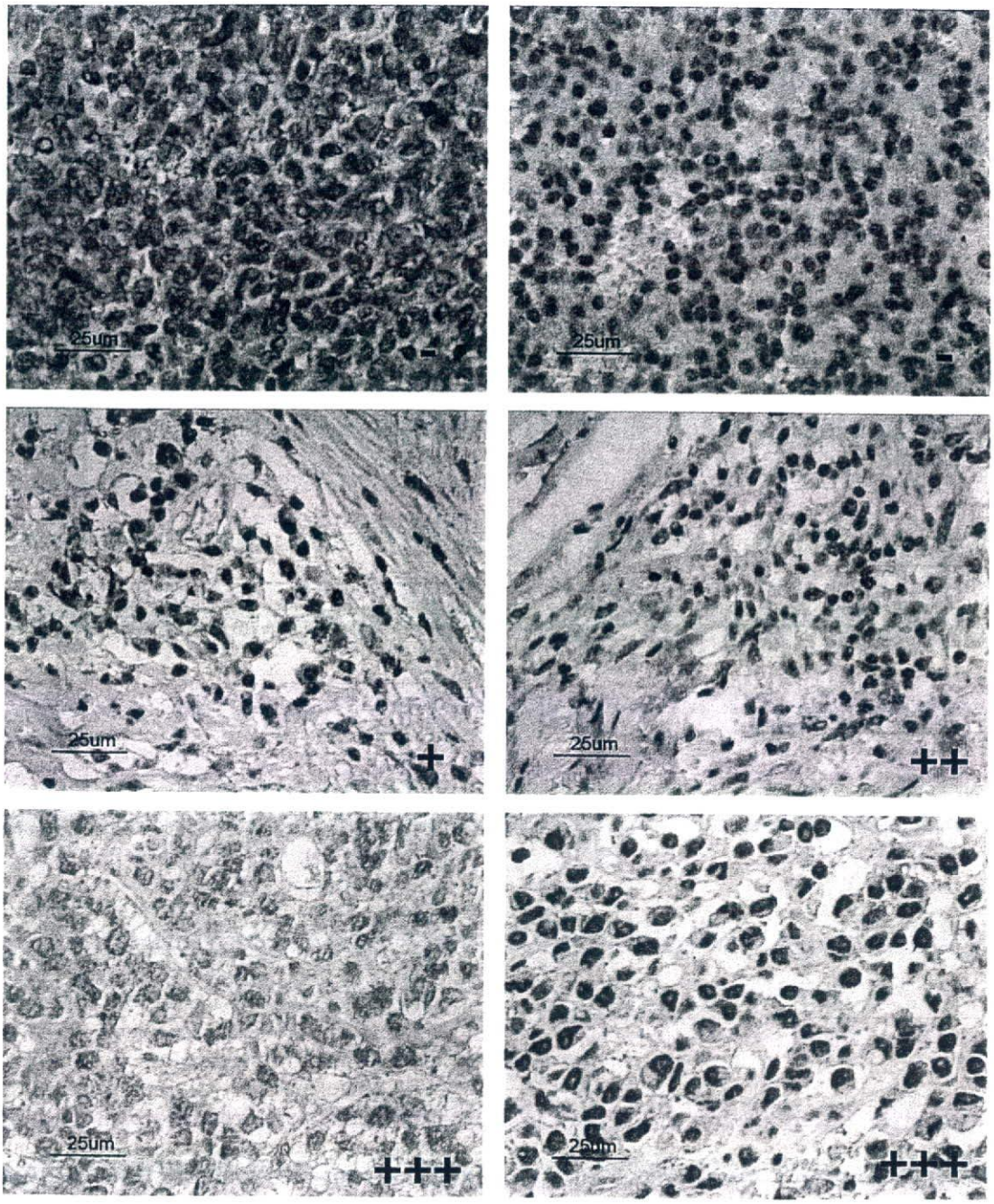


Figure 1. Immunohistochemical grading of PBF expression. Representative sections of ESFT specimens stained with anti-PBF antibody. Tumor cells showing positive reactivity in the nuclei were counted: -, ≤5% positive cells; +, 6-25% positive cells; ++, 26-60% positive cells; +++, >60% positive cells.

(19) was used to determine the prognostic significance of the following variables for disease-free and overall survival: age (≥30 or <30), gender (female or male), tumor site (trunk or limb), origin of tumor (bone or soft tissue), stage (I, II or III), laboratory parameters (within or higher than normal range), surgical margin (adequate or inadequate) and PBF expression status (-, +, ++ or +++). Laboratory parameters included LDH, alkaline phosphatase (ALP), C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) at 1 h. Analysis of the surgical margins was performed in 16 stage IIB patients. Wide excision and amputation were regarded as adequate margins, whereas intralesional and marginal excision were regarded as inadequate. A probability of <0.05 was considered to be statistically significant.

Results

PBF expression in ESFT. To determine the expression profiles of PBF in ESFT, we stained 20 ESFT biopsy specimens with anti-PBF antibody. Of these, 18 (90%) reacted positively to the anti-PBF antibody where the nuclei of tumor cells were stained (Fig. 1 and Table I). Two specimens were grade ++ and 1 was +. Fifteen specimens (75%) were graded as +++, indicating PBF overexpression.

Prognostic significance of PBF expression in ESFT. We then analyzed the prognostic significance of several variables, including the overexpression of PBF (grade +++). Overall survival rates of the 20 patients with ESFT were 53.1 and

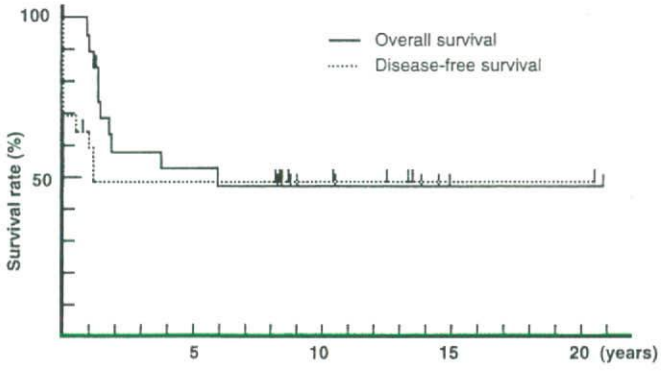
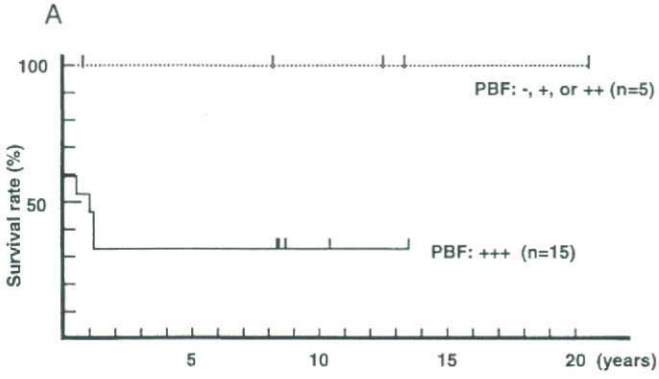


Figure 2. Overall survival of 20 patients with ESFT. Overall survival was estimated using Kaplan-Meier plots. The date of histological diagnosis was used as time 0.



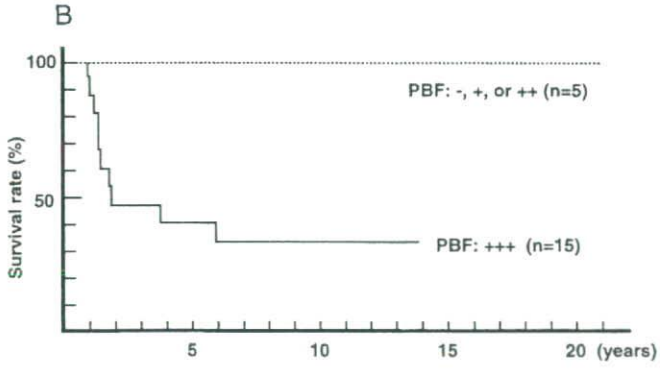


Figure 3. Survival curves of 20 patients with ESFT stratified by PBF expression status. (A) Disease-free survival curve. (B) Overall survival curve. Patients were divided according to PBF expression status into 2 groups (PBF expression of -, +, and ++, n=5; PBF expression of +++, n=15). Survival was estimated using Kaplan-Meier plots.

47.8% at 5 and 10 years, respectively (Fig. 2). Of the 11 variables analyzed, stage III, an inadequate surgical margin and PBF overexpression of were significantly associated with decreased disease-free and overall survival (Fig. 3 and Table II). Of note, the overall survival of the 15 patients with PBF overexpression was 33.3% at 10 years, whereas 5 patients remained continuously disease free during the entire follow-up period. None of the other variables, including age,

Table II. Univariate analysis of potential unfavorable prognostic factors.

Factor	No. of patients	P-value: Disease-free survival	P-value: Overall survival
Age (≥30 years)	5	0.71	0.54
Male sex	12	0.31	0.45
Soft tissue tumor	8	0.09	0.06
Trunk tumor	7	0.24	0.47
Stage III	4	0.006	P<0.001
High ALP	7	0.61	0.56
High LDH	8	0.34	0.51
High CRP	8	0.27	0.35
High ESR	8	0.30	0.46
Inadequate surgical margin[a]	6	P<0.001	P<0.001
PBF +++	15	0.04	0.04

[a]Marginal excision and intralesional excision were regarded as inadequate surgical margin and 16 stage IIB patients having undergone surgical treatment were analyzed.

gender, origin of tumor, tumor site and levels of LDH, ALP, CRP and ESR, showed a significant association to disease-free or overall survival.

Discussion

By staining 20 biopsy specimens of ESFT treated at a single institute with an antibody against PBF we found i) that PBF was expressed in 18 ESFT specimens (90%), including 15 specimens (75%) with grade +++ overexpression, and ii) that PBF overexpression was significantly associated with the decreased disease-free and overall survival of patients. These findings indicate that the overexpression of PBF is a factor of poor prognosis for ESFT. PBF, which was originally defined as an osteosarcoma-associated antigen (9), may also serve as a putative target antigen in immunotherapy for patients with ESFT and PBF overexpression, which confers a poor prognosis.

Compared to malignant melanoma and epithelial cancers, there is a marked delay in the identification of tumor-associated antigens in bone and soft tissue sarcomas (7,8). In ESFT, antigens proven to have specific T cell responses have been limited to EWS-FLI1 fusion gene products (6,20,21). More recently, cancer-testis antigens (also termed cancer-germline genes) were defined in 11 of 18 ESFT specimens and included MAGE-A3, A4, A6, A10, A12, C2 and GAGE-1, -2 and -8 (22). However, their expression levels were lower than those of other sarcomas, including osteosarcoma and rhabdomyosarcoma (22).

Among the tumor-associated antigens identified in malignant melanoma, some were later found in solid tumors in a significant association with poor prognosis (Table III). These include cancer-testis antigens MAGE-3 (23,24), MAGE-4

Table III. Tumor-associated antigens showing prognostic significance in solid tumors.

Antigen	Tumor	No. of samples (% positive)	Detection procedure	Refs.
Cancer testis				
MAGE-A3	NSCLC	523 (55.2)	RT-PCR	23
	Pancreatic cancer	57 (44.0)	qRT-PCR	24
MAGE-A4	NSCLC[a]	19 (36.8)	RT-PCR	25
	Squamous cell lung carcinoma	153 (56.9)	IHC	26
	Ovarian carcinoma	53 (57.0)	IHC	27
	Bladder carcinoma	908 (4.0)[b]	IHC	28
NY-ESO-1	NSCLC	523 (26.6)	RT-PCR	23
Overexpression				
PRAME	Neuroblastoma	95 (33.7)[b]	qRT-PCR	29
WT1	Soft tissue sarcomas	52 (32.7)[b]	qRT-PCR	30
	Osteosarcoma	37 (27.0)[b]	IHC	31
PBF	ESFT	20 (75.0)[b]	IHC	Present study

NSCLC, non-small cell lung carcinoma; qRT-PCR, quantitative real-time RT-PCR; IHC, immunohistochemistry. [a]Advanced stage cancers. [b]Percentage of samples with overexpression.

(25-28) and NY-ESO-1 (23), and an overexpression antigen, PRAME (29). Apart from melanoma-derived antigens, it has been reported that the overexpression of WT1 is associated with poor prognosis in bone and soft tissue sarcomas (30,31) (Table III). Though WT1 was originally defined as the tumor-suppressor gene responsible for Wilms' tumor, antigenic peptides derived from it have recently been used as vaccines for hematopoietic malignancies and solid cancers (32). PBF is classified as an overexpression antigen as it is detected in some normal tissues by RT-PCR (9). In addition to ESFT, expression of PBF was found to be significantly associated with poor prognosis in patients with osteosarcoma, with statistical significance (Tsukahara *et al*, unpublished data).

