Current progress and perspectives for human tumor immunotherapy

<Review>

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ABSTRACT

The investigation of human tumor immunotherapy has remarkably advanced in the past decade. In our laboratory, human tumor antigens and their HLA-A24-restricted immunogenic peptide epitopes were determined to develop therapeutic and prophylactic human cancer vaccines. Among these peptides, survivin 2 B peptide derived from survivin, an inhibitor of apoptosis protein (IAP), is immunogenic in more than 50 % of cancer patients with a wide variety of tumors, including colon, pancreas, lung, breast, urinary bladder and oral cancers. It is now under clinical trials, and with careful immunological monitoring we will finally be able to know if these vaccines can work clinically.

To develop a potent clinical therapeutic protocol, the immunological tumor escape mechanism should be more thoroughly examined in human tumor materials. To this end, anti-HLA-A, B, and C allele-specific monoclonal antibody EMR8-5, which can be used in routine paraffin-embedded sections, was successfully established. Unexpectedly, our data indicated that a high percentage of human cancers, par-

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ticularly breast and prostate cancers, lost HLA-class I molecules in their primary cancer tissues. We will discuss possibilities for resolution of this important old but yet new problem.

Although recent evidence has been accumulating for an important role of the heat shock proteins (HSPs) as so-called danger signals in initiating innate immunity and consequently activating acquired immunity, the precise immunological basis for this phenomenon remains to be elucidated. Our study indicated that certain HSP-chaperoned immunogenic peptides, particularly HSP90, could efficiently enter the cross-priming pathway in dendritic cells. Interestingly, this cross-priming was TAP-independent and followed endocytic pathways. We also showed that HSP90-chaperoned peptide complexes could work as a potential tumor therapeutic vaccine in the HLA-A24 transgenic mouse model.

Key words : Tumor antigens, Cancer vaccines, HSP, Cross-presentation

INTRODUCTION

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 Boon et al [1]. In the past decade many such melanoma tumor antigens and their peptides presented by each HLA-allele were discovered, as summarized in Table 1. Using antigenic peptides of these tumor antigens or protein antigens themselves, clinical trials for tumor immunotherapy have been performed in many institutes and hospitals in the United States, Europe and Japan [2-6].

In almost all trials when these vaccine candidates were injected into patients without adjuvants, there were overt side effects and toxicities, but clinical responses were not strong except for a few cases [5]. Immunological monitoring using tetramer and enzyme-linked immunospot (ELISPOT) analyses has indicated that some cases, but not many, show positive correlation between clinical and immunological responses.

These observations suggest that tumor antigenic peptides could work as anti-cancer vaccines in tumor immunotherapy as well as prophylactics to inhibit the tumor incidence. It is also highly likely that these anti-cancer vaccines can be used in strong anti-cancer therapeutic regimens if combined with adequate immuno-stimulatory adjuvants. Along with these trends in human tumor immunology research, a certain EU-based pharmaceutical company has already begun to undertake commercialization of tumor vaccines for cancer patients.

In our laboratory, we have principally investigated the molecular nature of human tumor antigens that are recognized by CTL for the past 10 years. We also determined the antigenic peptides of some of these tumor antigens, and, in collaboration with clinical departments, phase I clinical trials for assessing the toxicity and immunotherapeutic potential of these peptides have been performed [3, 4]. In the present article, the current status of these studies is reviewed, and future perspectives for human cancer immunotherapy and prophylaxis will be discussed.

Melanoma antigens recognized by CTL and immunotherapy

Human melanoma antigens that are recognized by CTL have been found in the past decade [7], and more than 20 melanoma antigens have been reported, as summarized in Table 1. Many of these antigens have undergone clinical trials, and their side effects and clinical responses were assessed. At the first stage of the trials there were positive clinical results in Europe and the US. However, in 2003 Rosenberg et al. reported that less than 5 % of patients who received peptide vaccines such as gp 100, Mart1 and tyrosinase plus IL-2 showed a complete response (CR) [5].

