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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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Original Paper

Tonsillar crypt epithelium of palmoplantar pustulosis secretes interleukin-6 to support B-cell development via p63/p73 transcription factors

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Abstract

Palmoplantar pustulosis (PPP) is an autoimmune disease characterized by psoriasis-like erythematous lesions on palms and/or soles due to an abnormal humoral immune response. Tonsillectomy is effectively employed for the treatment of PPP; however, how tonsils are involved in the aetiology of PPP remains unclear. Here we analysed surgically resected palatine tonsils from 36 cases of PPP as well as usual recurrent tonsillitis (RT) as a control. Histological examination revealed that a unique lesion, with lymphoid follicles surrounded by reticular crypt epithelial cells, was more frequently observed in tonsils of patients with PPP than in those with RT ($p < 0.0001$; PPP vs RT). Interestingly, crypt epithelial cells in primary cultures derived from PPP tonsils showed marked production of interleukin-6 (IL-6). Moreover, these epithelial cells from PPP tonsils expressed p53-related transcription factors in their nuclei that were found to contribute to the up-regulation of *IL-6* gene expression. These findings suggest that, at least in part, the specialized lymphoepithelial symbiosis of PPP tonsils, under the control of p53-related factors, may be relevant to the generation of the impaired micro-environment underlying the aberrant production of autoantibodies.

Keywords: autoimmunity; focal infection; p53 family; PPP; tonsillar epithelial cells

Introduction

Focal infection of palatine tonsils may cause palmoplantar pustulosis (PPP, synonymously recognized as palmoplantar pustular psoriasis) [1,2]. Patients with PPP display chronic relapsing inflammatory changes, with erythematous and scaly plaques on the palms and/or soles bilaterally, so that PPP has a profound impact on their quality of life. According to reports describing putative mechanisms leading to PPP, autoantibodies against a certain antigen(s) specifically presented by the epithelia of the palms and/or soles probably appear in certain lymphoid tissue(s) [3,4]. Once the autoantibodies have an opportunity to bind to epithelial cells of the palms and/or soles, subsequent cytopathic effects, including local activation of the complement system, might cause aseptic epidermal lesions in palmoplantar areas. To date, surgical resection of palatine tonsils combined with steroid therapy is considered to be one of the most effective therapeutic approaches for the treatment of PPP [5–7]. Thus, palatine tonsils have been considered to be a lymphoid tissue possibly responsible for generating such unusual immune responses in patients with PPP, although the mechanistic details in the patients' tonsils have not been fully investigated.

Tonsillar crypt epithelial cells (TCECs) constitutively form a unique reticular meshwork, which is often infiltrated by small lymphocytes such as B cells [8–11]. Such an association of TCECs and lymphocytes is specifically called lymphoepithelial symbiosis (LES), further characterizing the physiological features of palatine tonsils. Functional properties of the lymphocytes of PPP tonsils have been the focus of several studies, although knowledge about the function of LES in PPP tonsils is still limited in lymphocytes [12,13]. The biological significance of TCECs as another component of LES in the pathogenesis of PPP remains elusive.

The aim of this study was to shed light on TCECs of PPP tonsils and explore the role of TCECs in the pathogenesis of PPP. Our results provide the first evidence that TCECs of PPP tonsils may take part in the specialized formation of lymphoid follicles by secreting a high amount of interleukin-6 (IL-6), which is required for the development of B cells and plasma cells [14,15]. Moreover, we show that TCECs

of PPP tonsils abundantly express the epitheliotropic transcription factors p63 and p73, p53-related factors, in their nuclei. Surprisingly, p63 seems to be involved in the up-regulation of IL-6 at the transcriptional level through the NF-κB promoter of the *IL-6* gene. These results may open the way to the development of a new modality for the treatment of PPP as an alternative to surgical resection of the tonsils, in which quenching the activity of IL-6 produced by TCECs may be required.

Materials and methods

Patients of this study

A total of 74 patients were enrolled, as summarized in Table 1, including 36 with PPP, 36 with recurrent tonsillitis (RT), and two with IgA nephropathy (IgAN). All patients in this study underwent tonsillectomy at Sapporo Medical University Hospital in Japan from 1989 to 2006. Sole skin biopsies were performed in two patients with PPP and sera were collected from the same patients. All tissue was obtained with informed consent and the approval of the institutional review board.

Assessment of clinical status in patients with PPP

For determination of the clinical status of skin lesions in patients with PPP, the physician's global assessment (PGA) of change was used to assess treatment efficacy in this study. The PGA of change was categorized according to seven ratings: 'cleared' (100% improvement from baseline); 'excellent' (75–99%); 'good' (50–74%); 'fair' (25–49%); 'slight' (1–24%);

Table 1. Summary of PPP, RT, and IgAN cases in this study

PPP						RT			IgAN		
Case No	Age (years)	Sex	PGA (Palm)	PGA (Sole)	IP (months)	Case No	Age (years)	Sex	Case No	Age (years)	Sex
1	34	F	Unchanged	Unchanged	—	1	65	F	1	24	F
2	21	F	ND	ND	ND	2	53	M	2	20	F
3	50	F	ND	ND	ND	3	40	M	Average	22.0	
4	52	F	Cleared	—	1.5	4	40	M			
5	27	F	ND	ND	ND	5	52	F			
6	41	M	Cleared	Cleared	1	6	41	F			
7	46	M	Unchanged	Unchanged	—	7	44	F			
8	19	M	ND	ND	ND	8	54	M			
9	70	F	ND	ND	ND	9	53	F			
10	43	F	Cleared	Cleared	12	10	46	M			
11	32	F	ND	ND	ND	11	54	M			
12	58	F	Cleared	Cleared	3	12	46	F			
13	47	F	Good	Good	24	13	58	F			
14	50	F	Cleared	Cleared	1	14	9	M			
15	17	F	ND	ND	ND	15	5	M			
16	36	M	ND	ND	ND	16	24	F			
17	50	F	Cleared	Cleared	60	17	47	M			
18	49	M	Cleared	Cleared	6	18	5	M			
19	20	F	ND	ND	ND	19	25	F			
20	39	F	ND	ND	ND	20	41	F			
21	36	M	Cleared	Cleared	1	21	24	M			
22	51	F	Unchanged	ND	—	22	36	F			
23	55	F	Cleared	Cleared	1.5	23	5	F			
24	61	M	ND	ND	ND	24	31	M			
25	55	F	Cleared	Fair	1	25	5	F			
26	54	F	Cleared	Cleared	12	26	38	F			
27	51	F	Fair	Unchanged	2.5	27	53	F			
28	58	M	Cleared	Cleared	12	28	53	M			
29	43	F	Cleared	Cleared	2	29	37	F			
30	55	M	Cleared	—	1	30	53	F			
31	59	F	Unchanged	—	—	31	44	M			
32	48	F	Cleared	Excellent	18	32	26	M			
33	58	F	Unchanged	—	—	33	26	F			
34	62	F	Cleared	Cleared	3	34	53	F			
35	34	M	Cleared	Cleared	7	35	19	M			
36	31	F	ND	ND	ND	36	30	F			
Average	44.8				8.9	Average	37.1				

