mean fluorescence intensity (%MFI) increase of the HLA-A*2402 molecules in the calculation: %MFI increase: [(MFI with the given peptide − MFI without peptide)/(MFI without peptide)] × 100.

Limiting dilution/mixed lymphocyte peptide culture. Prior to frequency analysis and cytotoxicity assays, peripheral blood mononuclear cell (PBMC) of patients were subjected to mixed lymphocyte peptide culture under limiting dilution conditions (LD/MLPC) according to the method described by Karanikas *et al.*[29] with some modifications. For frequency analysis, peripheral blood samples (20 mL) were collected from nine patients with PBF^+ osteosarcoma (Patient 26, 36, 76, 78–83) (Table 1). PBMC were suspended in AIM-V (Invitrogen Crop., Carlsbad, CA, USA) supplemented with 1% human serum (HS) and incubated for 60 min at room temperature with PBF A24.2 peptide (25 μg/mL). Peptide-pulsed PBMC were seeded at 2×10^5 cells/200 μL/well into round-bottom 96-microwell plates in AIM-V with 10%HS, IL-2 (20 U/mL; a kind gift from Takeda Chemical Industries Ltd, Osaka, Japan) and IL-7 (10 ng/mL; R & D Systems, Minneapolis, MN, USA), and incubated. On day 7, half of the medium was replaced by fresh AIM-V containing IL-2, IL-7 and the same peptides. The cell cultures were maintained by adding fresh AIM-V containing IL-2. On days 14–21, they were subjected to tetramer-based frequency analysis.

For cytotoxicity assays, PBMC of Patient 36 were separated into $CD8^+$ cells and $CD8^-$ cells using magnetic anti-CD8 microbeads (Miltenyi Biotec, Gladbach, Germany). $CD8^-$ cells were pulsed with the PBF A24.2 peptide for 60 min. Half of the $CD8^-$ cells were cryopreserved at −80°C for the second stimulation. $CD8^+$ cells (2.5×10^5/well) and irradiated PBF A24.2 peptide-pulsed $CD8^-$ cells (5×10^5/well) were cocultured in 37 wells of a 48-well cell culture plate in 500 μL of AIM-V with 10%HS, IL-2 and IL-7. On day 7, the second stimulation was performed by adding irradiated peptide-pulsed $CD8^-$ cells to each culture well in 500 μL of freshly replaced AIM-V with 10%HS, IL-2 and IL-7. On day 14–28, they were subjected to tetramer-based cytotoxicity assays.

Tetramer-based frequency analysis. An fluorescein isothiocyanate-conjugated HLA-A24/HIV tetramer (here termed the control tetramer) and a phycoerythrin (PE)-conjugated HLA-A24/PBF A24.2 tetramer (A24/PBF A24.2 tetramer) were constructed by Medical & Biological Laboratories Co. Ltd. (Tokyo, Japan). PBMC from patients were stimulated with the PBF A24.2 peptide by LD/MLPC as described above. From each microwell containing 200 μL of the microculture pool, 100 μL was transferred to a V-bottom microwell and washed. On the spin-down pellets, the control tetramer and A24/PBF A24.2 tetramer (10 nM in 25 μL of phosphate-buffered saline (PBS)) were added in combination and incubated for 15 min at room temperature. Then a PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, CA, USA) was added (dilution of 1:30 in 25 μL of PBS containing the control tetramer and A24/PBF A24.2 tetramer) and incubated for another 15 min. The cells were washed in PBS twice, fixed with 0.5% formaldehyde, and analyzed by flow cytometry using FACScan and CellQuest software (Becton Dickinson, San Jose, CA, USA). $CD8^+$ living cells were gated and the cells labeled with the A24/PBF A24.2 tetramer and non-labeled cells with the control tetramer were referred to as tetramer-positive cells. The frequency of anti-PBF A24.2 CTLs was evaluated using the following calculation: (number of tetramer-positive wells)/([numbers of total tested wells] × [number of $CD8^+$ cells per well]).