This led to skepticism about immunother-

Table 1 Melanoma antigens/peptides recognized by autologous cytotoxic T lymphocytes

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		
beta-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
Overexpression antigens		
PRAME	A24	LYVDSLFFL
P15	A24	AYGLDFYIL

apy with peptide cancer vaccines. Furthermore, immunological monitoring for peptide-specific CTL using tetramers and ELISPOT, which can detect peptide-specific precursors and functional activated CTL, respectively, showed that the immunological response was not always parallel to the clinical response. However, in 2006 a UK-based pharmaceutical company announced that 3-year long observation after MAGE-A3 vaccine inoculation indicated a 33 % reduction of the post-operative recurrence in non-small lung cancers, as compared with a placebo group. This observation provides hope for current and future immunotherapies,and has accelerated many different investigations into human tumor immunology.

Tumor antigens identified in Sapporo

In addition to melanoma antigens, tumor antigens from non-melanoma tumors, such as colon, breast, lung, urinary tract, head and neck cancers and soft part sarcomas have been analyzed in various laboratories. Although the immunogenicity of these non-melanoma antigens was relatively weak, a certain number of tumor antigens from these non-melanoma tumors were determined.

In our laboratory, as shown in Table 2, we identified two genes of established autologous tumor − CTL pairs, C98[8, 9] and papilloma binding factor (PBF)[10, 11], from gastric cancer and osteosarcoma, respectively. In various immunological approaches the inhibitor of apoptosis

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, A2, A24	scheduled
Reverse immunology				
1) Apoptosis-related				
various	AYACNTSTL (2B)	survivin	A24	phase I
various	KWFPSCQFLL (L7)	livin	A24	phase I
2) Chromosome translocation				
synovial sarcoma				
	GYDQIMPKK (B)	SYT-SSX	A24	phase I
	GYDQIMPKI (K9I)	SYT-SSX	A24	phase I
Bioinformatic immunology				
variousn.d.		5 antigens		scheduled

protein (IAP) family members, survivin and livin, were shown to be powerful, highly-likely tumor antigens since these two antigens were selectively expressed in tumor tissues but not in normal counterparts [12-14]. In particular, the expression of survivin protein was very high; more than 90 % of colon, lung, pancreas and breast cancers had high expression of this protein [12]. Furthermore, survivin 2B peptide, which is HLA-A24 restricted and derived from the survivin splicing variant, survivin 2B, appeared to have strong immunogenicity for the induction of CTL from patients [13].

We also determined HLA-A24 restricted immunogenic peptides from SYT-SSX fusion protein of synovial sarcomas. This peptide, designated SYT-SSX B peptide, is derived from the fusion point at the SYT-SSX chromosomal translocation. When assessed in a peptide-specific tetramer study, we confirmed that this peptide-specific CTL was found in SYT-SSX chromosomal translocation (+) synovial sarcoma patients' PBL with relatively higher frequency than in non-synovial sarcoma patients [15, 16].

Clinical trials in Sapporo

In 2003, the general surgery and orthopedic surgery departments of our medical school began phase I clinical trials with survivin 2B peptide and SYT-SSX B peptide, respectively. As shown in Table 3, the HLA-A24 restricted survivin2B peptide was given subcutaneously to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, urinary bladder cancers and lymphomas. There were no side effects, and certain patients with colon and lung cancers showed reduction in tumor markers (minor response, MR) (Fig. 1) and growth arrest (stable disease, SD) as assessed by CT (3). However, these effects were not strong enough for the clinical requirements of cancer chemotherapy, since when assessed with the response criteria of RECIST, which requires more than 30 % regression of tumors by CT observation, only one patient each out of 15 colon cancers, three urinary bladder cancers and two lymphomas showed a positive clinical response. Meanwhile, breast and oral cancer patients exhibited resistance to survivin 2B administration. Administration of the HLA-A24-restricted SYT-