Patients with PPP ranged from 17 to 70 years old (median = 44.8 years) and the gender ratio was 1:2.6 (male:female). Patients with RT ranged in age from 5 to 65 years (median = 37.1 years) and had a gender ratio of 1:1.3. Patients with IgAN ranged in age from 20 to 22 years (median = 22.0 years) and were all female. The status of 12 cases ('ND' in the table) in the PPP group could not be surveyed after surgery.
PGA = physician's global assessment; IP = improvement period; ND = not determined.

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'unchanged' (no change in clinical signs and symptoms from baseline); and 'worse' (deterioration of clinical signs and symptoms from baseline).

Tissue and cell culture

For primary culture of TCECs, the surface epithelial cell layers were initially removed from the tonsil with micro-scissors, to prevent contamination of surface epithelial cells, under a research microscope (SZX7; Olympus). The procedures used for primary culture of TCECs were applied with modifications, as previously reported for primary culture of thymic epithelial cells [16]. Human HaCaT and HSC-3 epithelial cells were maintained in modified DMEM supplemented with 10% heat-inactivated bovine calf serum, 50 μg/ml streptomycin, and 100 units/ml penicillin. All cells were cultured at 37 °C in a humidified atmosphere in 5% CO_2.

Antibodies and immunohistochemistry

The antibodies used were a mouse anti-cytokeratin monoclonal antibody (MAb) (KL-1; Immunotech), rabbit anti-p63 polyclonal antibody (PAb) (H-137; Santa Cruz Biotechnology), rabbit anti-p73 PAb (ST-2G [17]), and mouse anti-IL-6 MAb (6708; Dako). The procedures for immunofluorescene have been previously described [16]. Signals were detected under an immunofluorescence microscope (IX71, Olympus) or a confocal laser microscope (R2100AG2, Bio-Rad).

RT-PCR and real-time PCR analyses

RT-PCR was conducted to detect certain transcripts, as previously reported [18]. Primer pairs were selected using Primer3 software on mRNA sequences based on the NCBI database. Sequences of the primer pairs were finally determined after reference to the original genomic organization presented in the Ensembl database (Sanger Centre), as summarized in Supplementary Table 1 (available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2266.html). Real-time PCR was performed as described in the manufacturer's protocol for Assays-on-Demand Gene Expression products (Applied Biosystems). To compare the levels of expression of IL-6, the ΔΔCT method was employed to analyse triplicate specimens according to the manufacturer's instructions.

Cytokine assays

Primary TCECs were seeded on collagen type I-coated dishes at a density of 5×10^6 cells for 3 days. The concentrations of cytokines in the culture supernatants were analysed in triplicate with ELISA kits, as described in the manufacturer's protocol (Biosource).

Luciferace activity assay

Human IL-6 promoter-reporter constructs in luciferase pGL3-basic vector were employed as previously described [19]. HSC-3 cells were seeded in 12-well plates at a density of 2×10^5 cells per well. After 24 h, 250 ng of a reporter gene plasmid, 250 ng of pcDNA3.1 harbouring cDNA encoding p63 (pcDNA3.1-p63), and 25 ng of pSV-β-galactosidase were transiently transfected using Lipofectamine 2000 reagent (Invitrogen). Transfections were performed in triplicate and cells were harvested after 48 h. Luciferase activity was measured according to the manufacturer's protocol (Promega) and normalized for transformation efficiency with the β-galactosidase assay as a relative luciferase unit (Stratagene).

Statistical analysis

The computer program Statview (Abacus Concepts) was used for all calculations. Values were expressed as the mean ± SD for each group. Comparisons between the two groups (PPP and RT) were performed with the Mann–Whitney U-test. Significance was assigned to $p < 0.05$. Spearman's rank-order correlations were used to test for a monotonic association of the CLG/GC (circular lymphoepithelial lesion with germinal centre/germinal centre) ratio and the improvement period.

Results

Clinical assessment after tonsillectomy in PPP patients

In 24 cases in the group with PPP, clinical responsiveness after tonsillectomy was categorized into the five classes of the physician's global assessment (PGA; see the Materials and methods section). Patients classified as 'cleared', 'excellent' or 'good' showed favourable recovery with noticeable improvements on the palms and soles in 18/24 (75.0%) and 15/19 (78.9%), respectively.

The sera of patients with PPP reacting to sole epidermis

We initially studied how the serum was involved in the development of the symptoms and signs of PPP as described in previous reports [3]. Immunofluorescence studies demonstrated strong signals in the epithelial cells of the sole tissue sections when using the patient's serum (Figure 1). This evidence indicated that the serum from the patient with PPP contained autoantibodies capable of binding to a sole epidermal cell antigen(s). Thus, it was thought that aberrant humoral immunity might induce the clinical manifestations of PPP. A possible candidate antigen might be a certain subtype of cytokeratin specifically expressed on palms and soles [20].