The antigenic role of PBF in patients with ESFT remains to be defined by T cell responses specific to PBF-derived peptides. To this end, we recently developed a limiting dilution/mixed lymphocyte peptide culture/tetramer/cytotoxicity assay by which the frequency and anti-tumor cytotoxicity of peripheral T lymphocytes directed against PBF were determined in patients with osteosarcoma (Tsukahara *et al*, unpublished data). This approach is also applicable to patients with ESFT. Another limitation of the present study is the small number of samples used, due mainly to the rare occurrence of ESFT in the Japanese population. It is, however, based on a consecutive series of patients treated at a single institute for more than 25 years.

In conclusion, the present analysis serves as a pilot study showing the prognostic significance of PBF for patients with ESFT. Large-scale analyses need to be conducted to verify the present findings if PBF-targeted immunotherapy for patients with ESFT is to be developed.

Acknowledgments

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References

1. Bernstein M, Kovar H, Paulussen M, Randall RL, Schuck A, Teot LA and Juergens H: Ewing's sarcoma family of tumors: current management. Oncologist 11: 503-519, 2006.
2. Miser JS, Krailo MD, Tarbell NJ, *et al*: Treatment of metastatic Ewing's sarcoma or primitive neuroectodermal tumor of bone: evaluation of combination ifosfamide and etoposide - a Children's Cancer Group and Pediatric Oncology Group study. J Clin Oncol 22: 2873-2876, 2004.
3. Bernstein ML, Devidas M, Lafreniere D, *et al*: Intensive therapy with growth factor support for patients with Ewing tumor metastatic at diagnosis: Pediatric Oncology Group/Children's Cancer Group Phase II Study 9457 - a report from the Children's Oncology Group. J Clin Oncol 24: 152-159, 2006.
4. Marcove RC, Mike V, Huvos AG, Southam CM and Levin AG: Vaccine trials for osteogenic sarcoma. A preliminary report. CA Cancer J Clin 23: 74-80, 1973.
5. Neff JR and Enneking WF: Adoptive immunotherapy in primary osteosarcoma. An interim report. J Bone Joint Surg Am 57: 145-148, 1975.
6. Dagher R, Long LM, Read EJ, *et al*: Pilot trial of tumor-specific peptide vaccination and continuous infusion interleukin-2 in patients with recurrent Ewing sarcoma and alveolar rhabdomyosarcoma: an inter-institute NIH study. Med Pediatr Oncol 38: 158-164, 2002.
7. Maki RG: Future directions for immunotherapeutic intervention against sarcomas. Curr Opin Oncol 18: 363-368, 2006.
8. Kawaguchi S, Wada T, Tsukahara T, Ida K, Torigoe T, Sato N and Yamashita T: A quest for therapeutic antigens in bone and soft tissue sarcoma. J Transl Med 3: 31, 2005.
9. Tsukahara T, Nabeta Y, Kawaguchi S, *et al*: Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. Cancer Res 64: 5442-5448, 2004.
10. Boeckle S, Pfister H and Steger G: A new cellular factor recognizes E2 binding sites of papillomaviruses which mediate transcriptional repression by E2. Virology 293: 103-117, 2002.
11. Urano F, Umezawa A, Yabe H, Hong W, Yoshida K, Fujinaga K and Hata J: Molecular analysis of Ewing's sarcoma: another fusion gene, EWS-E1AF, available for diagnosis. Jpn J Cancer Res 89: 703-711, 1998.
12. Enneking WF: A system of staging musculoskeletal neoplasms. Clin Orthop Relat Res: 9-24, 1986.
13. Jaffe N, Paed D, Traggis D, Salian S and Cassady JR: Improved outlook for Ewing's sarcoma with combination chemotherapy (vincristine, actinomycin D and cyclophosphamide) and radiation therapy. Cancer 38: 1925-1930, 1976.
14. Nesbit Jr ME, Gehan EA, Burgert Jr EO, *et al*: Multimodal therapy for the management of primary, nonmetastatic Ewing's sarcoma of bone: a long-term follow-up of the First Intergroup study. J Clin Oncol 8: 1664-1674, 1990.

15. Wilbur JR, Sutow WW, Sullivan MP and Gottlieb JA: Chemotherapy of sarcomas. Cancer 36: 765-769, 1975.
16. Rosen G: Current management of Ewing's sarcoma. Prog Clin Cancer 8: 267-282, 1982.
17. Kaneko M, Nishihira H, Mugishima H, *et al*: Stratification of treatment of stage 4 neuroblastoma patients based on N-myc amplification status. Study Group of Japan for Treatment of Advanced Neuroblastoma, Tokyo, Japan. Med Pediatr Oncol 31: 1-7, 1998.
18. Ahmed A, Gilbert-Barness E and Lacson A: Expression of c-kit in Ewing family of tumors: a comparison of different immunohistochemical protocols. Pediatr Dev Pathol 7: 342-347, 2004.
19. Kawaguchi S, Wada T, Nagoya S, *et al*: Extraskeletal myxoid chondrosarcoma: a multi-institutional study of 42 cases in Japan. Cancer 97: 1285-1292, 2003.
20. Mackall CL, Berzofsky JA and Helman LJ: Targeting tumor specific translocation in sarcomas in pediatric patients for immunotherapy. Clin Orthop 373: 25-31, 2000.
21. Meyer-Wentrup F, Richter G and Burdach S: Identification of an immunogenic EWS-FLI1-derived HLA-DR-restricted T helper cell epitope. Pediatr Hematol Oncol 22: 297-308, 2005.
22. Jacobs JF, Brasseur F, Hulsbergen-van de Kaa CA, *et al*: Cancer-germline gene expression in pediatric solid tumors using quantitative real-time PCR. Int J Cancer 120: 67-74, 2007.
23. Gure AO, Chua R, Williamson B, *et al*: Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. Clin Cancer Res 11: 8055-8062, 2005.
24. Kim J, Reber HA, Hines OJ, *et al*: The clinical significance of MAGEA3 expression in pancreatic cancer. Int J Cancer 118: 2269-2275, 2006.
25. Yoshida N, Abe H, Ohkuri T, *et al*: Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens and T cell infiltration in non-small cell lung carcinoma and their prognostic significance. Int J Oncol 28: 1089-1098, 2006.
26. Bolli M, Kocher T, Adamina M, *et al*: Tissue microarray evaluation of Melanoma antigen E (MAGE) tumor-associated antigen expression: potential indications for specific immunotherapy and prognostic relevance in squamous cell lung carcinoma. Ann Surg 236: 785-793, 2002.
27. Yakirevich E, Sabo E, Lavie O, Mazareb S, Spagnoli GC and Resnick MB: Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens in serous ovarian neoplasms. Clin Cancer Res 9: 6453-6460, 2003.
28. Kocher T, Zheng M, Bolli M, *et al*: Prognostic relevance of MAGE-A4 tumor antigen expression in transitional cell carcinoma of the urinary bladder: a tissue microarray study. Int J Cancer 100: 702-705, 2002.
29. Oberthuer A, Hero B, Spitz R, Berthold F and Fischer M: The tumor-associated antigen PRAME is universally expressed in high-stage neuroblastoma and associated with poor outcome. Clin Cancer Res 10: 4307-4313, 2004.
30. Sotobori T, Ueda T, Oji Y, *et al*: Prognostic significance of Wilms tumor gene (WT1) mRNA expression in soft tissue sarcoma. Cancer 106: 2233-2240, 2006.
31. Srivastava A, Fuchs B, Zhang K, *et al*: High WT1 expression is associated with very poor survival of patients with osteogenic sarcoma metastasis. Clin Cancer Res 12: 4237-4243, 2006.
32. Sugiyama H: Cancer immunotherapy targeting Wilms' tumor gene WT1 product. Expert Rev Vaccines 4: 503-512, 2005.

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Immunosuppressive effect on T cell activation by interleukin-16- and interleukin-10-cDNA-double-transfected human squamous cell line

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ABSTRACT

It is well known that induction of immunotolerance with allogeneic skin transplantation is generally difficult. This study attempted to find an immunosuppressive protocol for skin allograft rejection involving interleukin-16 (IL-16) and interleukin-10 (IL-10), because both are known to inhibit mixed lymphocyte reaction (MLR). The data indicated that IL-16 enhanced the immunosuppressive effect of IL-10. IL-16-cDNA- and IL-10-cDNA-double-transfected squamous cell carcinoma cell line were used as an *in vitro* model and they produced more than 20 ng/ml of IL-16 and 100 pg/ml of IL-10 in the supernatant, which significantly inhibited MLR and also the activation of allogeneic lymphocytes, which were stimulated directly by allogeneic double-cDNA-transfectant cells. Thus allogeneic skin graft producing IL-16 and IL-10 might have a local immunosuppressive action that could prolong graft survival.

1. Introduction

The transplantation of skin allograft has long been used to treat extensive burns; its biological effects on re-epithelialisation and neovascularisation have been investigated [1–3]. Skin allograft functions as a temporary barrier to bacterial invasion and also water and protein loss, compensating for skin function and improving the general condition of the burn victim. However, skin allograft rejection usually occurs 2–3 weeks after transplantation. Thus it is important to seek how to prolong skin allograft survival.

Previously, we confirmed that interleukin-16 (IL-16), which is considered one of the natural ligands of the CD4 molecule, enhanced an immunosuppressive effect of anti-CD4 monoclonal antibody (mAb). Furthermore, IL-16-cDNA-transfected keratinocyte-equivalent squamous cells directly inhibited allogeneic lymphocyte activation [4]. In this study, we evaluated the effect of another inhibitory cytokine, interleukin-10 (IL-10), which is known to inhibit mixed lymphocyte reaction (MLR) in the T cell response [5]. Our present data suggested that IL-10 in conjunction with IL-16 inhibited MLR more efficiently than either cytokine alone. This implies that allogeneic skin cells producing IL-16 and IL-10 might induce local immunosuppression or local tolerance, and consequently prolong allogeneic skin graft survival. We aimed to show whether IL-16-cDNA- and IL-10-cDNA-double-trans-

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fected keratinocyte-equivalent squamous cells inhibited allogeneic lymphocyte activation more efficiently, which suggests the possible development of an immunoregulatory skin allograft.

2. Materials and methods

2.1. *Recombinant human IL-16 and IL-10*

The preparation of recombinant human IL-16 (rIL-16) was described previously [4]. Briefly, to construct the *Escherichia coli* expression vector for His-tagged human IL-16, human IL-16-cDNA was amplified by polymerase chain reaction (PCR) and ligated into pET-16b *E. coli* expression vector (Novagen, Madison, WI, USA). The construct was transformed into *E. coli* BL21 (DE3); bacterial transformant was grown at 37 °C, and His-tagged rIL-16 was induced with 1 mM of isopropyl β-D-thiogalactopyranoside at 37 °C for 2 h. The recombinant protein was purified by His Trap™ (Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's protocol. Recombinant human IL-10 (rIL-10) was purified by the same procedure.

2.2. *Immunosuppressive effect of rIL-16 and rIL-10 on MLR*

Human peripheral blood lymphocytes (PBL) from healthy human volunteers were obtained by density centrifugation and washed with phosphate-buffered saline (PBS). Stimulator cells were prepared by incubating cells at 10^6 ml^{-1} with 25 μg/ml mitomycin C for 20 min. After four washes with PBS, they were suspended with RPMI1640 (Nissui, Tokyo, Japan) supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS; Filtron, Brooklyn, Australia) (complete medium) at 10^6 ml^{-1}. Responder PBL were supplemented in complete medium and incubated in a nylon wool column (Iwaki, Tokyo, Japan) for 60 min at 37 °C in 5% CO_2. The nylon wool non-adherent T cells, which were >97% T lymphocytes as determined by staining with anti-CD3 mAb (Becton–Dickinson, Franklin Lakes, NJ, USA), were then suspended in complete medium at 10^6 ml^{-1} and preincubated with 10^{-8} M of rIL-16 or bovine serum albumin (BSA) alone and, in some experiments, with 0.01 μg/ml of rIL-10, at 37 °C in 5% CO_2 for 1 h before the addition of stimulator cells. BSA, 10 μg/ml, was used as an irrelevant control protein. We confirmed that 10 μg/ml of BSA did not affect the MLR because there was no significant difference in the MLR with or without BSA (data not shown). Responder and stimulator cells were mixed in a 1:1 ratio and aliquoted into triplicate wells of 96-well round-bottom plates. The cells were cultured at 37 °C in 5% CO_2, pulsed with [^{3}H]-thymidine on day 4 after the cultivation, and harvested with a SKATRON harvester (Markham, ON, Canada). The radioactivity was counted in a LS6000 scintillation counter on day 5.