Tetramer-based cytotoxicity assay. CTL-mediated cytolytic activity was measured by a 6 h-^{51}Cr release assay.[30] Osteosarcoma cell lines (OS2000, HOS and U2OS), EB-transformed B cell line LCL-OS2000 and K562 were used as the target cells. OS2000 was treated with and without 100 U/mL interferon-gamma (R & D Systems, Minneapolis, MN, USA) for 48 h. LCL-OS2000 was also treated with and without peptides (25 μg/mL) for 2 h at room temperature before assay. Target cells were labeled with 100 μCi of ^{51}Cr for 1 h at 37°C. The labeled target cells were suspended in Dulbecco's modified eagle's medium containing 10% fetal calf serum and seeded to microwells (2×10^3 cells/well). Patient 36-derived $CD8^+$ CTL lines stimulated with the PBF A24.2 peptide by LD/MLPC were used as the effector cells. Tetramer-positive CTL lines were transferred to V-bottom microwells, suspended in AIM-V and mixed with the labeled target cells. In cold-target inhibition assays, a 100-fold excess of unlabeled PBF A24.2-pulsed target cells was added as cold target cells. After a 6 h incubation period at 37°C, the release of the ^{51}Cr level in the supernatant of the culture was measured by quantification in an automated gamma

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Table 3. Univariate analysis of variables in event-free survial and overall survival

Variables		*n*	Event-free survival (months in average)	*P*-value	Overall survival (months in average)	*P*-value
Age	≤15	41	49.6	0.215	66.8	0.949
	>15	37	54.7		63	
Gender	Male	46	48.5	0.244	64.4	0.101
	Female	32	57.1		70.8	
Stage	IIB	76	53.4	0.006	66.3	0.062
	IIIB	2	0		16.5	
Histological type	Osteoblastic	53	52.1	0.052*	60.7	0.461*
	Chondroblastic	12	38.2	<0.001*	67.7	0.105*
	Fibroblastic	12	61.0	0.899*	78.3	0.665*
	Teleangiectatic	1	103.4			
Response to chemotherapy	Grades 0,1	41	40.5	<0.001	60.2	0.007
	Grades 2,3	37	64.7		70.3	
PBF status	Positive	72	49.2	0.025	63.3	0.091
	Negative	6	85.2		85.2	

*P-value was determined in comparison with the survival of patients with other subtypes.

Table 4. Clinical picture and frequency of anti-PBF A24.2 peptide CTLs in PBMC of patients with PBF-positive osteosacoma

Participants	Status of tumor bearing	Chemotherapy	Total number of tested wells	Number of tetramer-positive wells	Number of PMBC	%CD8	Number of $CD8^+$ cells per pool	Frequency†
Patient								
26	(P)‡, M	underway	62	14	200 000	17	34 000	7×10^{-6}
36	P	not done	194	6	210 000	23	48 000	6×10^{-7}
76	(P)	underway	19	2	200 000	9	18 000	6×10^{-6}
78	(P)	underway	62	1	200 000	15	30 000	5×10^{-7}
79	(P), M	underway	28	0	200 000	15	30 000	$<1 \times 10^{-6}$
80	(P)	underway	160	15	290 000	20	58 000	2×10^{-6}
81	(P)	underway	149	5	200 000	3	6000	6×10^{-6}
82	(P)	underway	132	3	200 000	4	8000	3×10^{-6}
83	P	underway	40	5	200 000	25	50 000	3×10^{-6}

†Frequency of anti-PBF A24.2 CTLs among $CD8^+$ cells. ‡Parentheses indicate that the tumor had been present previously but was free at the time blood sample was taken. CTLs, cytotoxic T lymphocytes; M, metastatic tumor; P, primary tumor; PBF, papillomavirus binding factor; PMBC, peripheral blood mononuclear cell.

counter. The percentage of specific cytotoxicity was calculated as the percentage of specific ^{51}Cr release: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). The cytotoxicity rate to OS2000 was calculated as (%cytotoxicity to each target cells)/(%cytotoxicity to OS2000).

Results

Expression of PBF protein in osteosarcoma. To determine the prevalence of the osteosarcoma-derived antigen PBF protein in osteosarcomas, we stained formalin-fixed paraffin-embedded sections of 83 specimens with a polyclonal antibody against PBF (Table 1 and Fig. 1). Of these, 76 specimens (92%) were positively stained with the anti-PBF antibody, including 49 specimens (59%) with high-grade staining.

Prognostic impact of PBF expression in patients with osteosarcoma. We then analyzed the prognostic significance of several variables including expression of PBF, in 78 patients with osteosarcoma who completed chemotherapy protocols and had wide tumor excision (Table 1). As depicted in Table 3, patients with chondroblastic type osteosarcoma showed significantly poorer event-free survival rate than those with other histological types. Forty-one patients with osteosarcoma showing a poor response to preoperative chemotherapy (Grades 0 and 1) showed significantly more unfavorable event-free and overall survival rates than 37 good responders (Grades 2 and 3). With respect to PBF-expression status, 72 patients with positive expression of PBF in the primary tumor were significantly more unfavorable in event-free survival than six patients with PBF-negative osteosarcoma ($P = 0.025$). This finding was consistent in subgroup analysis with 46 patients with high-grade PBF expression and 26 patients with low-grade PBF expression ($P = 0.026$ and 0.032, respectively). In contrast, age and gender failed to have significant prognostic impacts on event-free and overall survival rates of the patients.