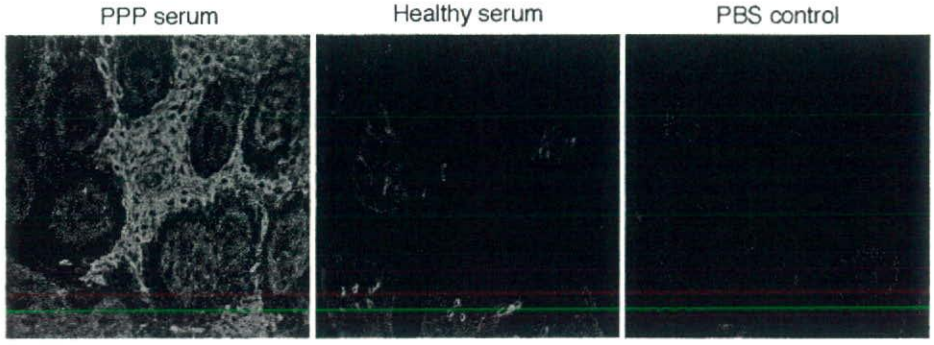

Figure 1. Immunofluorescene analysis of sole skin using the serum of a patient with PPP. The left panel shows high-level autoantibodies in the serum (PPP case 36) reacting to antigens in the sole epidermis. The middle and right panels show results using healthy serum and PBS, respectively, as controls. Original magnification: ×400

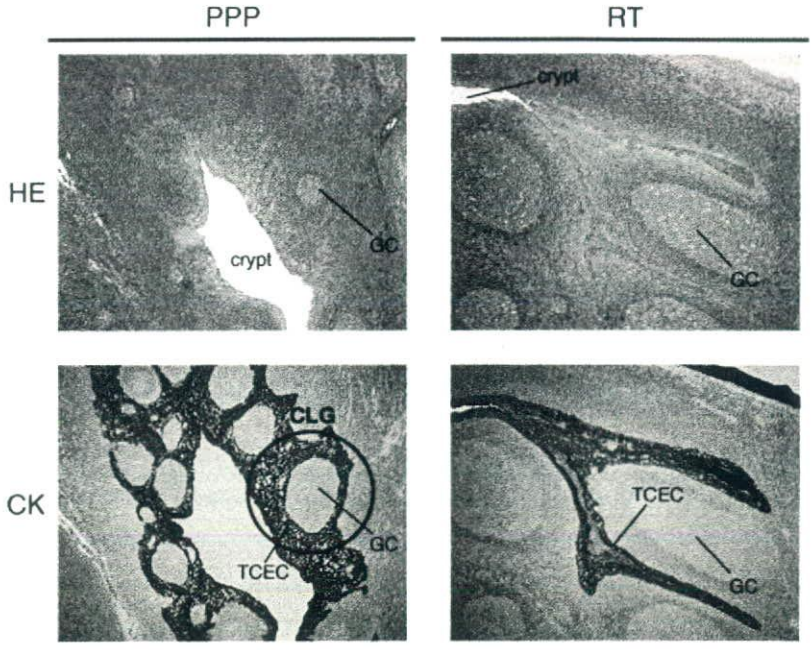

Figure 2. Histopathology focusing on relationships of the structures of lymphoid follicles and TCECs in tonsils of patients with PPP and RT. PPP tonsils (case 6) shown on the left contain lymphoid follicles with relatively small germinal centres (GC) along crypts, some of which were recognized as circular lymphoepithelial lesions with germinal centres (CLG) demarcated by TCECs, as indicated in the circle. In contrast, RT tonsils (case 19) on the right had lymphoid follicles with larger GCs that were infrequently surrounded by epithelial cells. PPP tonsils had thicker lymphoepithelial symbiosis (LES) than that of RT tonsils. Upper panels show haematoxylin and eosin (HE) staining of the tissue sections of tonsils. Lower panels depict immunostaining with the anti-cytokeratin (CK) MAb KL-1 of the respective areas of the upper panels. Original magnification: ×200

Histopathological characteristics of tonsils of PPP

We next investigated the features of surgically resected tonsils in PPP and RT cases as a control. In the tissue sections of PPP tonsils, we often found small lymphoid follicles along the crypts (Figure 2). In contrast, those around the crypts of RT tonsils seemed to be organized in larger and wider forms. Immunostaining analysis using anti-cytokeratin MAbs showed that lymphoid follicles with germinal centres in PPP tonsils were often surrounded by epithelial cells contiguous to the crypts (ie TCECs), whereas such structures were infrequently observed in RT tonsils. We designated this type of histological finding in PPP tonsils as 'a circular lymphoepithelial lesion with a germinal centre (CLG)'. To evaluate the functional significance of CLG in PPP cases, we counted the number of CLGs and also the total number of lymphoid follicles with a germinal centre (GC) in each case of PPP and RT (Table 2). Statistical analysis revealed that the raw number of GCs in PPP tonsils (average 60.0) was not significantly different from that of RT tonsils (average 75.5; $p = 0.305$). These probably accounted for the chronic inflammation similarly occurring in tonsils of both PPP and RT. In contrast to the situation for GCs, the raw number of CLGs in PPP tonsils (average 7.2) was larger than that in RT tonsils (average 2.1; $p < 0.001$). We therefore considered CLG to be a structure unique to the chronic inflammatory responses in PPP tonsils. To verify the results in terms of artefacts during the preparation of the tissue sections, the ratio of the raw numbers of CLGs to the total numbers of GCs (CLG/GC ratio)