2.3. *Squamous cell culture, plasmid construction and transfection*

The human oral squamous cell carcinoma cell line OSC-20 (HLA-A2, A11, B46, B55, Cw1, Cw9, DR5, DR8, DQ1, DQ7) was established in our laboratory [6]. Its appearance is epithelial in shape, it grows in a cobblestone pattern with scattered tonofilaments and desmosomes in the intercellular connection, and is positive for cytokeratins and vimentin [7]. It was cultured in RPMI1640 (Nissui, Tokyo, Japan) supplemented with 10% (v/v) FCS. We previously made the human IL-16-cDNA-transfected OSC-20 cell clone, OSC-20-IL16#4, which secreted 50 ng/ml of IL-16 as well as control plasmid (pcDSRα-E3 without IL-16-cDNA)-transfected OSC-20 cells, OSC-20-pcDSRα [4]. These were maintained in selective medium containing puromycin (1 μg/ml; Gibco BRL, NY, USA). Before DNA transfection, the cell monolayers were washed twice with PBS and detached by the treatment with 0.05% (w/v) trypsin with 0.02% (w/v) ethylene diamine tetraacetic acid (EDTA; Gibco BRL, NY, USA) in PBS. Cells were then washed with RPMI1640 containing 10% FCS.

Human IL-10-cDNA was constructed into the retrovirus expression vector pLXSN (Clontech, CA, USA). This construct was designated pLXSN-hIL-10. Approximately 10^6 cells of OSC-20-IL16#4 and OSC-20-pcDSRα were transfected with 10 μg of pLXSN-hIL-10 and control plasmids pLXSN without IL-10-cDNA, using the LipoFECTAMINE 2000 transfection reagent (Gibco BRL, NY, USA) following the manufacturer's protocol. The transfection mixture was replaced with a fresh medium, and after 48 h it was replaced again with a selective medium containing 1 μg/ml puromycin (Gibco BRL, NY, USA) and 500 μg/ml geneticin (Sigma, St. Louis, MO, USA). Half the medium was changed every 3 days and cells were grown for 3 weeks. Puromycin- and geneticin-resistant cells were transferred into a 96-well dish at a single cell per well, and the clones were obtained and maintained in the selective medium.

Quantitative determination of IL-10 production in these transfectants was performed by colorimetric enzyme-linked immunosorbent assay (ELISA), Cytoscreen hIL-10 (BioSource International, Camarillo, CA, USA) following the manufacturer's protocol. The supernatants of culture medium were collected 24 h after the cultivation of approximately 10^6 cells in culture flasks (Falcon #3024, Franklin Lakes, NJ, USA). Samples (100 μl) of the supernatant were added to ELISA plates to measure IL-10 content in the supernatant, following treatment with the blocking buffer. Colorimetric change was measured at 450 nm by a spectrophotometer and compared with a standard curve of rIL-10. To examine the biological activity of secreted IL-16 and IL-10, supernatants of the cells were added to the MLR experiment. The control comprised OSC-20 cells transfected with the vectors pcDSRα-E3, pBabe Puro [8] and pLXSN. In order to determine the additive effect of secreted IL-16 and IL-10, we used anti-IL-10 mAb (IgG1), kindly gifted by Dr. Ishida of Kyoto University School of Medicine, Kyoto, Japan, and anti-rat natural killer target molecule mAb 109 (IgG1) [9] as isotype-matched control mAb.

2.4. *Direct MLR between IL-16- and IL-10-cDNA-double-transfected OSC-20 lines and T cells*

The responder T cells were mixed at a 10:1 ratio with the stimulator cells: IL-16- and IL-10-cDNA-double-transfected OSC-20 clones, etc. The ratio when the responder T cells most proliferated in response to the control stimulatory squamous

cells was 10:1 (data not shown). Therefore we tested the ability of transfected squamous cells to suppress T cell responses at this ratio. Stimulator cells were irradiated with 6000 rad in advance. These mixtures were aliquoted into triplicate wells of a 96-well round-bottom plate. The cells were cultured at 37 °C in 5% CO_2, pulsed with [^{3}H]-thymidine on day 4, and harvested with a SKATRON harvester. The radioactivity was counted in a LS6000 scintillation counter on day 5.

In order to determine the level of expression of major histocompatibility complex (MHC) class I and class II molecules in these transfectant cells, we used W6/32 mAb [10] and L243 mAb [11], respectively. W6/32 mAb reacts with MHC class I molecules and L243 mAb reacts with human leukocyte antigen-DR (HLA-DR).

2.5. *Statistical analysis*

Student's t-test was used to assess the differences between data sets.

3. Results

3.1. *The additive effect of human rIL-16 and rIL-10 on human MLR*

We recently reported that IL-16-producing allogeneic skin graft might have a local immunosuppressive action that would prolong graft survival [4]. Following this line of study, in order to achieve this local immunosuppressive action more efficiently, we attempted to use IL-16 together with another well-known immunosuppressive cytokine, IL-10 [12], determining the immunosuppressive action of human IL-16 with or without human IL-10. To this end, we obtained recombinant human IL-16 and recombinant human IL-10, and their effects on human MLR were assessed using allogeneic lymphocytes of healthy volunteers. The HLA-haplotypes of responder and stimulator lymphocytes involved were HLA-A2, A33, B17, B61, Cw3, DR6, and HLA-A24, B52, B61, Cw3, DR2, DR8, respectively. As shown in Fig. 1, MLR was inhibited by the addition of 10^{-8} M rIL-16 alone or 0.01 μg/ml of rIL-10 alone; this inhibition was clearly enhanced when IL-10 and IL-16 were used together, whereas control protein BSA did not inhibit these MLRs. These data suggested that IL-16 and IL-10 acted additively to inhibit MLR.

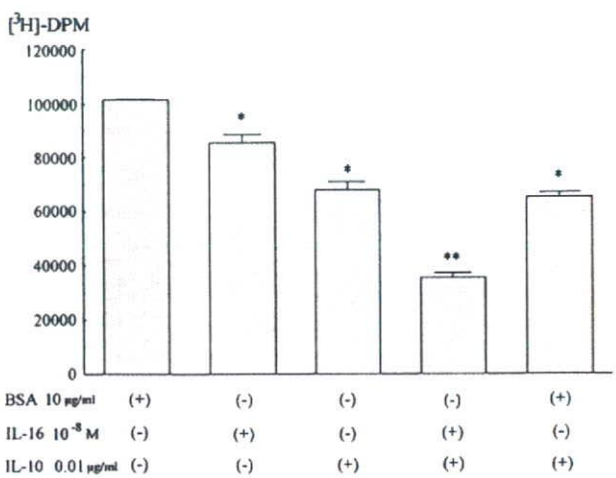

Fig. 1 – Additive action of rIL-16 and rIL-10 on MLR proliferation. Bars represent ±standard error. *$p < 0.05$ and **$p < 0.01$.

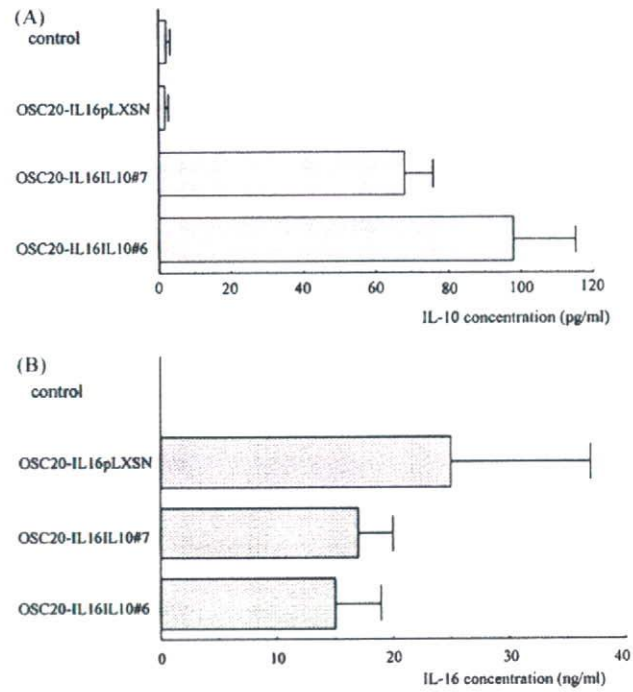

Fig. 2 – (A) IL-10 and (B) IL-16 production in the supernatant of the puromycin–geneticin-resistant squamous cell line OSC-20 clones IL16IL10#6 and #7. Bars represent ±standard error.

3.2. *Production and MLR inhibition of IL-16 and IL-10 secreted from a human skin keratinocyte-equivalent model, OSC-20 cells*

The above data led us to develop a model of tolerogeneic human skin substitute which produced IL-16 and IL-10. To this end, we used the human squamous cancer cell line OSC-20 as a model for the skin keratinocytes. We previously made the human IL-16-cDNA-transfected OSC-20 cell clone, OSC-20-IL16#4, which secreted 50 ng/ml of IL-16, and control plasmid (pcDSRα-E3 without IL-16-cDNA)-transfected OSC-20 cells, OSC-20-pcDSRα [4]. OSC-20-IL16#4 cells were transfected with a mixture of plasmids pLXSN-IL-10 and maintained in puromycin- and geneticin-containing medium. Several clones were obtained, and IL-10 production in the culture supernatant was determined by colorimetric ELISA. As a control, we used a mixture of plasmids pcDSRα-E3, pBabe Puro and pLXSN. We confirmed the insertion of the IL-10 gene by sequencing reverse transcriptase PCR products in all of transfectants #6 and #7 (data not shown). As depicted in Fig. 2A, it appeared that the OSC-20-IL16IL10#6 transfectant clone was the highest producer of IL-10 among selected transfectant clones OSC-20-IL16IL10#6 and #7. However, it seemed that the amounts of IL-16 production by each of these transfectants were the same, except for the control (Fig. 2B).

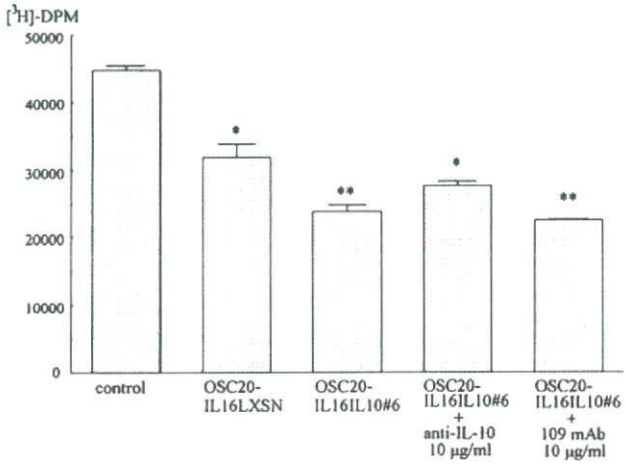

Fig. 3 – Inhibition of human mixed lymphocyte reaction by the supernatant of IL-16- and IL-10-producing OSC-20 clone. Bars represent ±standard error. *$p < 0.05$ and **$p < 0.01$.

By using the supernatant of an IL-16 and IL-10 double-producer clone, OSC-20-IL16IL10#6, we then assessed whether it could inhibit MLR. The responder lymphocytes were treated with the culture supernatant and mixed with mitomycin-C-treated stimulator cells. The reactivity of responder lymphocytes was then analysed as described above. As shown in Fig. 3, the supernatant of the transfectant clone OSC-20-IL16IL10#6, the highest IL-16 and IL-10 double-producer, inhibited MLR most efficiently. Inhibition was cancelled selectively only when anti-IL-10 mAb (IgG1) was added to the MLR assay, whereas isotype (IgG1)-matched control 109 mAb could not cancel the inhibition, compared with the inhibition of MLR by the supernatant of OSC-20-IL16pLXSN which secreted IL-16 only. These data indicated that the supernatant of transfectant clone OSC-20-IL16IL10#6 could inhibit MLR most strongly, because of the additive effect of secreted IL-16 and IL-10.