Subsequently, we analyzed the relationship between the PBF-expression status in osteosarcoma and other variables. PBF-expression status was not significantly related to any variables including age ($P = 0.472$), gender ($P = 0.184$), stage ($P = 0.694$), histological type ($P = 0.743$) and the response to chemotherapy ($P = 0.069$).

Affinity of PBF-derived synthetic peptides to HLA-A*2402 molecules. To determine the immunogenicity of PBF in patients with HLA-A24, we synthesized 10 peptides from the PBF sequence in accordance with the BIMAS score for HLA-A24 affinity (Table 2). Subsequently, we evaluated the affinities of these peptides to HLA-A24 molecules by the HLA class-I stabilization assay. As shown in Table 2, peptide PBF A24.2 showed the highest MFI increases in the context of HLA-A24.

Frequency of anti-PBF A24.2 CTLs in $HLA\text{-}A24^+$ patients with osteosarcoma. We then examined the frequency of peripheral $CD8^+$ T-lymphocytes that recognized the PBF A24.2 peptide in 9 $HLA\text{-}A24^+$ patients with PBF^+ osteosarcoma by LD/MLPC/tetramer analysis. As depicted in Table 4 and representatively shown in Fig. 2(a),

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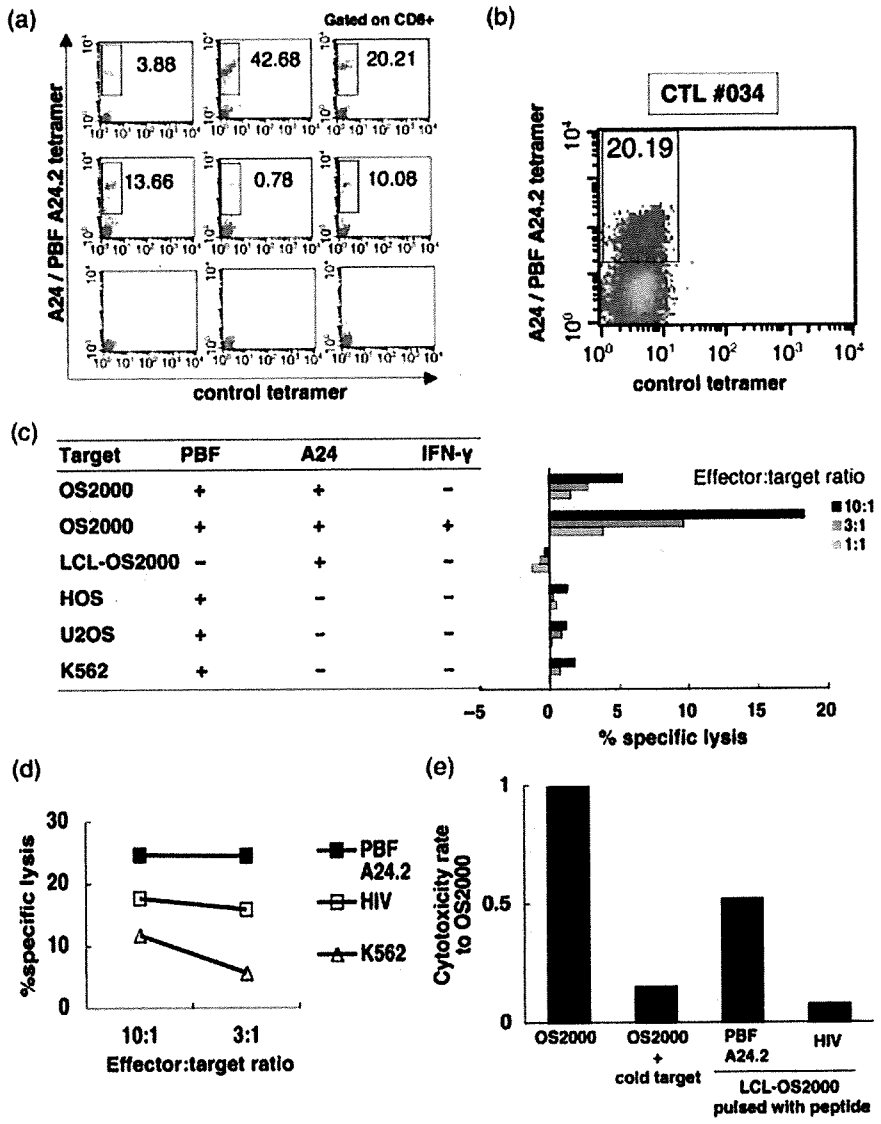