Table 2. The number of GCs and CLGs in each case of PPP and RT

PPP				RT			
Case No	GC (R No)	CLG (R No)	CLG/GC (%)	Case No	GC (R No)	CLG (R No)	CLG/GC (%)
1	24	1	4.2	1	21	0	0.0
2	130	10	7.7	2	86	1	1.2
3	23	0	0.0	3	19	0	0.0
4	14	5	35.7	4	71	2	2.8
5	101	6	5.9	5	22	0	0.0
6	160	54	33.8	6	180	4	2.2
7	110	40	36.4	7	18	1	5.6
8	68	9	13.2	8	46	0	0.0
9	21	5	23.8	9	40	3	7.5
10	20	0	0.0	10	52	3	5.8
11	135	8	5.9	11	82	2	2.4
12	49	8	16.3	12	24	1	4.2
13	92	16	17.4	13	44	0	0.0
14	16	7	43.8	14	132	1	0.8
15	67	4	6.0	15	21	0	0.0
16	98	8	8.2	16	108	0	0.0
17	6	0	0.0	17	32	1	3.1
18	21	4	19.0	18	56	1	1.8
19	70	12	17.1	19	69	0	0.0
20	42	3	7.1	20	90	9	10.0
21	123	11	8.9	21	38	1	2.6
22	106	4	3.8	22	18	0	0.0
23	25	1	4.0	23	49	1	2.0
24	9	0	0.0	24	118	13	11.0
25	40	5	12.5	25	128	5	3.9
26	46	1	2.2	26	116	4	3.4
27	35	6	17.1	27	71	0	0.0
28	120	8	6.7	28	262	2	0.8
29	18	2	11.1	29	3	0	0.0
30	47	3	6.4	30	56	0	0.0
31	44	2	4.5	31	73	1	1.4
32	23	0	0.0	32	118	1	0.8
33	12	0	0.0	33	106	0	0.0
34	97	12	12.4	34	225	11	4.9
35	95	2	2.1	35	15	0	0.0
36	53	2	3.8	36	108	6	5.6
Average	60.0	7.2	11.0	Average	75.5	2.1	2.3

CLG/GC ratio (%) means the raw number of CLGs per raw number of GCs. R No = raw number; GC = germinal centre; CLG = circular lymphoepithelial lesion with germinal centre.

was further examined in every case of PPP and RT. Interestingly, it was found that the CLG/GC ratio of PPP was clearly higher than that of RT tonsils, in agreement with the comparison of the raw numbers of CLGs in PPP and RT tonsils (Figure 3A). Collectively, these data implied that CLG, as a unique form of lymphoepithelial interaction, would markedly contribute to the inflammatory process of PPP tonsils rather than that of RT tonsils. Based on these results, the relationship of the CLG/GC ratio to the improvement period was investigated to determine the clinicopathological significance of CLG in PPP cases with a favourable prognosis for a PGA change to be 'cleared', 'excellent' or 'good'. We found, to our surprise, that the CLG/GC ratio was inversely related to the improvement period after tonsillectomy (Figure 3B), further indicating the active involvement of CLG in the pathogenesis of PPP, possibly in association with the preservation of abnormal humoral immune responses.

Functional characteristics of epithelial cells constituting CLG of tonsils of PPP

To address the question of whether there was any difference in the inflammatory process related to the formation of CLGs in PPP tonsils, we established primary cultures of TCECs derived from PPP and RT (PPP-TCECs and RT-TCECs, respectively) to investigate their functions (Figure 4A). We then examined the transcription levels of various cytokines including interleukins in these primary TCECs by using specific PCR primers. High levels of the transcripts of IL-6 were detected in primary PPP-TCECs in comparison to the levels of IL-6 in primary RT-TCECs (Figure 4B). Accordingly, quantitative PCR analysis of primary PPP-TCECs indicated levels of IL-6 transcripts higher than those of primary RT-TCECs (Figure 4C). These findings were further supported by ELISA of the culture supernatants of primary TCECs of PPP and RT tonsils (Figure 4D). Immunohistochemical analysis

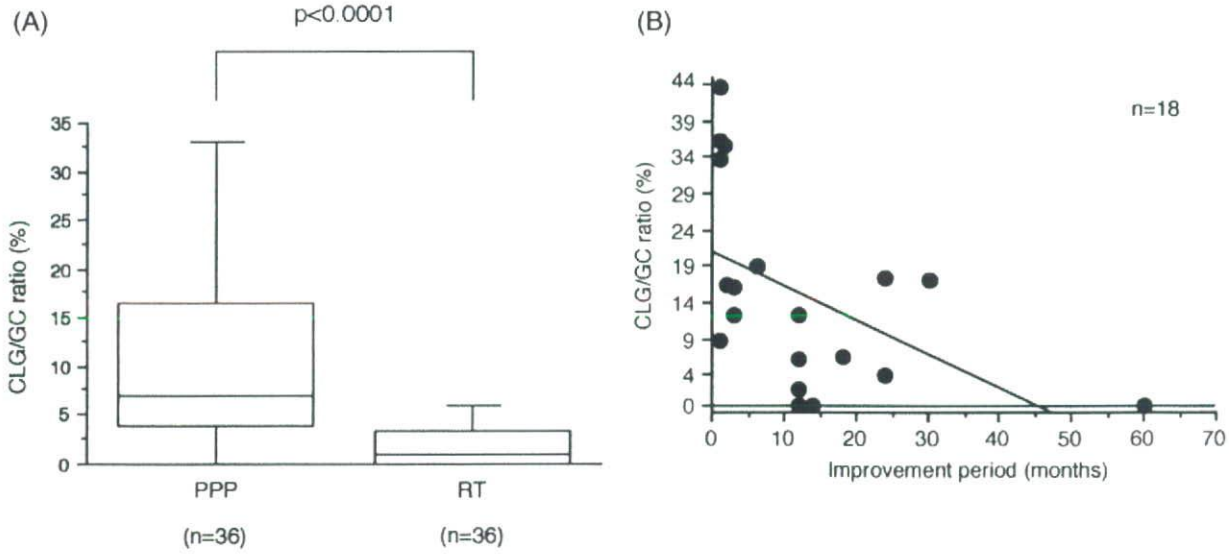

Figure 3. Involvement of tonsillar CLG in the pathogenesis of PPP. (A) The CLG/GC ratio of PPP tonsils was greater than that of RT tonsils, with statistical significance ($p < 0.0001$). Median values are shown by horizontal lines; the box represents values between the 25th and the 75th percentiles; and the lower and upper bars indicate the tenth and 90th percentiles, respectively. (B) The CLG/GC ratio was inversely related to the improvement period after surgical resection of tonsils (correlation coefficient: -0.511, $p < 0.024$). Favourable cases of patients with PPP were analysed ($n = 18$)

also showed high expression of IL-6 when compared with that of RT tonsils (Figure 4E). Thus, TCECs of PPP tonsils would secrete IL-6 to foster reacting B cells within CLGs. Factors affecting lymphoid follicle formation such as IL-7 and IL-11 were not significantly increased in PPP-TCECs (Figure 4B). The levels of other factors influencing Th2 responses such as IL-4 and IL-5 also showed insignificant differences between PPP- and RT-TCECs.