3.3. *Direct MLR inhibition by IL-16- and IL-10-cDNA-double-transfected OSC-20 lines with T cells*

Finally, we determined the direct interaction between allogeneic lymphocytes and transfected OSC-20 cells. In this experiment, the control plasmid (pcDSRα-E3 and pLXSN)-transfected OSC-20 (control), pcDSRα-E3-IL16 and pLXSN-transfected OSC-20 (OSC-20-IL16pLXSN) and pcDSRα-E3-IL16- and pLXSN-IL10-transfected OSC-20 cells (OSC-20-IL16IL10#6) as stimulator cells were irradiated with 6000 rad, and allogeneic lymphocytes (HLA-haplotype: HLA-A2, A33, B17, B61, Cw3, DR6) were added as the responder. Using colorimetric ELISA, we confirmed that OSC-20-IL16IL10#6 continued to release IL-16 and IL-10 after they had been irradiated (data not shown).

As depicted in Fig. 4A, OSC-20-IL16IL10#6 cells inhibited this MLR most strongly when compared with control cells and OSC-20-IL16pLXSN. In the direct MLR assay, the effects appeared to be irrelevant to the MHC class I expression on the cell surface as assessed by MHC class I-specific W6/32 mAb. However, they appeared to be relevant to the MHC class II expression on the cell surface, since IL-10-producing OSC-20-IL16IL10#6 cells exhibited a down-regulation of MHC class II expression as assessed by MHC class II-specific L243 mAb (Fig. 4B). Taken together, these data indicated that IL-16- and IL-10-double-producing allogeneic squamous cells, which are considered skin keratinocyte equivalents, work as direct inhibitors of allogeneic lymphocyte activation.

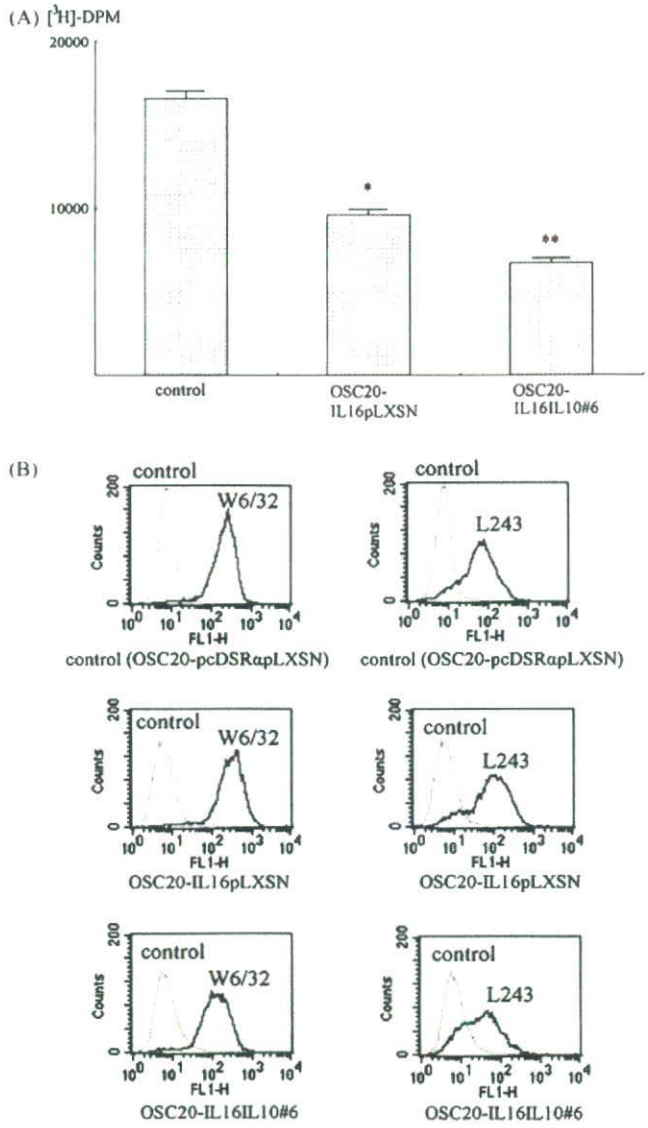

Fig. 4 – (A) Inhibition of the reactivity of allogeneic lymphocytes with IL-16- and IL-10-producing OSC-20 clone. Bars represent ±standard error. *$p < 0.05$ and **$p < 0.01$. (B) Fluorescence-activated cell sorter (FACS) profile of the expression of major histocompatibility complex class I and class II molecules of OSC-20 transfectants as determined by W6/32 mAb and L243 mAb, respectively. Control denotes the FACS pattern of cells stained without W6/32 or L243.

4. Discussion

Although many synthetic skin substitutes have been produced [13–15], the mainstay of treatment for extensive severe burns is still transplantation of skin allografts. However, the therapeutic effect of these grafts ceases with their immunological rejection. Therefore, prolongation of skin allograft survival has been a major aim of clinical treatment. It is recognised that in the field of allogeneic organ transplantations it is relatively difficult to delay the immunological rejection of skin. Perhaps these phenomena may have something to do with the ample presence of MHC class II-positive professional antigen-presenting cells, such as dermal dendritic cells, in the skin. Furthermore, certain epidermal keratinocytes express MHC class II antigen on the cell surface as well [16]. These characteristics indicate that the prolongation of skin allograft survival may require a more potent additional therapeutic regimen.

In the *in vitro* studies, anti-rat CD4 mAb RTH7 [17,18] significantly reduced rat allogeneic MLR (data not shown). In our study, however, this mAb prolonged *in vivo* skin allograft survival only minimally. This might be due to antibody neutralisation and rapid clearance *in vivo* or to insufficient delivery of mAb to the skin tissue. We previously assessed the effect of topically applied 0.5% FK506 ointment in the rat skin allograft model. The treatment was clearly effective for local immunosuppression and consequent delay of skin allograft rejection by up to 1 week compared with placebo treatment, even though the blood concentration of FK506 was mostly below levels detectable by ELISA with a mouse anti-FK506 monoclonal antibody [19]. From the same point of view, we speculate that an efficient way to prolong skin allograft survival might be to develop an *in situ* immunosuppressive mechanism in the skin. Thus, we planned the experiment in which the cytokines inhibiting MLR were one of the *in situ* immunosuppressive agents.

IL-16 was first described in 1982 as a lymphocyte chemoattractant factor generated by mitogen-stimulated human peripheral mononuclear cells [20]. The functional bioactivities of IL-16 towards CD4 T cells involve the migration and upregulation of surface-expressed IL-2 receptor, the G0 to G1 transition of the cell cycle and increased cell growth [21]. On the other hand, it was also reported that IL-16 acted as the natural ligand of the CD4 molecule and inhibited MLR, although it did not influence MHC class II expression on the cell surface [22].

Meanwhile, IL-10 has been identified as a cytokine synthesis inhibitory factor capable of suppressing the production of Th1 cytokines including IL-2 and IFN-γ [23–26]. In addition, IL-10 inhibits the production of most cytokines produced by monocytes/macrophages [27,28] and polymorphonuclear neutrophils [29,30]. IL-10 also strongly reduces antigen-specific T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of MHC class II expression [31]. Because IL-10 is well known as an immunosuppressive cytokine, attempts have been made to more thoroughly investigate its function in a number of *in vivo* models including endotoxemia [32–36], transplantation [37–39], autoimmune diseases [40], diabetes in non-obese mice [41] and allergen-induced lung inflammation [42].

In this study, we demonstrated that IL-16 enhanced or added independently to the immunosuppressive effect of IL-10 in allogeneic MLR. It was therefore concluded that if the skin equivalent was reconstituted with skin cells which secreted both IL-16 and IL-10, it might induce the most efficient local immunosuppressive effect and so prolong skin allograft survival. It is known that skin keratinocytes express MHC class II molecules on the cell surface [16], and allogeneic skin graft rejection will be triggered by the interaction of dendritic cells, MHC class II-positive keratinocytes and lymphocytes. Thus we compared the immunosuppressive effects of IL-16 and IL-10 on the MLR between responder lymphocytes and MHC class II-positive skin keratinocyte-equivalent cells, such as the OSC-20 line.

We transfected the IL-16 and IL-10 expression vector to OSC-20, and IL-16- and IL-10-double-producing OSC-20 clones were obtained. Consequently IL-16 and IL-10, secreted in the culture supernatant of these clones, inhibited MLR most significantly. Furthermore, the reactivity of allogeneic lymphocytes directly cultured with these OSC-20 clones was also inhibited. It was confirmed that the MHC class II expression on the cell surface of these OSC-20 clones was down-regulated. Such observations have previously been reported in the literature [43–48] and may be due to the biological effects of the secreted IL-10. Our results suggest that the inhibition of allogeneic lymphocyte reactivity might be due to direct action of secreted IL-16 and IL-10, and indirect action through down-regulation of MHC class II expression by the secreted IL-10. Taken together, our results suggest that, in clinical practice, gene therapy based on the introduction of IL-16-cDNA and IL-10-cDNA to the skin cells might confer the capability of prolonging survival of this allogeneic skin substitute.

Transfection to normal cells *in vivo* by methods such as a gene gun with naked DNA, or an adenovirus vector, is not stable; it is predicted that the amount of cytokines secreted from the transfectants would decrease or the transfected gene expression itself would disappear. Therefore, if the current transfection method is used, it seems difficult for differentiated skin cells to achieve the concentrations of cytokines secreted by *in vitro* cell lines. Normal human keratinocyte culture is usually maintained only up to passages 6–8, and it is difficult for these cells to be stably transfected with double-cDNA. However, normal human fibroblasts can be maintained up to passage 50 at least, so we think a stable double transfection can be achieved in primary fibroblasts. Moreover, if normal human keratinocytes are immortalised by the transfection with genes such as hTERT, we think that stable double transfection could be achieved in this cell line as well.

We consider that normal keratinocytes and fibroblasts harvested from allogeneic skin can be stably transfected with immunosuppressive cytokine cDNA *in vitro* and then the allogeneic skin equivalent could be reconstituted from these stable transfectants, which would maintain high amounts of cytokine secretion. These cells could be transplanted to severe burns as allografts. To this end we are now studying the *in vivo* effects of IL-16- and IL-10-cDNA-double-transfected skin cells.

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REFERENCES

[1] Brown JB, Minot PF, Randall P. Postmortem homograft as "biological dressing" for extensive burns and denuded areas. Ann Surg 1953;138:618–29.

[2] Zaroff LI, Mills W, Duckett JW, Switzer WE, Moncrief JA. Multiple uses of viable cutaneous homografts in the burned patient. Surgery 1966;59:368–72.

[3] Spence RJ, Wong L. The enhancement of wound healing with human skin allograft. Surg Clin North Am 1977;77: 731–45.

[4] Fujita T, Matsumoto Y, Hirai I, Ezoe K, Saito T, Yagihashi A, et al. Immunosuppressive effect on T cell activation by interleukin-16-cDNA-transfected human squamous cell line. Cell Immunol 2000;202:54–60.

[5] Bejarano MT, de Waal Malefyt R, Abrams JS, Bigler M, Bacchetta R, de Vries JE, et al. IL-10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary MLR. Int Immunol 1992;4:1389–97.

[6] Miyazaki A, Sato N, Takahashi S, Sasaki A, Kohama G, Yamaguchi A, et al. Cytotoxicity of histocompatibility leukocyte antigen-DR8-restricted CD4+ killer T cells against human autologous squamous cell carcinoma. Jpn J Cancer Res 1997;88:191–7.

[7] Yokoi T, Hirata S, Nishimura F, Miyakawa A, Odajima T, Kohama G, et al. Some properties of a newly established human cell line derived from an oral squamous carcinoma. Tumor Res 1990;25:93–103.

[8] Morgenstein JP, Land H. Advanced mammalian gene transfer; high titer retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res 1990;18:3587–96.

[9] Cho JM, Sato N, Yagihashi A, et al. Natural killer target molecules associated with the transformation of the oncogene-transformed fibroblast. Cancer Res 1991;51: 4250–6.

[10] Brodsky FM, Bodmer WF, Parham P. Characterization of a monoclonal anti-beta 2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. Eur J Immunol 1979;9: 536–45.

[11] Smith LM, Petty HR, Parham P, McConnell HM. Cell surface properties of HLA antigens on Epstein–Barr virus-transformed cell lines. Proc Natl Acad Sci USA 1982;79: 608–12.

[12] Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993;11:165–90.

[13] Peters WJ. Biological dressings in burns—a review. Ann Plast Surg 1980;4:133–7.

[14] Alexander JW, Wheeler LM, Rooney RC, McDonald JJ, MacMillan BG. Clinical evaluation of epigard, a new synthetic substitute for homograft and heterograft skin. J Trauma 1973;13:374–83.

[15] Friedman SJ, Su WPD. Management of leg ulcers with hydrocolloid occlusive dressing. Arch Dermatol 1984;120:1329–36.