Fig. 2. Tetramer-based detection of anti-PBF A24.2 peptide CTLs in peripheral blood of patients with osteosarcoma. (a) peripheral blood mononuclear cell of Patient 26 were seeded into 62 microwells and stimulated with the papillomavirus binding factor (PBF) A24.2 peptide by mixed lymphocyte peptide culture under limiting dilution conditions (LD/MLPC). The resultant cytotoxic T lymphocytes (CTL) pools were stained with the phycoerythrin (PE)-conjugated A24/PBF A24.2 tetramer, fluorescein isothiocyanate-conjugated control tetramer, and a PE-Cy5-conjugated anti-CD8 mAb. Cells reacting with the anti-CD8 mAb were gated. The reactivity of gated cells with the A24/PBF A24.2 tetramer and the control tetramer are shown. The upper and middle columns display six representative pools with positive reactivity to the A24/PBF A24.2 tetramer. The cells labeled with the A24/PBF A24.2 tetramer and non-labeled cells with the control tetramer were considered to be tetramer-positive cells and are boxed to show their proportion among $CD8^+$ cells. The bottom row shows three negative pools. (b) $CD8^+$ cells (2.5×10^5 cells/well) of Patient 36 were seeded into 37 wells of a 48-well culture plate and stimulated with irradiated peptide-pulsed $CD8^-$ cells (5×10^5 cells/well) by LD/MLPC. Tetramer analysis was performed on day 14. The results of one of the four tetramer-positive CTL line (CTL #034) in the 37 pools are shown. Cells reacting with the anti-CD8 mAb were gated. The tetramer-positive cells are boxed to show their proportion among $CD8^+$ cells. (c) The cytotoxicity of CTL #034 against allogeneic osteosarcoma cell lines (OS2000, HOS, U2OS), LCL-OS2000 and K562 was assessed by a 6 h standard ^{51}Cr release assay at the indicated effector:target ratios. OS2000 was assayed in the presence and absence of 48 h-interferon-gamma pretreatment. (d) The cytotoxicity of CTL #034 against peptide-pulsed LCL-OS2000 and K562 was assessed by a 6 h standard ^{51}Cr release assay at the indicated effector:target ratios. LCL-OS2000 was pulsed with 25 μg/mL of PBF A24.2 peptide or HIV control peptide for 2 h at room temperature before labeling with ^{51}Cr. (e) Cold target inhibition assay. The cytotoxicity of CTL #034 against interferon-gamma-treated, ^{51}Cr-labeled OS2000 was assessed in the presence and absence of a 100-fold excess of PBF A24.2 peptide-pulsed, cold LCL-OS2000 at a 10:1 effector-target ratio. ^{51}Cr-labeled LCL-OS2000 cells pulsed with the PBF A24.2 peptide or HIV peptide were also used as control target cells.

anti-PBF A24.2 CTLs were detected as tetramer-positive cells in eight of the nine patients with osteosarcoma. The frequencies of anti-PBF A24.2 CTLs were between 5×10^{-7} and 7×10^{-6} (4×10^{-6} in average) in eight tetramer-positive patients.

Tetramer-based cytotoxicity of anti-PBF A24.2 CTLs against osteosarcoma cell lines. Finally we assessed the cytotoxic activity of tetramer-positive cells against allogeneic osteosarcoma cell lines. We induced tetramer-positive anti-PBF A24.2 CTLs from 1×10^7 $CD8^+$ cells of Patient 36 by LD/MLPC using 48-well culture plates. Irradiated peptide-pulsed $CD8^-$ cells were used as stimulator cells. As a result, 4 of 37 tetramer-positive CTLs were detected by tetramer analysis on day 14. Four tetramer-positive CTLs contained 3.47%, 0.03%, 15.26% and 20.19%. One of four CTL lines (CTL #034) was shown in Fig. 2(b).

The cytotoxicity against OS2000 (PBF^+, $A24^+$), LCL-OS2000 (PBF^-, $A24^+$), HOS (PBF^+, $A24^-$), U2OS (PBF^+, $A24^-$) and K562 (PBF^+, HLA class I loss) was comparatively assessed by ^{51}Cr release assay. As depicted in Fig. 2(c), CTL #034 showed specific cytotoxicity against OS2000 and the cytotoxicity was enhanced by interferon-gamma pretreatment. In contrast, none of these CTL lines exhibited cytotoxic activity against LCL-OS2000, HOS, U2OS, or K562 cells. The other three tetramer-positive CTL lines also showed specific cytotoxicity against OS2000 (data not shown).