Role of p53-related transcription factors in TCECs in PPP tonsils

We further investigated regulatory factors determining the functional properties of TCECs of PPP tonsils. In recent investigations, the p53-related molecules p63 and p73 have been studied as epitheliotropic transcription factors [21]. When we examined the expression of p63 and p73 in the tissue sections of tonsils, we found high expression of these factors in the nuclei of epithelial cells around CLGs in PPP tonsils (Figures 5A and 5B). On the other hand, p63 and p73 were scattered more faintly and were localized in the nuclei of epithelial cells in RT tonsils. When transcription analysis was employed on primary TCECs, transcripts of p63 and p73 were clearly detected in PPP-TCECs rather than in RT-TCECs under PCR conditions with a low number of cycles (25 cycles) (Figure 5C). These findings suggested that p63 and p73 of TCECs might have a role in the histogenesis of CLG in PPP tonsils.

To determine the regulatory roles of p63 and p73 in the expression of IL-6, we studied two different types of human epithelial cells into which cDNA encoding p63 or p73 was transiently introduced. Intriguingly, both p63 and p73 had the capacity to up-regulate the transcription levels of IL-6 in these epithelial cells (Figure 5D). These findings were compatible with our previous results showing that when p63 was transfected into thymic epithelial cells, a large amount of IL-6 was produced in the culture supernatant [16]. We next conducted reporter analysis of the *IL-6* gene, which indicated that p63 could up-regulate the transcription levels of IL-6 through sites including NF-κB recognition sequences (Figure 5E). Taken together, these results suggest that p63 and p73 may have a role in the up-regulation of the level of IL-6 in epithelial cells.

Discussion

The present study is, to the best of our knowledge, the first to describe the particular tissue structure of CLG and the histogenesis of LES in PPP tonsils. The CLG of the PPP tonsil is considered to represent a unique form of lymphoid follicle in the TCEC meshwork. This situation of lymphoid follicles in PPP tonsils may reflect their appearance, with a relatively smaller conformation than those of RT tonsils. To facilitate the formation of lymphoid follicles within the restricted area of the TCEC meshwork, B cells probably require a stimulating factor(s) in the PPP tonsil. It is well known that various epithelial cells secrete IL-6 [22]. In this context, we could identify the surrounding TCECs as a potential producer of IL-6, an important cytokine for B-cell maturation, by employing primary culture techniques. Such B cells might be related to the production of autoantibodies reacting to a certain antigen(s) specifically presented by palmoplantar epithelial cells. T cells are considered in the pathogenesis of PPP tonsils. IL-6 is also thought as an inducer of certain T-cell populations including T_H-17 cells which