[16] Albanesi C, Cavani A, Girolomoni G. Interferon-γ stimulated human keratinocytes express the genes necessary for the production of peptide-loaded MHC class II molecules. J Invest Dermatol 1988;110:138–42.

[17] Tamura Y, Tsuboi N, Sato N, Kikuchi K. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. J Immunol 1993;151:5516–24.

[18] Yamaki T, Uede T, Shijubo N, Kikuchi K. Functional analysis of mononuclear cells infiltrating into tumors. III. Soluble factors involved in the regulation of T lymphocyte infiltration into tumors. J Immunol 1988;140:4388–96.

[19] Fujita T, Takahashi S, Yagihashi A, Jimbow K, Sato N. Prolonged survival of rat skin allograft by treatment with FK506 ointment. Transplantation 1997;64:922–5.

[20] Cruikshank WW, Kornfeld H, Center DM. Signaling and functional properties of interleukin-16. Int Rev Immunol 1988;16:523–40.

[21] Cruikshank WW, Center DM, Nisar N, Wu M, Natke B, Theodore AC, et al. Molecular and functional analysis of a lymphocyte chemoattractant factor; association of biological function with CD4 expression. Proc Natl Acad Sci USA 1994;91:5109–13.

[22] Theodore AC, Center DM, Nicoll J, Fine G, Kornfeld H, Cruikshank WW. CD4 ligand IL-16 inhibits the mixed lymphocyte reaction. J Immunol 1996;59:1958–64.

[23] Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 1989;170:2081–95.

[24] Vieira P, de Waal-Malefyt R, Dang MN, Johnson KE, Kastelein R, Fiorentino DF, et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein–Barr virus open reading frame BCRFI. Proc Natl Acad Sci USA 1991;88:1172–6.

[25] Fiorentino DF, Zlotnik A, Vierira P, Howard M, O'Garra A. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 1991;146:3444–51.

[26] Taga K, Tosato G. IL-10 inhibits human T cell proliferation and IL-2 production. J Immunol 1992;148:1143–8.

[27] de Waal Malefyt R, Abrams J, Bennet B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209–20.

[28] Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147:3815–22.

[29] Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1β in mediating the production of IL-8 triggered by lipopolysaccharide. J Exp Med 1993;178:2207–11.

[30] Kasama T, Strieter RM, Lukacs NW, Burdick MD, Kunkel SL. Regulation of neutrophil derived chemokine expression by IL-10. J Immunol 1994;152:3559–69.

[31] de Waal-Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med 1991;174:915–24.

[32] Drazan KE, Wu L, Bullington D, Shaked A. Viral IL-10 gene therapy inhibits TNF-a and IL-1b, not IL-6, in the newborn endotoxemic mouse. J Pediatr Surg 1996;31:411–4.

[33] Xing Z, Ohkawara Y, Jordana M, Graham FL, Gauldie J. Adenoviral vector-mediated interleukin-10 expression in-vivo: intramuscular gene transfer inhibits cytokine responses in endotoxemia. Gene Ther 1997;4:140–9.

[34] Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, et al. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. J Exp Med 1993;177:547–50.

[35] Pajkrt D, Camoglio L, Tiel-van Buul MC, de Bruin K, Cutler DL, Affrime MB, et al. Attenuation of proinflammatory responses by recombinant human IL-10 in human endotoxemia. J Immunol 1997;158:3971–7.

[36] Van der Poll T, Jansen PM, Montegut WJ, Braxton CC, Calvano SE, Stackpole SA, et al. Effects of IL-10 on systemic inflammatory responses during sublethal primate endotoxemia. J Immunol 1997;158:1971–5.
[37] Suzuki T, Tahara H, Narula S, Moore KW, Robbins PD, Lotze MT. Viral interleukin-10 (IL-10), the human herpes virus 4 cellular IL-10 homologue, induces local anergy to allogeneic and syngeneic tumors. J Exp Med 1995;182:477–86.
[38] Qin L, Chavin KD, Ding Y, Tahara H, Favaro JP, Woodward JE, et al. Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. J Immunol 1996;156:2316–23.
[39] Zheng XX, Steele AW, Nickerson PW, Steurer W, Steiger J, Strom TB. Administration of noncytolytic IL-10/Fc in murine models of lipopolysaccharide-induced septic shock and allogeneic islet transplantation. J Immunol 1995;154:5590–600.
[40] Mathisen PM, Yu M, Johnson JM, Drazba JA, Tuohy VK. Treatment of experimental autoimmune encephalomyelitis with genetically modified memory T cells. J Exp Med 1997;186:159–64.
[41] Zheng XX, Steele AW, Hancock WW, Stevens AC, Nickerson PW, Roy-Chaudhury P, et al. A noncytolytic IL-10/Fc fusion protein prevents diabetes, blocks autoimmunity, and promotes suppressor phenomena in NOD mice. J Immunol 1997;158:4507–13.
[42] Grunig G, Corry DB, Leach MW, Seymour BWP, Kurup VP, Rennick DM. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. J Exp Med 1997;185:1089–99.
[43] Kooy AJ, Prens EP, van Heukelum A, Vuzevski VD, van Joost T, Tank B. Interferon-γ-induced ICAM-1 and CD40 expression, complete lack of HLA-DR and CD80 (B7.1), and inconsistent HLA-ABC expression in basal cell carcinoma: a possible role for interleukin-10? J Pathol 1999;187:351–7.
[44] Redpath S, Angulo A, Gascoigne NR, Ghazal P. Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10. J Immunol 1999;162:6701–7.
[45] Chadban SJ, Tesch GH, Foti R, Lan HY, Atkins RC, Nikolic-Paterson DJ. Interleukin-10 differentially modulates MHC class II expression by mesangial cells and macrophages in vitro and in vivo. Immunology 1998;94:72–8.
[46] Koppelman B, Neefjes JJ, de Vries JE, de Waal Malefyt R. Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. Immunity 1997;7:861–71.
[47] Boorstein SM, Elner SG, Bian ZM, Strieter RM, Kunkel SL, Elner VM. Selective IL-10 inhibition of HLA-DR expression in IFN-gamma-stimulated human retinal pigment epithelial cells. Curr Eye Res 1997;16:547–55.
[48] Yue FY, Dummer R, Geertsen R, Hofbauer G, Laine E, Manolio S, et al. Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules. Int J Cancer 1997;71:630–7.

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Review Article

Molecular pathological approaches to human tumor immunology

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Research on human tumor immunology has greatly advanced in the past two decades. Many immunogenic tumor antigens have been identified, and some of these antigens entered in clinical trials. Consequently, it has been shown that these antigens can inhibit tumor growth in patients to some extent, indicating that they act as potent immunogenic therapeutic vaccines in cancer patients with malignancies originating from various tissues. These patients had antigen-specific cytotoxic T-lymphocyte (CTL) responses when assessed on tetramer, enzyme-linked immunospot (ELISPOT), T-cell clonotype and CTL induction efficiency. Thus, it has become clear that human tumor vaccines can evoke clinical and immunological anti-tumor responses in patients. The tumor regression effects of tumor vaccines, however, are generally low, and it is obvious that current vaccination protocols are generally too weak to provide substantial and satisfactory clinical benefits. This means that other drastic and more potent clinical and immunological protocols are required in cancer immunotherapy. To find such efficient protocols the basic immunological and biological properties of cancers must be investigated. In the present review the identification of human tumor antigens recognized on CTL and the clinical trials are introduced. Next, the most recent analysis of human cancer-initiating cell (cancer stem cell)-associated antigens is described. These antigens might be able to act as 'universal, general and fundamental' tumor antigens. Also present is the authors' recent study for increasing cross-presentation efficiency in dendritic cells and subsequent enhancement of human leukocyte antigen (HLA)-class I-restricted peptide antigenicity by using HSP90 and ORP150 molecular chaperones that act as endogenous Toll-like receptor ligands. In addition to the aforementioned manipulation of the positive loop of tumor immunity, it is necessary to regulate and intervene in the negative loop. In particular, the potential of the expression of HLA class I molecule regulation by epigenetic mechanisms will be discussed. Finally, the type of basic and clinical tumor immunology research highly required currently, and in the very near future, are described.

Key words: antigenic peptide, cross-presentation, epigenetics, human leukocyte antigen, heat shock protein, T cell, tumor immunology, tumor immunotherapy

The exploitation of human cancer vaccines has been one of the main aims in basic cancer research and clinical studies. Although a huge number of immunological studies using animal tumor models has been reported, human tumor immunology research has advanced since the first human melanoma tumor antigen recognized by CD8 (+) cytotoxic T lymphocytes (CTL) was identified in 1992 by van der Bruggen *et al.*[1] In the past decade many such melanoma tumor antigens and their peptides presented by each human leukocyte antigen (HLA) allele have been discovered, and subsequently many tumor antigens of epithelial cancer origin have also been identified. These antigens were found using molecular cloning of tumor antigens with human autologous pairs of established tumor lines and CTL clones, reverse immunology and complementary DNA microarray devices. Using antigenic peptides derived from tumor protein antigens or protein antigens themselves as anti-tumor vaccines, clinical trials for tumor immunotherapy were subsequently performed in many institutes and hospitals in the USA, Europe and Japan.[2–11]

When these vaccine candidates were injected into patients without any addition of adjuvants, there were no overt side-effects or toxicity, but clinical responses were generally not strong except for a few cases.[5] Immunological monitoring using tetramer, enzyme-linked immunospot (ELISPOT), CTL induction efficiency, and T-cell receptor clonotype expansion has indicated that in some cases, but not many, there is a positive correlation between clinical and immunological responses.

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These observations suggested that tumor antigenic peptides could work as anti-cancer vaccines in tumor immunotherapy as well as for immunoprophylaxis without severe adverse effects. It is also highly likely that these anti-cancer vaccines can be used for strong anti-cancer therapeutic regimens if combined with adequate immunostimulatory adjuvants, including dendritic cells (DC). Along with these trends in human tumor immunology research, a certain European Union-based pharmaceutical company has already begun to undertake commercialization of tumor vaccines for patients with diseases such as lung cancers and gliomas.

Immune responses generally involve both positive and negative regulation, and this has proved to be true for tumor immunology. Current and future tumor immunology studies require much deeper understanding of these conflicting aspects. Obviously, there needs to be an efficient link between innate and acquired (specific) immunity. Efficient activation of antigen-presenting cells (APC), particularly DC, is indispensable for the ample induction of antigen-specific CTL. Meanwhile, although the role of CD4+, CD25+, Foxp3+ regulatory T cells (Treg) was investigated to a certain extent in human cancers, a large part of the negative regulation of tumor immunity remains to be elucidated.[12–15]

In our laboratory at Sapporo Medical University, for the past 10 years we have principally investigated the molecular nature and pathological characteristics of human tumor antigens that are recognized by CTL. In collaboration with clinical departments, phase I clinical trials for assessing the toxicity and immunotherapeutic potential of antigenic peptides have been performed.[3,4,10,11] Along with these studies, others were conducted on positive regulation of tumor immunity with chaperone-assisted peptide vaccination and on negative regulation, namely, immunoescape by downregulating HLA class I. In the present article, the current status of these studies is reviewed, and future perspectives for human cancer immunotherapy and prophylaxis are discussed from the viewpoint of molecular pathology.

POSITIVE REGULATION OF HUMAN TUMOR IMMUNITY

Melanoma antigens recognized by CTL and immunotherapy

Various human melanoma antigens that are recognized by CTL have been identified since the discovery in 1992,[1] and more than 20 melanoma antigens have been reported, as shown in Table 1.[16,17] Some antigens and HLA class I-restricted antigenic peptides underwent clinical trials, and their side-effects and clinical and immunological responses were assessed. At the first stage of the trials there were positive clinical results in Europe and the USA. In 2003, however, Rosenberg *et al.* reported on a large number of melanoma patients and found that <5% of patients who received peptide vaccines such as gp100, Mart1 and tyrosinase plus interleukin (IL)-2 had a complete response.[5]

Table 1 Melanoma antigens/peptides recognized by autologous CTL

Antigens	HLA	Peptides
Cancer-testis antigens		
BAGE	Cw16	AARAVFLAL
GAGE	Cw6	YRPRPRRY
MAGE-1	A1	EADPTGHSY
MAGE-3	A1	EVDPIGHLY
NY-ESO-1	A31	ASGPGGGAPR
Melanoma-melanocyte differentiation antigens		
MAERT-1/Melan-A	A2	AAGIGILTV
gp100 (pmel-17)	A2	LLDGTATLRL
Tyrosinase	A1	SSDYVIPIGTY
TRP-1 (gp75)	A31	MSLQRQFLR
TRP-2	A31	LLPGGRPYR
Mutated (unique) antigens		
β-Catenin	A24	SYLDSGIHF
MUM-1	B44	EEKLIVVLF
MUM-2	B44	SELFRSGLDSY
	C6	FRSGLDSYV
MUM-3	A28	EAFIQPITR
CDK-4	A2	ACDPHSGHFY
MART-2	A1	FLGGNEVGKTY
Over-expression antigens		
PRAME	A24	LYVDSLFFL
P15	A24	AYGLDFYIL

CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen.