We subsequently determined the specificity of cytotoxicity with the PBF A24.2 peptide by using peptide-pulsed LCL-OS2000. As shown in Fig. 2(d), CTL #034 lyzed PBF A24.2 peptide-pulsed LCL-OS2000 more than control peptide (HIV)-pulsed LCL-OS2000 or K562 cells. Peptide-specific cytotoxicity of CTL #034 was also assessed by cold-target inhibition assay (Fig. 2e). Cytotoxicity against OS2000 pretreated with interferon-gamma was inhibited by adding a 100-fold excess of cold LCL-OS2000 pulsed with PBF A24.2.

Discussion

In the present study, we examined the distribution profile, prognostic impact, and immunogenicity of the novel tumor-associated antigen PBF in osteosarcoma. We found: (i) that 92% of 83 osteosarcoma specimens expressed PBF protein; (ii) that PBF-positive osteosarcomas conferred a significantly poorer prognosis than those with negative expression of PBF in event-free

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survival of 78 patients who completed the standard treatment; (iii) that $CD8^+$ T cells reacting with a PBF-derived HLA-A24-binding peptide (PBF A24.2 peptide) were detected in eight out of nine HLA-A24-positive patients with osteosarcoma at the frequency from 5×10^{-7} to 7×10^{-6}; and (iv) that PBF A24.2 peptide induced CTL lines from an HLA-A24-positive patient, which specifically killed an osteosarcoma cell line that expresses both PBF and HLA-A24. These findings suggest the oncogenic and antigenic role of PBF in patients with osteosarcoma, especially those with HLA-A24. The proof of immunogenicity of PBF has been limited to an HLA-B55-positive patient with osteosarcoma.[12] Wide distribution of PBF in osteosarcoma and the immunogenicity of PBF seen in patients with HLA-A24 extended the possibility of PBF-targeted immunotherapy against osteosarcoma. Patients with negative expression of PBF in osteosarcoma can be treated successfully by the current chemotherapy-based treatment protocols.

To date, peptide-based immunotherapy in patients with bone and soft tissue sarcomas has been reported only with the use of fusion gene-derived peptides.[31,32] This approach has been available for tumors in which specific chromosomal translocations have been identified, including synovial sarcoma and Ewing sarcoma. However, for other sarcomas including osteosarcoma, where chromosomal translocation and a resultant fusion gene have not been identified, novel tumor-associated antigens need to be defined. With this aim, we developed autologous pairs of tumor cells and CTLs from patients with sarcomas. Consequently, PBF was identified from an autologous osteosarcoma-CTL pair. PBF protein was defined in 89% of the various bone and soft tissue sarcomas (Tsukahara *et al.* 2004, unpub. data). Therefore, the PBF A24.2 peptide might be applicable to immunotherapy against bone and soft tissue sarcomas without known chromosomal translocations, other than osteosarcoma.

Our univariate analysis revealed prognostic significance of PBF in event-free survival. Such prognostic values need to be verified by multivariate analysis. In this regard, all of the six patients with PBF-negative osteosarcoma analyzed are continuously disease-free. Unfortunately, the disproportional profile of these patients caused failure in multivariate analysis. In contrast, poor response to chemotherapy and chondroblastic subtype remained significant in the multivariate analysis (Tsukahara *et al.* 2007, unpub. data), indicating the validity of the patient population in the present analysis. The unfavorable prognostic value of PBF was also seen in our analysis with 20 patients with Ewing sarcoma.[33]

The frequency of anti-PBF CTL precursor was determined between 5×10^{-7} and 7×10^{-6}. In melanoma patients, the anti-MAGE3.A1 CTL precursor frequency was estimated to be $<10^{-7}$ in normal donors and prevaccinated patients, and 10^{-6} in postvaccinated patients.[34] Although the anti-PBF A24.2 peptide CTL precursor frequency defined in the present study was relatively higher than the anti-MAGE3.A1 CTL frequency, it was still under the detection level of the standard tetramer analysis and thus required the LD/MLPC/tetramer procedure for detection. The LD/MLPC/tetramer procedure was advantageous with its high sensitivity for the frequency analysis of peptide-specific CTLs in preclinical studies and clinical trials.[35,36] Also, this procedure served as prescreening of CTLs for subsequent cytotoxicity analysis. However, the multistep procedure of LD/MLPC/tetramer analysis requires labor-intensive laboratory work and long-term cell culture.[37,38] This gives rise to a concern about possible changes in the effector function and differentiation status of CTLs during the analysis.[39,40]

Cytotoxicity of CTL induced with PBF A24.2 peptide was proved only in an osteosarcoma cell line that is positive for PBF and HLA-A24. It would be ideal to conduct cytotoxicity assays with more number of cell lines. However, limited cell numbers of CTL lines expanded after LD/MLPC/tetramer analysis made it difficult to carry out. Instead, we examined the specificity of the cytotoxicity by peptide-pulsation as well as cold inhibition assays.