Table 3 Summary of phase I clinical trials

tumors	No. of patients	side effect	clinical response	
			tumor maker/SD	RECIST
1) Surviving 2B peptide				
colon	15	no	7/15(47%)	1/15(7%)
breast	12	no	2/12(17%)	0/12(0%)
lung	10	no	5/10(50%)	0/10(0%)
oral cavity	9	no	1/9(11%)	0/9(0%)
urinary bladder	3	no	2/3(67%)	1/3(33%)
lymphoma	2	no	1/2(50%)	1/2(50%)
2) SYT-SSX B peptide				
Synovial sarcoma	6	no	0/6(0%)	0/6(0%)

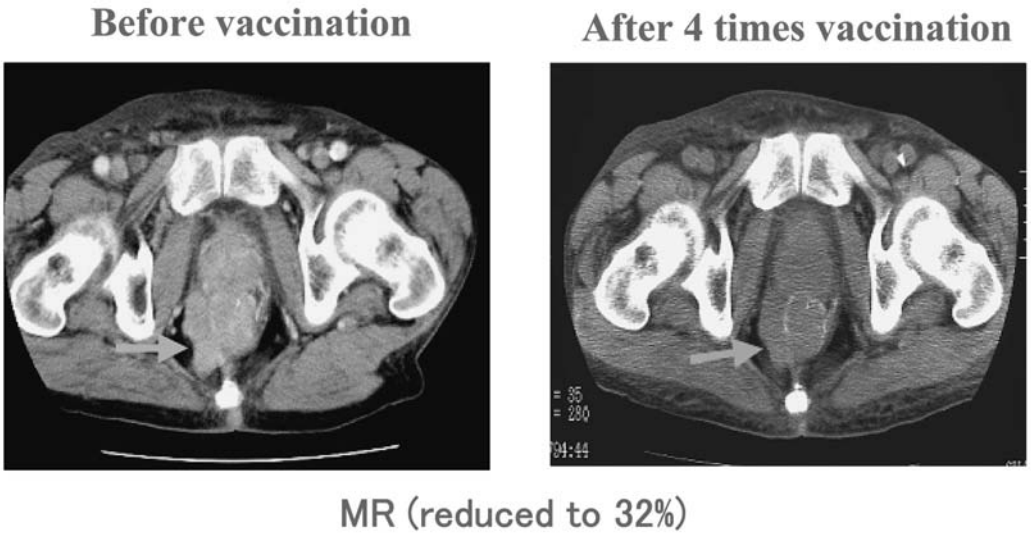

Fig.1 A representative CT view of a colon cancer patient subcutaneously administered survivin 2B cancer peptide vaccine, showing 32 % regression of colon cancer (arrows). Left and right CT views show before and after 4 vaccinations, respectively.

SSX translocation-derived B peptide to six synovial sarcoma patients also showed no side effects, but it resulted in little clinical response as well [4].

These data strongly indicated that the current protocol of single use of survivin 2B peptide alone or SYT-SSX B peptide alone was not sufficient for clinical application. We also studied the immunological responses of the patients. With survivin 2B peptide, certain patients exhibited approximately ten-to fifteen fold increases in the CTL precursor frequency and number of functional CTLs as determined by peptide-specific tetramer and ELISPOT analyses, respectively. However, there was no clear correlation between these immunological and clinical responses.

Lessons from these clinical studies suggested that we obviously need other protocols for inducing greater clinical effectiveness. In our laboratory such new approaches have been investigated. One of these candidates is to use heat shock proteins (HSP) complexed with peptide antigens as discussed below [17-19].

Analysis of HLA class I expression in cancer tissues and immunological and clinical outcomes

Although determination of immunogenic tumor peptides is the fundamental issue in tumor immunology, it is also extremely important to study the tumor immuno-escape mechanism. Recent studies clarified an obvious negative action in the tumor immune response by so-called CD25 (+) FOXP3 (+) regulatory T cells (Treg). Indeed, the selective elimination of Treg by a bacterial toxin (pseudomonas exotoxin A) conjugated with an anti-CD25 monoclonal antibody (mAb) enhanced the tumor regression of melanoma patients who received anti-melanoma peptide vaccines [20].