This led to skepticism about immunotherapy with peptide cancer vaccines. Furthermore, immunological monitoring for peptide-specific CTL using tetramers and ELISPOT, which can detect peptide-specific precursor CTL and activated functional CTL, respectively, showed that the immunological response was not always parallel to the clinical response. Regardless of such problems, however, in 2006 a UK-based pharmaceutical company announced that a 3 year-long observation after melanoma antigen family A, 3 (MAGE-A3) vaccine inoculation indicated a 33% reduction of the postoperative recurrence in non-small lung cancers as compared with a placebo group. This observation provides strong hope for current and future immunotherapy, and has accelerated many different investigations for the establishment of human tumor immunotherapy and immunoprophylaxis.

Tumor antigens identified in Sapporo

In addition to melanomas, human tumor antigens of non-melanoma tumors such as colon, breast, lung, urinary tract, head and neck cancers and soft-tissue sarcomas have been analyzed extensively in various laboratories. Although the immunogenicity of these non-melanoma antigens was

Table 2 Candidates for cancer vaccines identified in Sapporo

Tumors	Peptides	Proteins	HLA	Clinical study
Autologous system				
Stomach	YSWMDISCWI (F4.2)	c98	A31	
Osteosarcoma	CTACRWKKACQR	PBF	B55	
	AYRPVSRNI		A24	Scheduled
			A2	Scheduled
Reverse immunology				
Apoptosis-related				
Various	AYACNTSTL (survivin 2B80-88)			
		Survivin	A24	Phase I
Various	KWFPSCQFLL (L7)	Livin	A24	Phase I
Chromosome translocation				
Synovial sarcoma				
	GYDQIMPKK (B)	SYT-SSX	A24	Phase I
	GYDQIMPKI (K9I)	SYT-SSX	A24	Phase I
Bioinformatic immunology				
Various	RYAMTVWYF (HIFPH3-8)	HIFPH3		Scheduled
	VYVKGLLAKI (Cep55-10)	CEP55		
	NMVEGTAYL (AMARCR2)	AMACR		
	QYFKKIPIL (STEAP-B)	STEAP		
	N. D.	Lengsin		

AMACR, α-methylacyl-coenzyme A racemase; Cep55, centrosome protein 55 kDa; HIFPH3, hypoxia-inducible factor prolyl hydroxylase 3; HLA, human leukocyte antigen; Lengsing, lens protein with glutamine synthetase domain; STEAP, six-transmembrane epithelial antigen of prostate.

relatively weak as compared with that of melanoma-associated tumor antigens, a certain number of tumor antigens from these non-melanoma tumors were identified.

In our laboratory at Sapporo Medical University, as shown in Table 2, we have identified tumor antigens using several different experimental systems, namely (i) autologous established tumor line-CTL pairs; (ii) reverse immunology; and (iii) bioinformatics. In established autologous tumor line-CTL pairs, two tumor antigen genes, C98[18,19] and papilloma binding factor (PBF),[20–24] were identified from gastric signet ring cell cancer and osteosarcoma, respectively. Using reverse immunology the inhibitor of apoptosis protein family members survivin and livin were shown to be highly immunogenic tumor antigens in addition to that fact that these two antigens were selectively expressed in tumor tissues of different tissue origins but not in normal counterparts.[25–30] In particular, the expression of survivin protein was very high: >90% of colon, lung, pancreas and breast primary cancers had high expression of this protein.[25] Furthermore, HLA-A24-restricted survivin 2B80-88 nonamer peptide, which was derived from the survivin splicing variant survivin 2B from cancer patients, appeared to have strong immunogenicity when assessed for CTL induction efficiency, tetramer CTL frequency and on ELISPOT.[26]

Meanwhile, HLA-A24-restricted immunogenic peptide from immunologically non-self SYT-SSX fusion protein of synovial sarcomas was also identified. This peptide, designated SYT-SSX B peptide, is derived from the fusion point at the SYT-SSX t(X;18) (p11;q11) chromosomal translocation. When assessed in a peptide-specific tetramer study, it was confirmed that this peptide-specific CTL was found in SYT-SSX chromosomal translocation (+) synovial sarcoma patients' peripheral blood lymphocytes with a relatively higher frequency than in non-synovial sarcoma patients.[31,32] Subsequently, we also identified variant antigenic peptide K9I, in which the C-terminal ninth agretope residue required for the binding to HLA class I molecules was substituted to isoleucine from lysine.[33]

Very recently, using bioinformatics approaches, several interesting, highly immunogenic tumor antigens have been found. Hypoxia-inducible factor prolyl hydroxylase 3 (HIFPH3), centrosome protein 55 kDa (Cep55), α-methylacyl-coenzyme A racemase (AMACR), six-transmembrane epithelial antigen of prostate (STEAP) and lens protein with glutamine synthetase domain (Lengsin) are expressed most preferentially in renal cell, colon, prostate, oral and lung cancers, respectively.[34,35] We routinely developed mAb to each of all these tumor antigens, through work on paraffin-embedded tissue sections, and subsequent immunohistochemical data indicated that the expression status of antigens in tumors was highly parallel to the induction efficiency of CTL from the patients. Therefore it is highly likely that these tumor antigens could work as tumor vaccines.

Clinical trials

In 2003 the General Surgery, Oral Surgery, Urology and Orthopedic Surgery Departments of Sapporo Medical University Hospital and affiliated hospitals began phase I clinical trials with survivin 2B80-88 peptide and SYT-SSX B peptide.

Table 3 Summary of phase I clinical trials

Tumors	No. patients	Side-effect	Clinical response	
			Tumor marker/SD *n* (%)	RECIST *n* (%)
Survivin 2B peptide				
Colon	15	no	7/15 (47)	1/15 (7)
Breast	12	no	1/17 (6)	0/17 (0)
Lung	10	no	5/10 (50)	0/10 (0)
Oral cavity	9	no	3/11 (27)	1/11 (9)
Urinary bladder	3	no	2/3 (67)	1/3 (33)
Lymphoma	2	no	1/2 (50)	1/2 (50)
SYT-SSX B peptide				
Synovial sarcoma	6	no	1/6 (17)	0/6 (0)

RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.

As shown in Table 3, the HLA-A24-restricted survivin2B peptide was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, urinary bladder cancers and lymphomas. There were no severe side-effects and, clinically, certain patients with colon and lung cancers showed reductions in tumor markers (minor response) and growth arrest (stable disease) as assessed on CT.[9–11] These effects, however, were not strong enough for the clinical requirements as judged by the criteria of cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors (RECIST), which requires >30% regression of tumors on CT, only one patient each out of 15 with colon cancers, three with urinary bladder cancers and two with lymphomas had a positive clinical response. Meanwhile, breast cancer patients exhibited rare clinical responses to the current protocol of survivin2B80-88 administration. Interestingly, however, these breast cancer patients showed enhancement of peptide-specific CTL frequency, indicating a high possibility that certain negative immunoregulatory mechanisms existed in the breast tumors.

Our group studied immunological characteristics of products derived from chromosomal translocation that occurred in synovial sarcomas, and identified immunogenic SYT-SSX B peptide in the context of the HLA-A24 molecule. This peptide was also entered into clinical trials. Consequently, administration of this SYT-SSX translocation-derived B peptide to six synovial sarcoma patients also produced no side-effects, but resulted in little clinical response.[7,8]

These data strongly indicated that the current protocol of single use of survivin 2B80-88 peptide alone or SYT-SSX B peptide alone was not sufficient for clinical application. We also studied the immunological responses of almost all the patients. With survivin 2B80-88 peptide vaccinations, certain patients exhibited approximately 10–15-fold increases in the CTL precursor frequency and number of functional CTL as determined on peptide-specific tetramer and ELISPOT, respectively. There was no clear statistical correlation, however, between immunological and clinical responses.[10,11] Despite such observations it was also evident in colon cancer patients given the survivin 2B80-88 peptide vaccine that the tetramer-detected survivin 2B80-88 peptide-specific CTL precursor frequency was obviously increased in accordance with peptide plus incomplete Freud adjuvant (IFA) compared to the peptide alone, and it was interesting that the peptide plus IFA plus interferon (IFN)-α resulted in the strongest enhancement of CTL precursor frequency. In this protocol we used IFN-α for maturating DC,[36,37] and on this protocol patients displayed more overt clinical responses as well, suggesting that additional clinical trials should be undertaken for exact assessment in a larger number of patients.

Side population technology and identification of cancer-initiating cell tumor antigens

It has long been disputed whether tumor cells are homologous. Many recent studies indicated that tumor cells are not homogeneous but rather different in several biological aspects such as growth potential, differentiation status and invasive or metastatic characteristics. Histologically it is obvious that tumors are composed of very different cellular and structural morphologies with diverse neoplastic characteristics. This complex nature has also been one of the main interests for pathologists for many years, and two models for the origin of tumor cell populations, stochastic and hierarchic, have been advocated, as shown in Fig. 1. The stochastic model indicates that the tumor is composed of homogeneous cells, whereas the hierarchic model is of heterogeneous cells, and in this latter model there must be 'cancer stem' or 'cancer-initiating' cells in tumors.[38–40]

The concept of 'cancer stem cells' (CSC) or 'cancer-initiating cells' (CIC) is highly intriguing and important for development of efficient cancer immunotherapy and immunoprophylaxis protocols, because tumor antigens expressed in these cells must be the most suitable immunological

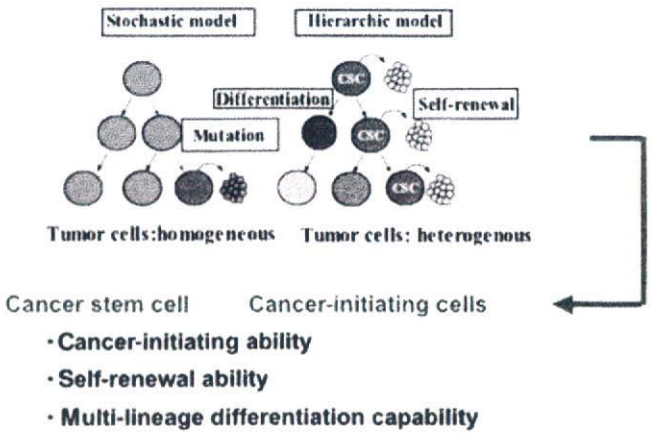

Figure 1 Models of origins for cancer stem cells and cancer-initiating cells.