In conclusion, the present study demonstrated the feasibility and population of candidates for PBF-targeted immunotherapy for osteosarcoma. The combination of LD/MLPC with tetramer labeling as well as ^{51}Cr release cytotoxicity assay enables us to concurrently determine the frequency and function of CTL precursors, and thus serves as a useful tool for identification of novel antigenic peptides and immunomonitoring in clinical immunotherapy trials.

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ORIGINAL ARTICLE

Disruption of the association of 73 kDa heat shock cognate protein with transporters associated with antigen processing (TAP) decreases TAP-dependent translocation of antigenic peptides into the endoplasmic reticulum

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List of Abbreviations: ATPase, ATP phosphatase; CHAPS, 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; 15-DSG, 15-deoxyspergualin; ER, endoplasmic reticulum; HSC73, 73 kDa heat shock cognate protein; HSP70, 70 kDa heat shock protein; HSP90, 90 kDa heat shock protein; MeDSG, methyldeoxyspergualin; MHC, major histocompatibility complex; RCMLA, reduced carboxymethylated lactalbumin; TAP, transporters associated with antigen processing.

Key words
antigen presentation, deoxyspergualin, heat shock protein, major histocompatibility complex.

ABSTRACT

Major histocompatibility complex class I-bound antigenic peptides generated in the cytosol are translocated into the ER by TAP. In the present study, the physical association of HSC73 with TAP in human lymphoblastoid T1 cells was demonstrated. The dissociation was induced in the presence of 10 mM ATP, indicating that the ADP-binding form of HSC73 might be associated with TAP. We found that HSC73-binding immunosuppressant, MeDSG disrupted the HSC73-TAP association, whereas it did not affect the binding of HSC73 to a substrate protein. MHC class I expression on the cell surface was also downregulated. Then, the effect of MeDSG on the TAP-mediated ER translocation was examined using two homologous model peptides, NGT-Bw4 and NGT-Bw6, which had distinct binding affinity to HSC73. Although high-affinity peptide NGT-Bw4 was translocated by TAP, low-affinity peptide NGT-Bw6 was not. The TAP-dependent translocation of NGT-Bw4 was abolished in the presence of MeDSG. Decreased presentation on the cell surface was shown for the human leukocyte antigen (HLA)-A31-restricted natural antigenic peptide F4.2, which had high affinity to HSC73, in the presence of MeDSG. It was indicated that disruption of the HSC73-TAP association resulted in inhibition of TAP-dependent translocation of HSC73-bound peptides. Our findings highlighted an important role of HSC73 for feeding antigenic peptides to TAP, and suggested a possibility that a synthetic polyamine might inhibit the function of HSC73, thereby suppressing MHC class I-restricted presentation of HSC73-bound antigenic peptides.

Major histocompatibility complex class I molecules bind to endogenous antigenic peptides and present them to CD8-positive T cells. Most of the peptides are produced by proteasome-mediated degradation of cytosolic or nuclear proteins (1). The degradation products are transported from the cytosol into the endoplasmic reticulum (ER) by heterodimeric transmembrane molecules called TAP, and then loaded onto MHC class I molecules (2–4). The mechanism of peptide transfer from TAP to MHC class I has been well documented. TAP1 and TAP2 belong to a family of ATP-binding cassette transporters, and can translocate peptide fragments into the ER through hydrolysis of ATP (5, 6). The transfer of translocated peptides to MHC class I requires a complex formation among TAP, empty MHC

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heavy chain and tapasin (7–11). Although tapasin does not bind to peptides, it is an essential molecule for the physical association between TAP and MHC heavy chain (12). It is known that some molecular chaperones such as calnexin (9), calreticulin (13, 10), ERp57 (14, 15) and GRP94 (16) have important roles during assembly and maturation of MHC class I complex. In contrast to the ER event, the mechanism of peptide transfer in the cytosol remains to be elucidated. How can peptide fragments produced by proteasome be transported to the TAP? What molecules are associated with the cytosolic domain of TAP? It has been speculated so far that the cytosolic peptide fragments should be accompanied by some molecular chaperones before TAP-dependent translocation so that: (i) hydrophobic peptides become soluble in the cytosol; and (ii) peptides can be protected from degradation by cytosolic peptidases (17). Moreover, it is possible that antigenic peptides may be transported through an energy-dependent active mechanism rather than through a passive diffusible mechanism.