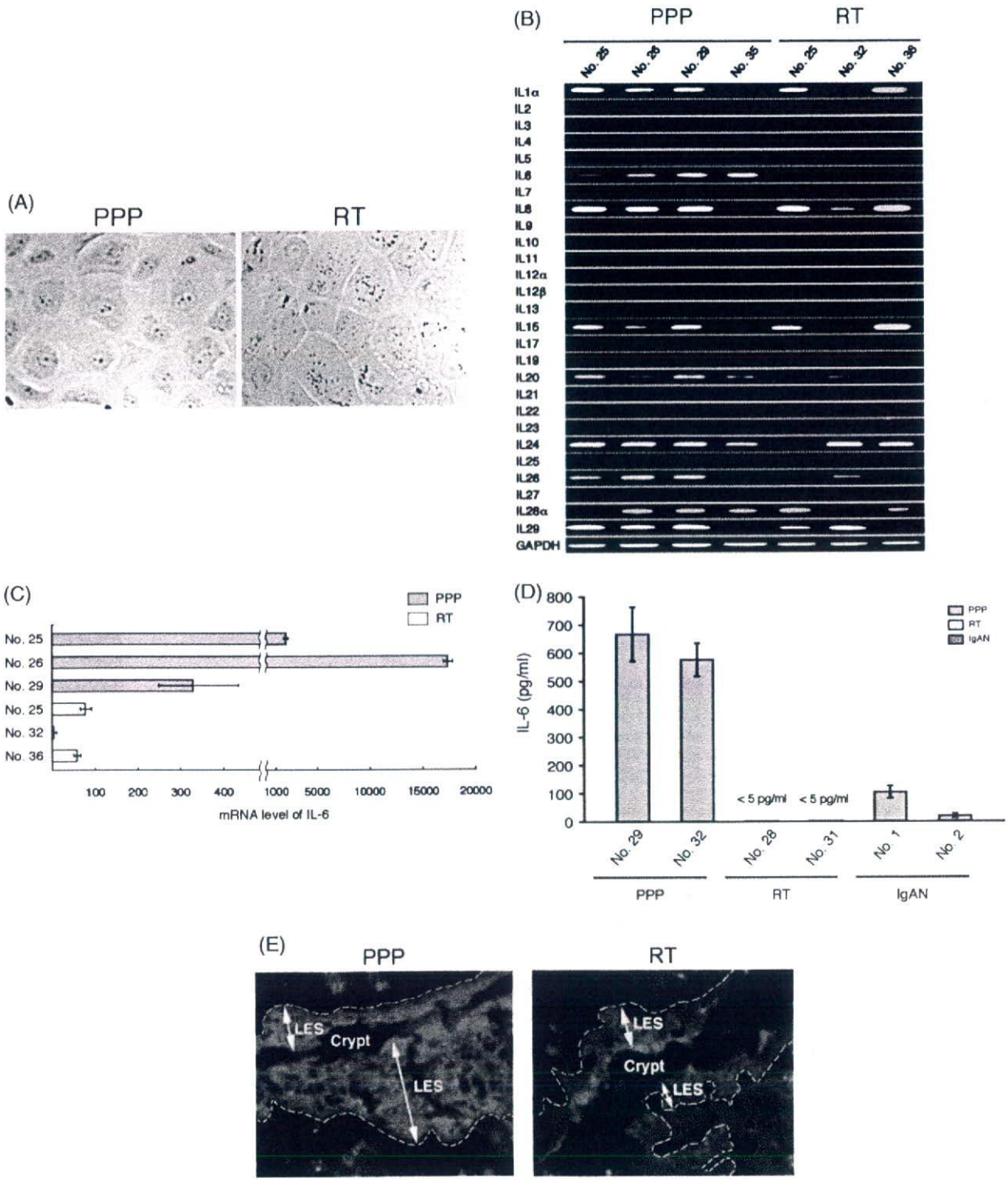

Figure 4. Expression and production of IL-6 in TCECs of PPP tonsils. (A) Representative images of PPP-TCECs (case 26) and RT-TCECs (case 32) obtained from day 5 after the initial primary culture are shown on the left and right, respectively. The morphology of PPP- and RT-TCECs of the primary culture was not significantly different, both showing flat and polygonal shaped cells with round nuclei. Original magnification: ×450. (B) Expression of various types of interleukin in primary cultures of PPP- and RT-TCECs as assessed by RT-PCR analysis. PCR was performed for 35 cycles and GAPDH was used as a control. (C) Quantification of IL-6 at the transcription level in PPP- and RT-TCECs by real-time PCR analysis. IL-6 transcripts were detected in PPP-TCECs at higher levels than in RT-TCECs. (D) Quantification of IL-6 at the protein level in the culture supernatants of PPP- and RT-TCECs as assessed by ELISA. Two cases of TCECs from IgAN tonsils were also employed as a control. (E) Immunohistochemistry of frozen sections of PPP (case 30) and RT (case 33) tonsils after staining with anti-IL-6 MAbs. IL-6 was detected in TCECs of LES of PPP tonsil rather than in those of RT tonsil. Original magnification: ×400. Case numbers are depicted for PPP and RT cases in B, C, and D

are thought to serve autoimmunity [23,24]. In fact, enlargement of the T-cell-dependent area in PPP tonsils has been previously reported [25]. In accordance, our flow cytometric analysis showed that the number of $CD4^+$ T-cell populations was larger in the PPP tonsil than in the RT tonsil (data not shown).