Obviously it can be easily speculated that the downregulation of HLA molecules could affect the outcome of tumor growth. Since 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 reports indicating loss of heterozygosity in chromosome region 6p12 [21, 22].

However, a mAb that could detect HLA class I molecules in routine paraffin-embedded sections was not yet available. Such a mAb is pivotal to study the expression in patients' cancer tissues. To this end, we succeeded in establishing EMR8-5 mAb. This mAb detects the HLA class I heavy chain of all HLA-A, B and C alleles. As shown in Fig. 2, this mAb immuno-histochemically detects HLA class I molecules of colon cancer cells (Fig. 2a), and there is a clear finding between positive staining of vessels, leukocytes and lymphocytes, and negative staining of colon cancer cells (Fig. 2b).

Surprisingly we found that breast and prostatic cancers showed obvious down-regulation of HLA class I molecules. The breast and prostatic cancers had only 15 % and 18 % positive HLA class I expression, respectively. The expression in soft part sarcomas, oral cancers, and renal cell cancers was less than 50 %,

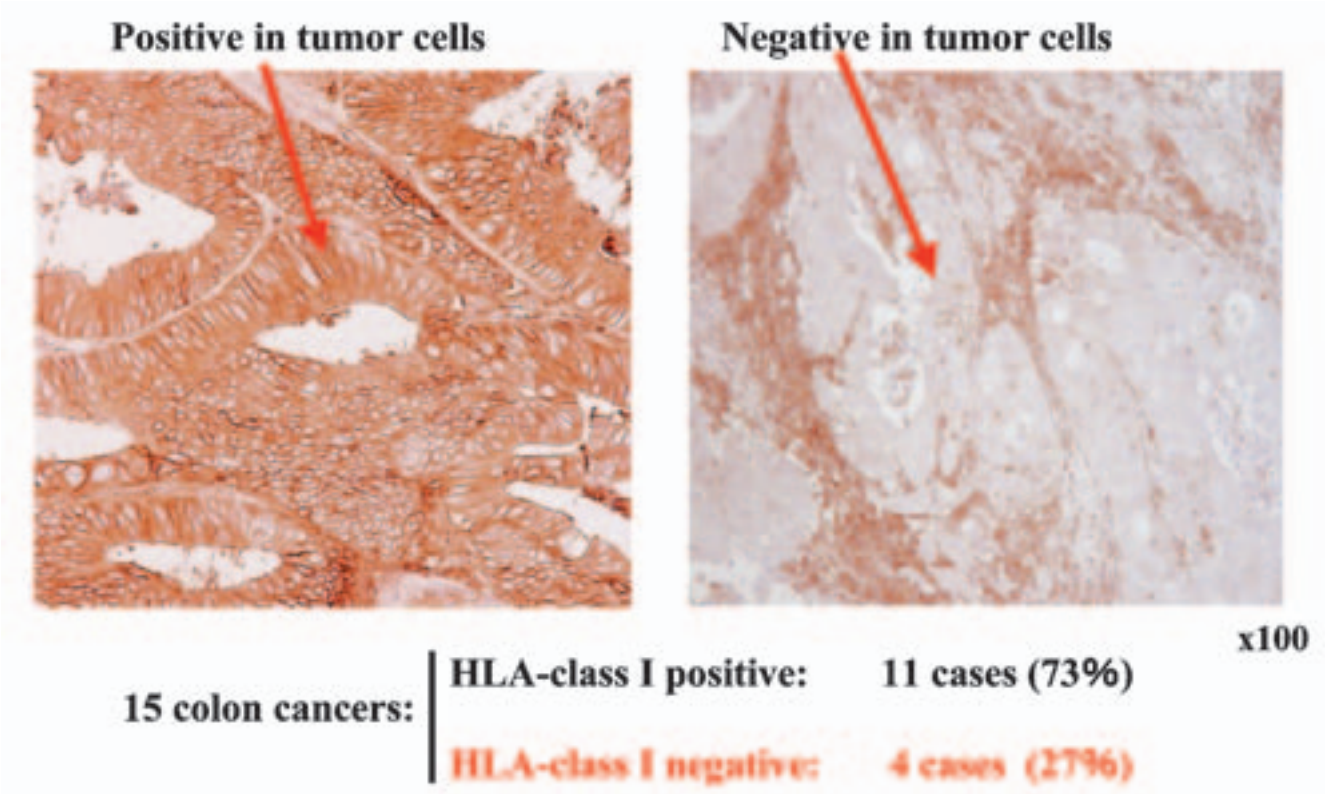

Fig.2 A representative view of immunohistochemical analysis of colon cancers as assessed by anti-pan HLA class I mAb, EMR8-5. Left and right arrows indicate positive and negative staining of colon cancer cells, respectively. Of 15 colon cancers studied, 11 were positive (73 %), and 4 were negative (27 %).

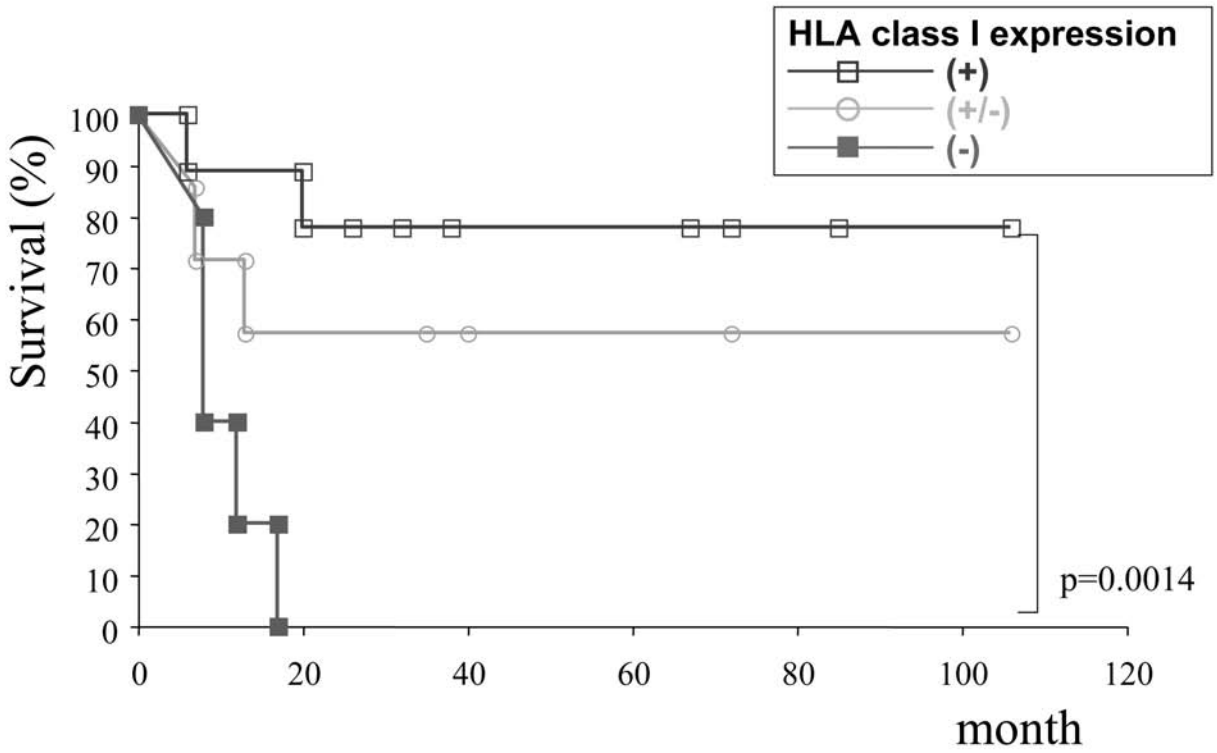

Fig.3 Survival rate of 21 patients with osteosarcoma stratified by anti- pan HLA class I mAb EMR8-5. The numbers of cases with high, low and negative expression were 9, 7 and 5, respectively.

whereas that of urinary bladder, colon and kung cancers nearly 70 %. It is particularly interesting that 90 % of metastatic breast cancers lost the expression of HLA class I, although their primary cancer cells showed strong expression.