Previously our group and others have shown that heat shock proteins (HSP) were associated with antigenic peptides, and that some of them were presented to T cells (18–22). It was indicated that HSP-chaperoned peptides might be efficiently presented by MHC class I (23). One of the 70 kDa HSP family proteins, HSC73, is a cytoplasmic protein serving as a molecular chaperone. HSC73 can bind to various peptides or proteins (24, 25), preferentially containing hydrophobic amino acids, and functions to regulate localization, conformation, and degradation of these molecules (26). HSC73 has an ATP-binding domain, and its function is dependent on the intrinsic ATPase activity (27). These data led us to speculate that HSC73 might be involved in the feeding of cytosolic peptides to TAP.

In the present study, we focused on the functional significance of the interaction between TAP1 and HSC73 for TAP-dependent peptide translocation. We found that HSC73 was associated with TAP1 in an ATP-dependent manner and that the association was disrupted by HSC73-binding polyamine compound, MeDSG (28, 29). 15-DSG is an immunosuppressant that has been used clinically to protect against rejection after organ transplantation (30, 31). MeDSG induced the downregulation of cell surface MHC class I levels. The consequences of the dissociation of HSC73 from TAP were analyzed by ER translocation assay of synthetic peptides and by cytotoxic T lymphocyte (CTL) assay against endogenous human leukocyte antigen (HLA)-A31-restricted natural antigenic peptides (32). We demonstrated that TAP-mediated translocation and presentation of HSC73-bound peptides were abolished in the presence of MeDSG. Our study provided evidence for the direct involvement of a molecular chaperone, HSC73, in the feeding of antigenic peptides into TAP and the inhibitory role of polyamine compound.

MATERIALS AND METHODS

Cells, antibodies, peptides and reagents

Human lymphoblastoid cell line T1 cells and TAP-deficient cell line T2 cells (33) were purchased from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 10% FCS (Filtron, Brooklyn, Victoria, Australia). In some cases, T1 cells were cultured in the complete media containing 150 U/mL -interferon (IFN- ; Chugai Pharmaceutical, Tokyo, Japan).

A gastric signet ring cell carcinoma line, HST-2, and $CD8^+$ CTL clone, TcHST-2, were established and characterized previously (34). TcHST-2 is specifically cytotoxic to autologous HST-2 cells in the context of HLA-A31 restriction, as this cytotoxicity was completely blocked by anti-HLA-A31 mAb, as previously reported. TcHST-2 was maintained in an AIM-V serum-free medium (Gibco BRL) supplemented with 100 U/mL recombinant interleukin (IL)-2. We also used HLA-$A31^+$ lines, such as C1R-A31 (B lymphoma line C1R transfected with genomic HLA-A 31012 DNA) and HOBC8-A31-12 (HOBC8 cells transfected with HLA-A 31012 cDNA). Establishment and characterization of these cells were reported previously (32).

Affinity purified rabbit anti-human TAP1 polyclonal antibody R.RING4C was kindly provided by Dr P. Cresswell (Yale University, New Haven, CT, USA). Anti-HSP70 mAb 3a3 was purchased from Affinity BioReagents (Neshanic Station, NJ, USA).

Amino acid sequences of NGT-Bw4 peptide (sequence, NGTRENLRIALRY) and NGT-Bw6 peptide (sequence, NGTRESLRNLRGY) are derived from the common epitope of 1-domain of human MHC class I heavy chain (35) with additional N-terminal *N*-glycosylation motif. F4.2 peptide (sequence, YSWMDISCWI) was identified as HLA-A31-restricted tumor antigen peptide recognized by TcHST-2 cells, as previously reported (32). These peptides were synthesized, purified by high performance liquid chromatography (HPLC) and confirmed by mass spectrometry (Iwaki Glass Life Science Center, Chiba, Japan).

MeDSG (28) was provided by Nippon Kayaku Co. (Tokyo, Japan). Spermidine was purchased from Wako Chemical Co. (Osaka, Japan). RCMLA was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Bovine

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brain 70 kDa heat shock protein consisting mainly of HSC73 was purchased from StressGen (Victoria, BC, Canada).