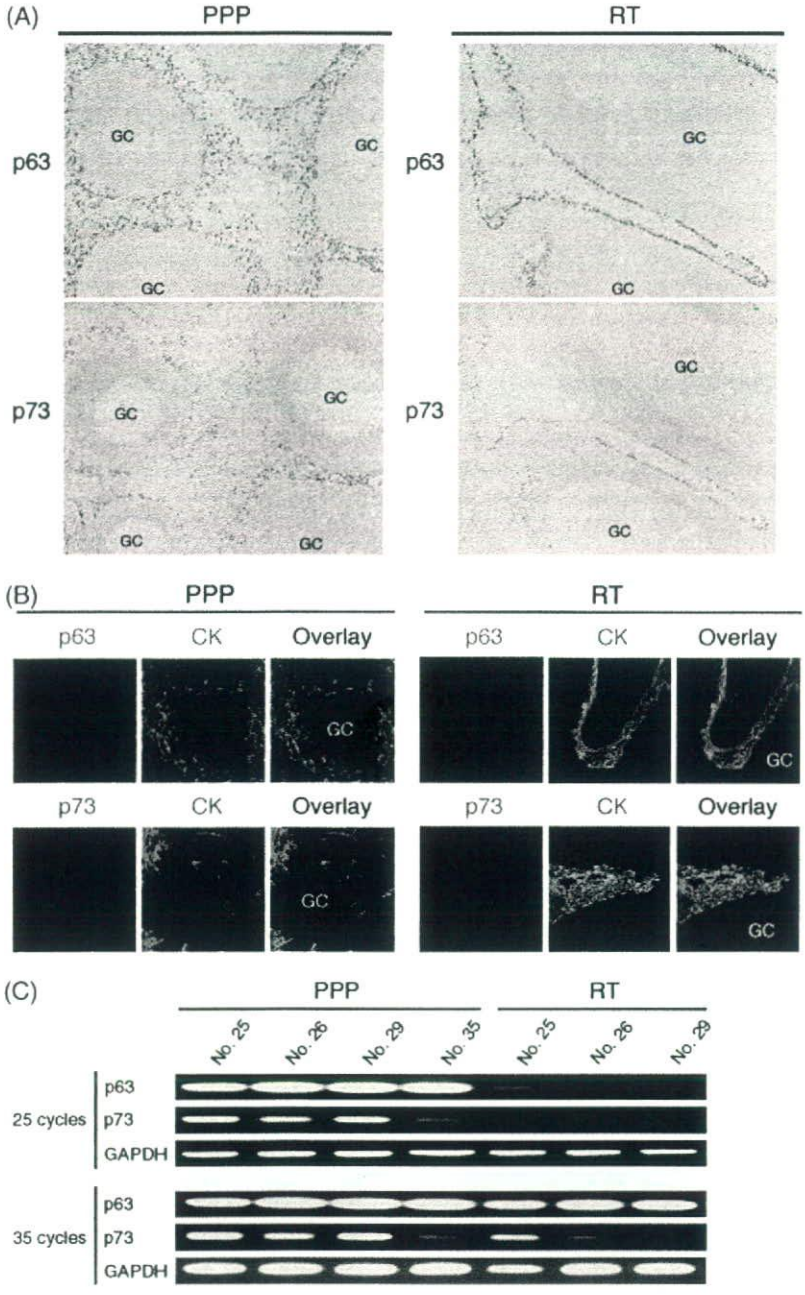

Figure 5. Expression of p63 and p73 of p53-related factors in TCECs of PPP tonsils. (A) p63 and p73, shown in the upper and lower panels, respectively, were extensively expressed in cells around lymphoid follicles with germinal centres (GC) in PPP tonsil (case 6), whereas there seemed to be much less expression of p63 and p73 in RT tonsil (case 19). Formalin-fixed, paraffin-embedded tissue sections of tonsils were immunostained and signals were detected by using DAB. Original magnification: ×400. (B) Confocal laser microscopy after double immunostaining of frozen sections of PPP (case 30) and RT (case 33) tonsils shown in the left and right panels, respectively. TCECs of PPP tonsil showed high expression of p63 and p73 in the nuclei of PPP-TCECs, while low expression of p63 and p73 in the nuclei of TCECs of RT tonsil was observed. CK = cytokeratin. Original magnification: ×400. (C) After a low number of PCR cycles (25 cycles), p63 and p73 were preferentially detected in PPP-TCEC rather than in RT-TCEC primary cultures as assessed by RT-PCR analysis. When the number of PCR cycles was increased to 35, p63 and p73 were detectable in primary RT-TCECs. GAPDH was used as a control. (D) Quantification of IL-6 at the transcriptional level in HSC-3 and HaCaT cells transfected by plasmid DNAs encoding p63 or p73 as assessed by real time-PCR analysis. VC = vector control. (E) Reporter analysis of the promoter region of the *IL-6* gene using HSC-3 cells. Promoter activity of the parental pIL6-luc651 construct (containing a full-length promoter, −651/+1), which contains 651 bp directly upstream of the transcription initiation site of the *IL-6* gene, was enhanced by the introduction of p63. While promoter activity of the deletion construct of the AP-1 site (pIL6-luc65ΔAP-1) was still enhanced by the introduction of p63, promoter activity of the deletion construct of the NF-κB site (pIL6-luc65ΔNF-κB) by p63 was clearly abrogated. Data shown are from three independent experiments. RLU = relative luciferase unit; VC = vector control

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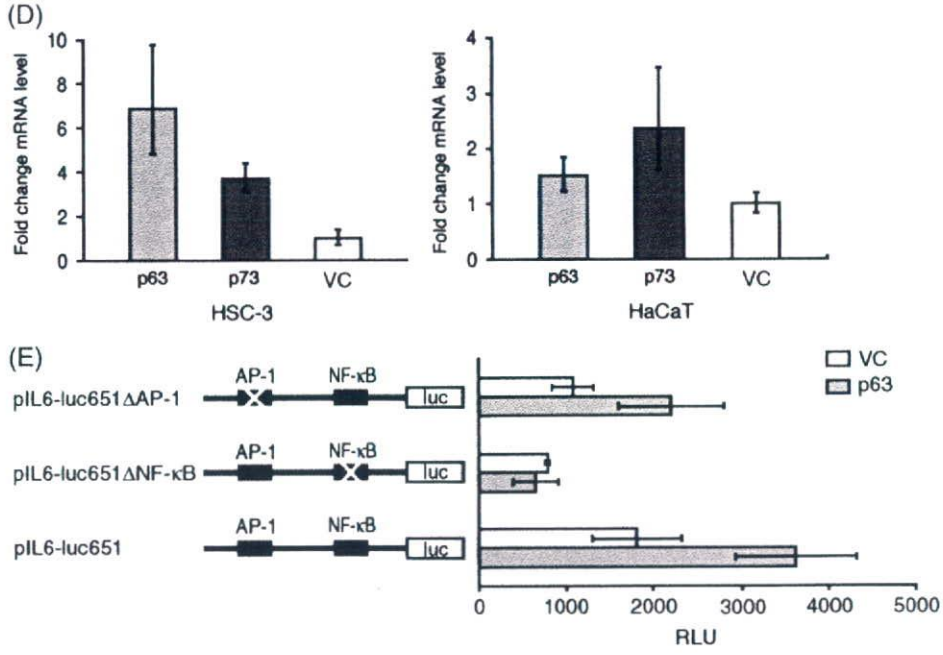

Figure 5. Continued

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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Overexpression of papillomavirus binding factor in Ewing's sarcoma family of tumors conferring poor prognosis

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Abstract. Ewing's sarcoma family of tumors (ESFT) is comprised of highly malignant bone and soft tissue tumors in children and young adults. Despite intensive treatments for patients with ESFT, disease which presents with metastatic spread or relapses after primary treatment remains incurable in the majority of cases, indicating the importance of efforts to develop new treatment modalities, including immunotherapy. The present study was designed to examine the expression profile of papillomavirus binding factor (PBF), which we previously defined as an osteosarcoma-associated antigen, and its prognostic significance for patients with ESFT. Biopsy specimens from 20 ESFT were stained with an anti-PBF antibody. Survival was estimated using Kaplan-Meier plots and the prognostic significance of several variables, including the expression status of PBF, on disease-free and overall survival was determined by univariate analysis using the log-rank test. Of 20 specimens, 18 (90%) reacted positively to the anti-PBF antibody. Fifteen specimens (75%) were graded as PBF overexpression. Of the 11 variables analyzed, stage III disease, inadequate surgical margins and PBF overexpression were significantly associated with decreased disease-free and overall survival. None of the other variables, including age, gender, origin of tumor, tumor site or levels of LDH, ALP, CRP and ESR, showed any significant association. These findings indicate that the overexpression of PBF is a factor indicative of poor prognosis in ESFT. PBF may also serve as a putative target antigen in immunotherapy for patients with ESFT that have a poor prognosis and PBF overexpression.

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Introduction

Ewing's sarcoma family of tumors (ESFT) is comprised of highly malignant bone and soft tissue tumors in children and young adults (1). Although systemic adjuvant chemotherapy has significantly improved the prognosis for patients with ESFT, disease which presents metastatic spread or relapses after primary treatment remains incurable in the majority of cases (2,3).