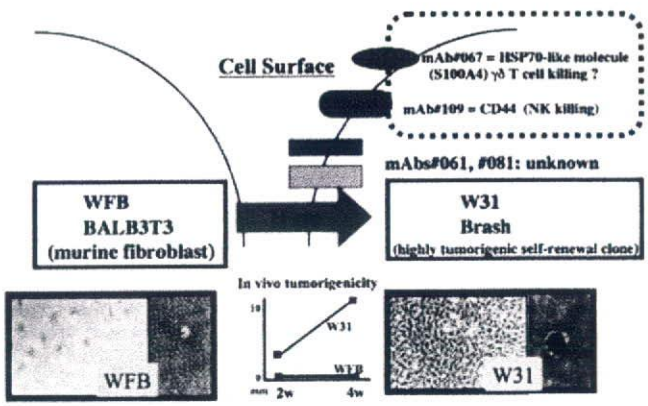

Figure 2 W31 and Brash cells as cancer stem cell/cancer-initiating cell (CSC/CIC) counterparts and the expression of cell surface antigens associated with activated H-ras-induced cell transformation. HSP, heat shock protein; NK, natural killer; WFB, WKA-H rat fetal fibroblast.

targets for inducing tumor regression. It is suggested that CSC/CIC maintain cancer-initiating ability, self-renewal ability and multilineage differentiation capability. Among these characteristics cancer-initiating ability is considered to be the most critical biological factor of tumor development; hence, this could be the most suitable characteristic to target for the development of immunotherapeutic effectiveness. Therefore, our group has attempted, for the past two decades, to identify specific tumor-associated antigens the expression of which is paralleled by the cancer-initiating property, namely antigens associated with CSC/CIC. To this end we developed rat and mouse models by using various activated oncogenes, as shown in Fig. 2. Consequently, mAb 109 was successfully established, and this detected the cell-surface antigen that is expressed on activated H-ras transformant W31, which is compatible with highly tumorigenic CSC/CIC, but not on its parental WKA-H rat fetal fibroblast (WFB) non-transformant.[41,42] W31 cells are not only extremely highly

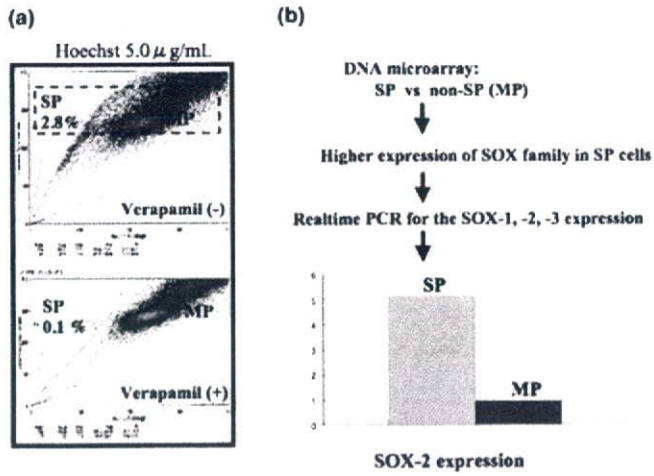

Figure 3 (**a**) Side population (SP) of LHK2 human lung carcinoma line and (**b**) detection of antigens expressed preferentially in the SP rather than the main population (MP) cells assessed on DNA microarray and real-time polymerase chain reaction (PCR).

tumorigenic *in vivo*, because inoculation of syngeneic rats or nude mice with even as few as 100 cells results in rapid tumor development, but they also show very high anchorage-independent growth, implying that the cells exhibit the most critical characteristics of CSC/CIC. Subsequent molecular cloning of mAb 109-defined antigen has clarified that it is CD44.[43] This investigation, although in an animal model, was presumably one of the earliest of CSC/CIC marker studies. In fact several subsequent recent reports in early 2000 supported CD44 as one of the critical human CSC/CIC markers in cancers of the breast, pancreas, prostate, ovarium and head/neck in addition to other markers, including CD133.[44–47] CD133 is amply expressed in glioma and hepatocellular, pancreatic, prostate and colon carcinomas.[48,49]

Although these cell-surface markers are useful to determine the biological nature of CSC/CIC, it is difficult to use immunological targets because their expression is distributed widely even in normal tissues. It is well known that CD44 and CD133 are both expressed in normal tissues and cells, including vascular endothelial cells and hematopoietic stem cells, and this may indicate strong side effects in clinical trials. Therefore, more sophisticated techniques to isolate CSC/CIC and for tumor-specific antigen analysis of CSC/CIC are required.

The side population (SP) is the fraction of cells in fluorescence-activated cell sorting that can exclude pigment dyes such as Hoechst 33342 because the cells express ATP binding cassette (ABC) transporters such as ABCG2, which directly transport chemotherapeutic agents and dyes from inside to outside the cells.[50] The majority of CSC/CIC are known to be resistant to chemotherapy, and this could be attributable to the expression of ABC transporters.[51,52] As shown in Fig. 3, the evidence that verapamil functionally

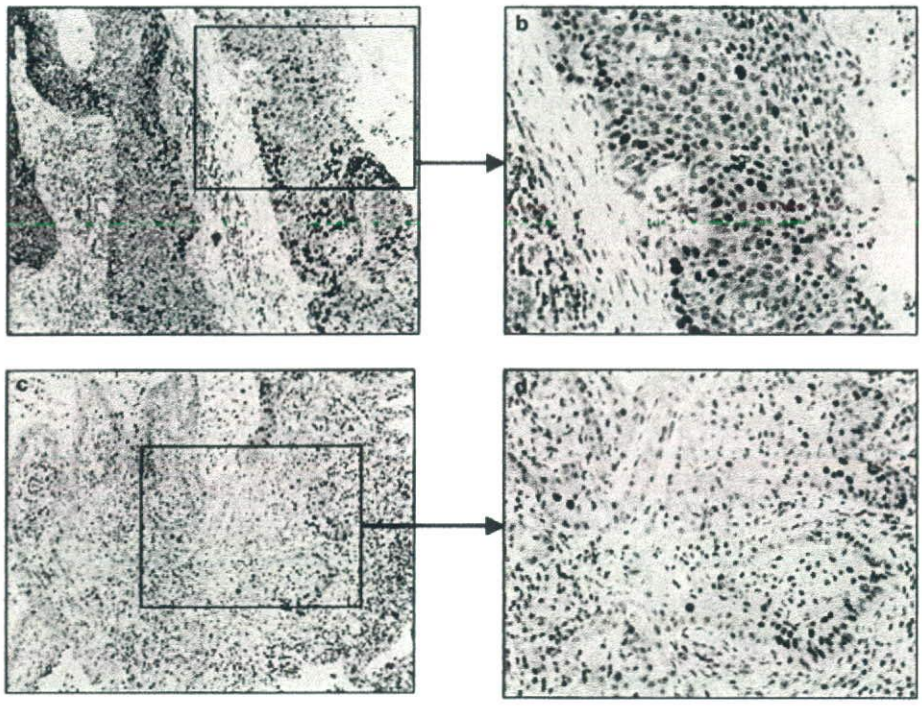

Figure 4 Immunohistochemistry of SOX-2 antigen expression in primary human lung cancers. (**a**,**b**) Patient 1, 74-year-old man; (**c**,**d**) patient 2, 59-year-old man.

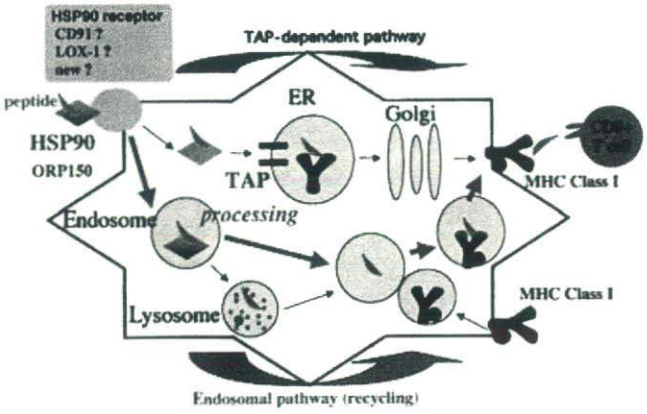

Figure 7 Cross-presentation of extracellularly administered heat shock protein (HSP)90-peptide complex to cytotoxic T-lymphocyte (CTL). HSP90-chaperoned peptides preferentially enter via putative cell surface receptors into the early endosomal pathway rather than TAP-ER, and consequently can be presented to CTL. ER, endoplasmic reticulum; MHC, major histocompatibility complex; ORP, oxygen regulated protein; TAP, transporter associated with antigen processing.

blocks ABC transporters, particularly ABCG2, contributed to CSC/CIC isolation as an SP. We demonstrated that we could isolate SP cells from malignant fibrous histiocytoma (MFH2003), lung cancer (LHK2), breast cancer (MCF7), and colon cancers (SW480, KM12LM, HCT15, Colo206 and HT29). *In vivo* tumorigenicity assays clearly indicated overt tumor development induced by inoculations into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice with as few as 100–150 SP cells, whereas 100 000–150 000 cells were required for the same size tumor development with the non-SP or main population (MP) fraction.

Clearly, it is highly reasonable and intriguing to compare antigens of SP and MP cells, because tumor antigens selectively expressed in SP cells are considered to be ideal immunological targets. We recently isolated such antigens and, as shown in Fig. 3, SOX-2 in particular appeared to be a very potent candidate. This molecule is expressed in many tumor tissues, and especially in basal portions of many pulmonary squamous cell carcinomas (Fig. 4) and breast carcinomas. The HLA-A24-restricted antigenic epitope of (sex determining region Y)-box 2 (SOX-2) was also determined, and it is highly worthwhile to undertake clinical trials for the development of CSC/CIC-based immunotherapy. Obviously, as other potent CSC/CIC tumor antigens are increasingly required for future use in immunotherapy, there is a demand for rapid and substantial research progress.

Link and cooperation between innate and specific tumor immunity: Piloting by endogenous chaperones to anti-tumor peptide-specific immunity

Lessons from our clinical studies suggested that we obviously need other protocols for inducing greater and satisfactory clinical effectiveness that corresponds with immunological responses. It can be easily hypothesized that peptide vaccines alone are not sufficient to induce tumor regression because the activation of APC such as DC is

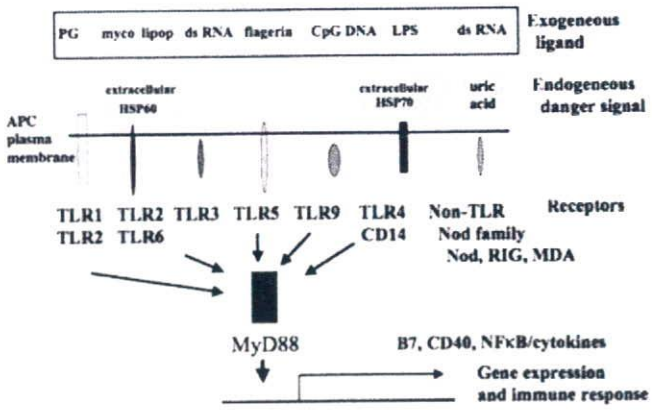

Figure 5 Innate immunity activation in antigen-presenting cells (APC) by exogenous and endogenous danger ligands and their receptors. CpG, unmethylated CG dinucleotides; dsRNA, double-strand RNA; HSP, heat shock protein; LPS, lipopolysaccharide; MDA, melanoma differentiation-associated gene; myco lipop, mycoplasma lipoprotein; RIG, retinoic acid-inducible gene; TLR, Toll-like receptor.

pivotal for the induction and proliferation of activated functional CTL.[53,54] To this end, in our laboratory at Sapporo Medical University, such new approaches have been investigated since 1989 when we found that heat shock protein (HSP)-like molecules were expressed along with the neoplastic cell transformation by the introduction of the activated ras oncogene into cells.[42,55,56] In this experiment the HSP70-like molecule was expressed on the surface of W31 cells, an activated H-ras-induced transformant of rat fetal fibroblast WFB cells. Interestingly, this molecule was also relevant to recognition by certain T cells.

HSP is known functionally as a molecular chaperone. It assists in conformational maturation and stabilization of client substrates, or sometimes directs their disruption and denaturation for further molecular degradation. In addition to its intracellular function, HSP could play a role even when released to the external cellular milieu. Based on further analysis of the immunological roles of these external HSP, several interesting observations were recently reported, implying that HSP complexed with peptide antigens can work as very potent antigen-specific CTL stimulators, as discussed below.[57–62]

Meanwhile, for the past decade, the whole mechanism of APC activation was largely clarified by the findings on Toll-like receptors (TLR) and non-TLR such as NOD family proteins as well as non-NOD proteins.[63–65] The ligands of TLR were also clarified as illustrated in Fig. 5. In addition to exogenous bacterial and viral ligands, certain endogenous molecules such as uric acid, work as ligands for these innate immunity receptors, and Matzinger *et al.* proposed that these endogenous ligands to TLR were danger signal models.[66] HSP are typically considered to be strong endogenous ligands for certain TLR. In fact, extracellular addition of HSP70 to APC can induce production of inflammatory cytokines such as tumor necrosis factor-α and IL-6 in DC.[57,58,60]

In our laboratory at Sapporo Medical University we investigated whether HSP could induce APC activation and maturation and consequent effective CTL activation.[37,58] We assessed immunogenic potentiation by using HSP70 and HSP90. We found that certain forms of the HSP-peptide complex could strongly induce peptide-specific CTL activation.[60,61] Such activation was particularly efficient with an HSP90-peptide complex.[67] Immunization with the HSP90–survivin 2B80-88 peptide complex (which was formed in 30 min at 45°C with purified HSP90 and HLA-restricted survivin 2B80-88 peptide) into the HLA A*2402 transgenic mouse resulted in highly efficient *in vitro* induction of HLA A24-restricted survivin 2B80-88-specific CTL. This was not possible with immunization using the survivin 2B80-88 peptide alone. Very importantly, the extent of immunogenic potentiation with the HSP90-survivin 2B80-88 peptide vaccine was almost the same as with the immunization using an emulsion composed of a mixture of complete Freud adjuvant, currently the strongest available adjuvant, and survivin 2B80-88 peptide.[61,67]

These observations strongly suggest that the HSP90-survivin 2B80-88 peptide complex is worth using for *in vivo* immunotherapy. To this end we established a mouse immunotherapy model using fibrosarcoma line TG3, which was obtained from *in vivo* tumor induced by methylcholanthrene treatment of HLA-A*2402 transgenic mouse skin, and then the TG3-sur2B line was obtained by transfecting the survivin 2B gene into the TG3 line. As shown in Fig. 6, subsequent experiments indicated that immunization with the HSP90–survivin 2B80-88 peptide complex, but not with the peptide alone or HSP90 alone, clearly reduced TG3-sur2B tumor growth in HLA-A*2402 transgenic mice.