Immunoprecipitation

5×10^6 cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 500 μL of 0.5% CHAPS lysis buffer (0.5% 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS), 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.2 TIU/mL aprotinin). After incubation on ice for 45 min, nuclei and cell debris were removed by centrifugation (15 000 × g, 10 min). The lysates were pre-cleared by incubating for 1 hr with rabbit immunoglobulin G (IgG) conjugated with fixed *Staphylococcus aureus* (Sigma-Aldrich Co.). Then, the lysates were incubated with 2.7 μg anti-TAP1 antibody at 4 C for 30 min, followed by incubation with 25 μL Protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 18 hr. After washing four times with lysis buffer, immunoprecipitates were boiled for 5 min with reducing sodium dodecylsulfate (SDS) sample buffer (3% SDS, 0.5 M 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8)), and subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE). In some cases, MeDSG or spermidine was included in the cell lysates at a final concentration of 200 μg/mL before incubating with the antibody.

Western blotting

Proteins separated by SDS-PAGE were transferred onto Immobilon membranes (Millipore, Bedford, MA, USA). The membranes were soaked in a blocking buffer (PBS, 10% non-fat dry milk) for 2 hr at room temperature. Then, the blots were incubated for 90 min with anti-TAP1 antibody or anti-HSP70 antibody 3a3. After washing in a washing buffer (PBS, 0.1% Tween-20), the membranes were incubated with horseradish peroxidase-labeled anti-rabbit (KPL, Gaithersburg, MD, USA) or anti-mouse antibodies (KPL) for 30 min, followed by incubation in ECL detection fluid (Amersham, Birmingham, AL, USA) for 1 min. The bands were visualized using X-ray films (Fuji Photo Film, Tokyo, Japan). In some cases, membranes were incubated in a stripping buffer (62.5 mM Tris-HCl, pH 7.5, 2% SDS, 0.1 M 2-mercaptoethanol) for 30 min at 50 C, rinsed in a washing buffer and soaked in a blocking buffer again for 1 hr before incubating with antibody.

Flow cytometry

Cells were cultured for 36 hr in an AIM-V serum-free medium (GIBCO, Invitrogen, Carlsbad, CA, USA) containing various concentrations of MeDSG. After washing once with PBS, cells were incubated with anti-human MHC class I antibody W6/32 for 45 min at 4 C, followed by incubation with fluorescein-isothiocyanate (FITC)-labeled anti-mouse IgG antibody (KPL) for 45 min and analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Complex formation between HSC73 and RCMLA, and competitive HSC73-binding assay of synthetic peptides

The binding affinity of HSC73 to a substrate protein was assessed as described by Fourie *et al.*, based on observations that HSC73 binds to an unfolded form of lactalbumin (RCMLA) (36). RCMLA (250 μg) was incubated with 37 mBq of 125-iodine (MEN Life Science Products, Boston, MA, USA) and two drops of Iodo-beads (Pierce, Rockford, IL, USA) in 250 μL PBS for 15 min at 20 C. Then, labeled RCMLA was separated from free 125-iodine by a Micro Spin G-25 column (Pharmacia Biotech). The final concentration of the labeled RCMLA was analyzed by micro BCA assay (Pierce). The specific radioactivity was 4.8×10^6 c.p.m./μg. HSC73 (2 μg; 2.8 μM final concentration) was incubated with 40 ng labeled RCMLA in 10 μL PBS (HSC73/RCMLA mole ratio of 10:1) at 37 C for 1 hr in the absence or presence of various concentrations of MeDSG or spermidine. In the case of competitive binding assay of peptides, 2.8 μM, 28 μM, 84 μM or 140 μM of each peptide was incubated with the HSC73-RCMLA mixture. Free RCMLA was separated from HSC73-bound RCMLA by native PAGE (resolving gel: 7% acrylamide, 0.4 M Tris-HCl, pH 8.8; running buffer: 25 mM Tris-HCl, 192 mM glycine, pH 8.3), followed by autoradiography using X-ray films.

Radiolabeling and ER translocation assays of synthetic peptides

Synthetic peptides (750 μg) were resolved in 250 μL PBS containing 20% acetonitril. The peptides were labeled with 125-iodine and purified as described above. The specific radioactivities of the peptides were 1×10^6 c.p.m./μg for both NGT-Bw4 and NGT-Bw6 peptides.

ER translocation assay was carried out as described by Androlewicz *et al.* (37). Briefly, 1×10^7 cells were washed with serum-free RPMI-1690 medium and permeabilized by incubation in the medium containing 4 mM dithiothreitol and 1 U/mL streptolysin O (Murex, Norcross, GA, USA) at 4 C for 10 min. After washing cells with serum-free medium three times, the permeabilized cells were incubated with 19 μM radiolabeled peptide and 4 mg/mL BSA in 1 mL transport buffer (50 mM Hepes, pH 7.0, 78 mM KCl, 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM

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