After a nearly 30-year interval from the initial immunotherapeutic trials for osteosarcoma (4,5), immunotherapy has recently re-emerged as a targeted therapy for bone and soft tissue sarcomas, including ESFT (6,7). In a clinical trial with ESFT patients, peptides derived from the junction regions of EWS-FLI1 fusion genes were used as ESFT-specific vaccines (6). However, clinical response was seen in only 1 of the 12 patients enrolled, suggesting the importance of finding further efficacious antigens and of developing antigenic peptide vaccines (8).

Recently, papillomavirus binding factor (PBF) was identified as an autologous cytotoxic T-lymphocyte-defined osteosarcoma antigen (9). It was originally defined as a transcriptional regulator of genomic DNA of human papillomavirus type 8 (10). The antigenic and oncologic roles of PBF in ESFT remain uncertain. In this study, with the aim of gaining basic information on these aspects of PBF, we examined its expression in 20 cases of ESFT and analyzed its prognostic significance.

Materials and methods

This study was approved according to institutional guidelines for the use of human subjects in research. Patient specimens were analyzed after informed written consent was obtained from the patients or their families.

Patients and samples. Between 1979 and 2005, 20 consecutive patients with ESFT were treated at Keio University hospital. The clinical picture is summarized in Table I. There were 12 male and 8 female patients, with an average age at diagnosis of 23.9 years (range, 1-63 years). Twelve tumors arose from bone and the remaining 8 from soft tissue. Eight tumors were located in the trunk and 13 in the extremities. Fusion genes, including

Table I. Clinical characteristics and expression status of PBF in patients with ESFT.

Patient	Age (years)	Gender	Location	Stage[a]	Fusion gene	Treatment	PBF status	Event-free survival (mos)	Overall survival (mos)	Prognosis
Bone tumor										
1	1	M	Right tibia	IIB	ND	Chx (VAC) + Amp + Rx	++	179	179	CDF
2	36	F	Right femur	IIB	ND	Chx (A-VAC) + Amp+ Rx	-	174	174	CDF
3	16	M	Right humerus	IIB	ND	Chx (A-VAC) + WE + Rx	+	250	250	CDF
4	13	M	Left tibia	IIB	ND	Chx (CYVADIC) + WE + Rx	+++	166	166	CDF
5	17	M	Thoracic spine	IIB	EWS/ERG	Chx (T11) + ME + Rx	+++	8	45	DOD
6	16	M	Sacrum	IIB	ND	Chx (T11) + WE + Rx	++	99	99	CDF
7	16	F	Left tibia	IIIB	EWS/FLI-1	Chx (KS-1) + WE	+++	0	16	DOD
8	22	F	Right fibula	IIB	EWS/FLI-1	Chx (KS-1) + WE	+++	108	108	CDF
9	18	M	Left 5th rib	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	+++	105	105	CDF
10	18	M	Right fibula	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	+++	7	21	DOD
11	24	M	Left tibia	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	-	15	15	CDF
12	23	M	Pelvis	IIIB	ND	Chx (KS-1) +Rx	+++	0	17	DOD
Soft tissue tumor										
13	29	M	Right thigh	IIIB	EWS/FLI-1	Chx (KS-1) + ILE +Rx	+++	0	12	DOD
14	63	F	Paraspine	IIB	ND	Chx (KS-1) + ILE +Rx	+++	0	71	DOD
15	20	F	Right tibia	IIB	EWS/FLI-1	Chx (KS-1) + ME +Rx	+++	9	14	DOD
16	56	F	Right forearm	IIIB	ND	Chx (CYVADIC + KS-1) + WE	+++	0	11	DOD
17	7	M	Paraspinal cord	IIB	Not detected	Chx (KS-1) + ME	+++	14	16	DOD
18	11	F	Paraspinal cord	IIB	EWS/FLI-1	Chx (KS-1) + ILE +Rx	+++	17	22	DOD
19	35	M	Left femur	IIB	ND	Chx (KS-1) + WE	+++	126	126	CDF
20	36	F	Right upper arm	IIB	Not detected	Chx (KS-1) + WE	+++	101	101	CDF

[a]Stage was determined according to Enneking's surgical staging system. ND, not determined; Chx, chemotherapy; Rx, radiotherapy; ILE, intralesional resection; ME, marginal excision; WE, wide excision; CDF, continuous disease free; DOD, death of the disease.

EWS/FLI-1, EWS/ERG, EWS/ETV1 and EWS/E1AF, were determined by RT-PCR (11) in 9 of the 11 cases for which frozen biopsy specimens were available. According to Enneking's surgical stage (12), 16 patients were in stage IIB and 4 in IIIB. Treatment consisted of chemotherapy and surgery for 6, chemotherapy, surgery, and radiotherapy for 13, and chemotherapy and radiotherapy for 1. The chemotherapy protocols used were VAC (13), A-VAC (14), CYVADIC (15), T11 (16) and KS1, which is a modified protocol of New A3 (17). Radiotherapy (50-60 Gy) was instituted postoperatively. The average follow-up period after diagnosis was 78.4 months (range, 11-250 months).

Immunohistochemistry. Polyclonal antibody against PBF was generated previously (9). Formalin-fixed paraffin-embedded sections of biopsy specimens were boiled for 20 min in a microwave oven for antigen retrieval. Sections were blocked with 1% non-fat dry milk and stained with streptavidin-biotin-complex (Nichirei), followed by hematoxylin staining as previously described (9). The reactivity of the anti-PBF polyclonal antibody was determined by staining the nuclei. The expression status of PBF was estimated based on the number of tumor cells according to Ahmed *et al* (18) (Fig. 1): the presence of ≤5% of positively-stained tumor cells was represented by a minus (-), 6-25% by a plus (+) and 26-60% by ++. Overexpression, a number of positive tumor cells >60%, was represented by +++.

Clinicopathological analysis. Survival was estimated using Kaplan-Meier plots. Univariate analysis with the log-rank test