Our concomitant experiments have also suggested that enhanced peptide immunogenicity to CTL conferred by an extracellularly administered HSP-peptide complex is possibly mediated by increased cross-presentation of the peptide in DC, as illustrated in Fig. 7.[61] We observed that HSP90-assisted peptides can enter very quickly into early endosomes, rather than the transporter associated with antigen processing–endoplasmic reticulum (TAP-ER) pathway, of DC and stay there for a while, thereby transferring peptides to cell-surface recycled HLA class I. Consequently, such DC could efficiently induce peptide-specific CTL. From a practical point of view, approaches for the *in vivo* immunogenic potentiation of peptide vaccines are critically important for future clinical use. From this point of view, the HSP90 peptide complex immunization strategy is intriguing for developing human tumor immunotherapy.

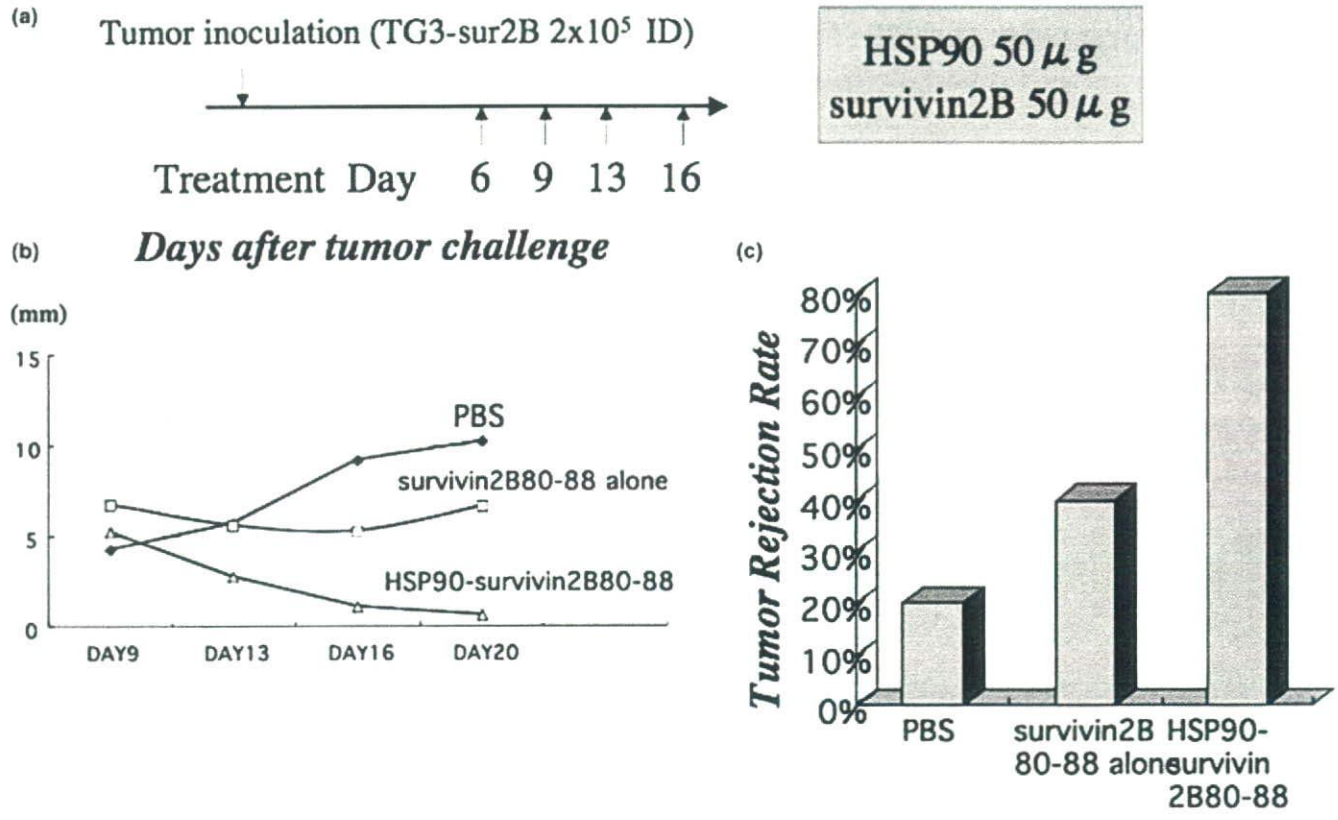

Figure 6 Potentiation of *in vivo* immunogenicity of survivin 2B80-88 cancer peptide vaccine by molecular chaperone heat shock protein (HSP)90. HSP90 and the HLA-A24-restricted survivin 2B80-88 peptide were complexed (HSP90/survivin 2B complex) *in vitro* at 45°C for 30 min, and the complex was administered s.c. four times at days 6, 9, 13 and 16 after inoculation of TG3-sur2B fibrosarcoma cells into HLA-A*2402/K^b transgenic mice. Tumor size was measured at 9, 13, 16 and 20 days after inoculation.

NEGATIVE REGULATION

There is no question that many different ways to overcome the negative regulation of tumor immunity, that is, tumor immunoescape mechanisms, will be required for more efficient clinical and immunological outcomes of immunotherapy. Perhaps immunoescape exists to a greater or lesser extent in almost all cancer patients although its extent and mechanism depend on the patient. As indicated in Table 4, immunoescape in tumor immunity is categorized into two aspects, one of which is attributable to tumor cells. The downregulation of tumor antigens, antigenic peptides and HLA heavy and light chains (B2M) directly affects recognition of CTL.[68,69] The peptide processing and presentation machinery such as proteasomes and non-proteasomes, ER aminopeptidase associated with antigen processing (ERAPP),[70] TAP and different kinds of molecular chaperones, is also critical for the expression of HLA class I and peptide complexes on the tumor cell surface, and subsequent recognition by T-cell receptors. It is known that tumor cell-derived immunosuppressive cytokines, for example transforming growth factor-β, can induce regulatory T cells. Tumor cells sometimes express FAS ligand, and are thereby protected from FAS-mediated apoptotic cell death by CTL. Other types of immunoescape are caused by various mechanisms in host lymphocytes and APC. It is widely known that T cells become tolerant when exposed to several sets of immunoinhibitory molecules such as CTLA4 (CD152) and PD-1. Some CTL

Table 4 Putative mechanism of tumor immunoescape

Level of cancer cells
1 Reduction of antigenicity
HLA class I heavy chain, light chain (B2M)
TAP, proteasome
Peptide processing chaperones
2 Immunoregulatory molecules
3 Fas ligand, anti-granzyme
4 Others
Level of T cells, APC and relevant immune cells
1 Immune tolerance
2 Killer inhibitory receptor
3 Treg
4 Tolerogenic state of APC
5 Inhibition of recruitment
6 Blocking antibody
7 Others

APC, antigen-presenting cells; HLA, human leukocyte antigen; TAP, transporter associated with antigen processing; Treg, regulatory T cells.

express killer inhibitory receptors, resulting in loss of their cytotoxic function. One particular T-cell subpopulation, the so-called CD25 (+) Foxp3 (+) regulatory Treg cells, is also involved in negative regulation even in anti-tumor immunity. Recent studies clarified an obvious countervailing action to Treg in the tumor immune response. Indeed, the selective elimination of Treg by a bacterial toxin (pseudomonas exotoxin A) conjugated with an anti-CD25 mAb enhanced the tumor regression of melanoma patients who received anti-melanoma peptide vaccines.[71] We should understand the immunobiological details of lymphocyte recruitment into or from tumors as well. In APC it is clear that the differentiation status of DC affects the presentation of antigens and activation of CTL.

Each of the aforementioned subjects must be studied in greater detail with new tools and methods, and the information obtained will contribute to provision of more efficient clinical protocol development for tumor immunotherapy. Nevertheless, analysis of HLA class I antigen expression remains pivotal and fundamental for understanding tumor immunity. This became clear when clinical trials with tumor vaccines were undertaken in many hospitals and institutes.

Molecular pathology of HLA class I expression

Obviously it can be easily speculated that the downregulation of HLA molecules could affect the outcome of tumor growth. Because HLA class I molecules present antigenic tumor peptides to CTL, the expression of HLA class I is believed to be critically important. In studies using tumor cell lines and primary live tumor tissues, there were multiple reports indicating loss of heterozygosity (LOH) in chromosome region 6p12,[68,69] which contains the HLA genome.

Although HLA class I is the most pivotal molecule in tumor immunity, its whole scope of expression in tumors has been controversial. This was partly due to the lack of an anti-HLA mAb. Until very recently an mAb that could detect HLA class I molecules in routine paraffin-embedded sections was not available. We recently succeeded in establishing such an antibody, mAb EMR8-5.[72] As shown in Fig. 8, this mAb detects the HLA class I heavy chain of all HLA-A, B and C alleles. Furthermore, as shown in Fig. 9, this mAb immunohistochemically detects HLA class I molecules of colon cancer cells, and there is a clear contrast between positive staining of vessels, leukocytes and lymphocytes, and negative portions of colon cancer cells.

Using this mAb we found, surprisingly, that breast and prostatic cancers had obvious downregulation of HLA class I molecules. The breast and prostatic cancers had only 15% and 18% positive HLA class I expression, respectively. In soft-tissue sarcomas, oral cancers, and renal cell cancers expression was detected in <50%, whereas in urinary

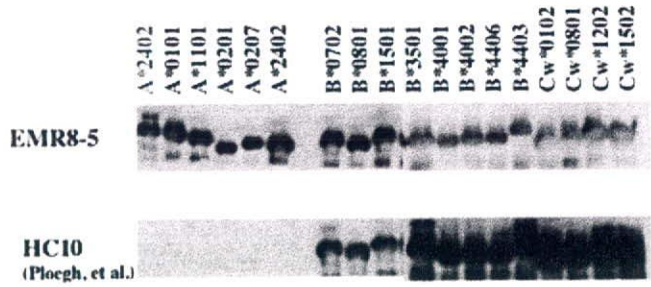

Figure 8 Anti-human leukocyte antigen (HLA) class I heavy chain mAb EMR8-5 can detect all recombinant proteins of HLA-A, B and C alleles on western blot. HC10 mAb reacts with HLA-B and C, but scarcely with HLA-A alleles.

bladder, colon and lung cancers the rate was nearly 70%. It is particularly interesting that 90% of metastatic breast cancers lost the expression of HLA class I, even in cases in which primary cancer cells showed strong expression.

These observations are critically important from the prognostic point of view for cancer patients. For example, there is a strong correlation between the expression of HLA class I as assessed by mAb EMR8-5 and the mortality rate in osteosarcoma patients.[72] Approximately 80% of patients with high expression of HLA class I remained alive even at 100 months after surgical resection. This rate fell to 60% of patients with heterogenous expression of HLA class I, in which some but not all tumor cells had reduced expression of HLA class I. Patients without the expression of HLA class I in osteosarcoma cells, however, had a very rapid clinical course, and all patients died before 20 months after resection. These clinical features also hold true for other neoplasms such as carcinomas of the kidney, urinary bladder, colon, pancreas, breast and lung.[73,74]

These findings have led to important investigations into ways to restore the expression of HLA class I molecules in HLA class I-reduced or deficient tumors. To this end we also developed the anti-β2 microglobulin (B2M; HLA class I light chain) mAb EMR-6. This mAb can clearly detect B2M in paraffin sections. Our analysis indicated that in breast cancers almost all patients with reduced HLA class I heavy chain expression had reduced or deficient expression of B2M, confirming the central role of B2M in the HLA class I expression of breast cancers.

We investigated the molecular mechanism of downregulation of B2M in breast cancers. Previously LOH was detected, but the frequency was relatively low. Other genetic changes including point mutations were also low in frequency. These observations suggest that non-genetic, possibly epigenetic, changes could occur.[75–77] We first studied the DNA methylation status of the promoter area of the B2M genome using methylation-specific polymerase chain reaction in HLA class I-deficient breast cancer cell lines and primary cancer