These observations are critically important from the prognostic point of view for cancer patients. As shown in Fig. 3, there is a strong correlation between the expression of HLA class I as assessed by EMR8-5 mAb and the mortality rate in osteosarcoma patients [23]. Approximately 80% of patients with high expression of HLA class I remained alive even at 100 months after resection. This rate fell to 60% of patients with heterogenous expression of HLA class I, in which some but not all tumor cells express HLA class I. However, patients without the expression of HLA class I in osteosarcoma cells 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 and lung [24, 25].

These findings have led to important investigations into ways to increase the expression of HLA class I molecules in HLA class I-reduced or deficient tumors. To this end we also developed the anti-b2 microglobulin (HLA class I light chain) mAb EMR-6. This mAb can detect b2 microglobulin in paraffin sections. In breast cancers, almost all cases with reduced HLA class I heavy chain expression had reduced or deficient expression of b2 microglobulin, suggesting the central role of b2 microglobulin in the HLA class I expression of breast cancers. Interestingly, although the results are preliminary so far, a chromatin immunoprecipitation experiment showed that a histone deacetylation inhibitor increased or restored the expression of b2 microglobulin. Thus, we may in the future be able to increase the cell surface expression of HLA class I molecules in tumor

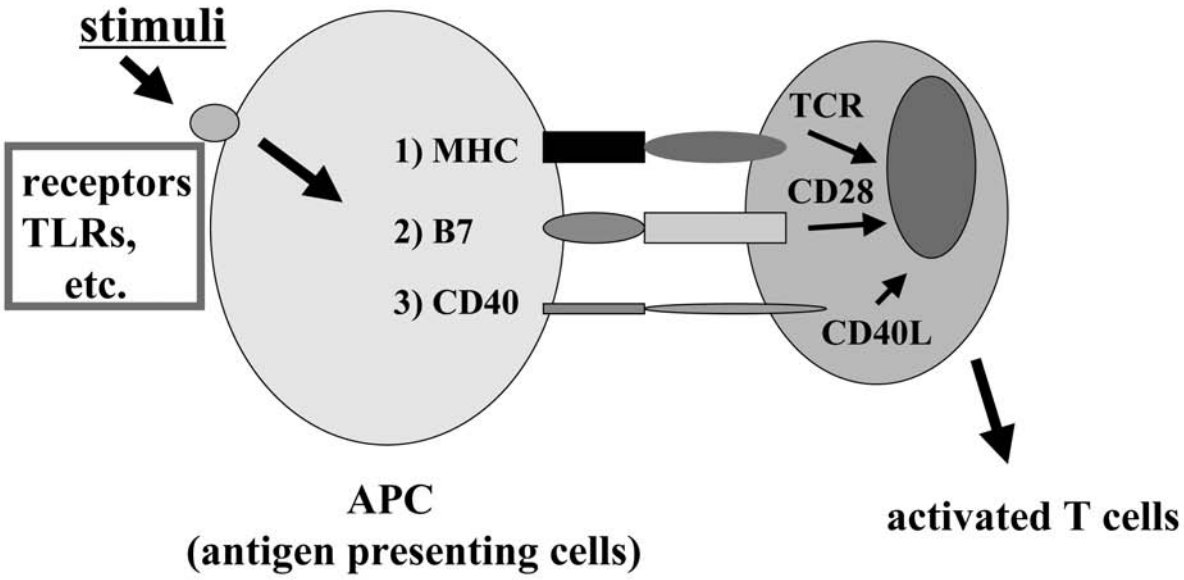

Fig.4 A diagram illustrating the activation of APC and consequent T cell activation.

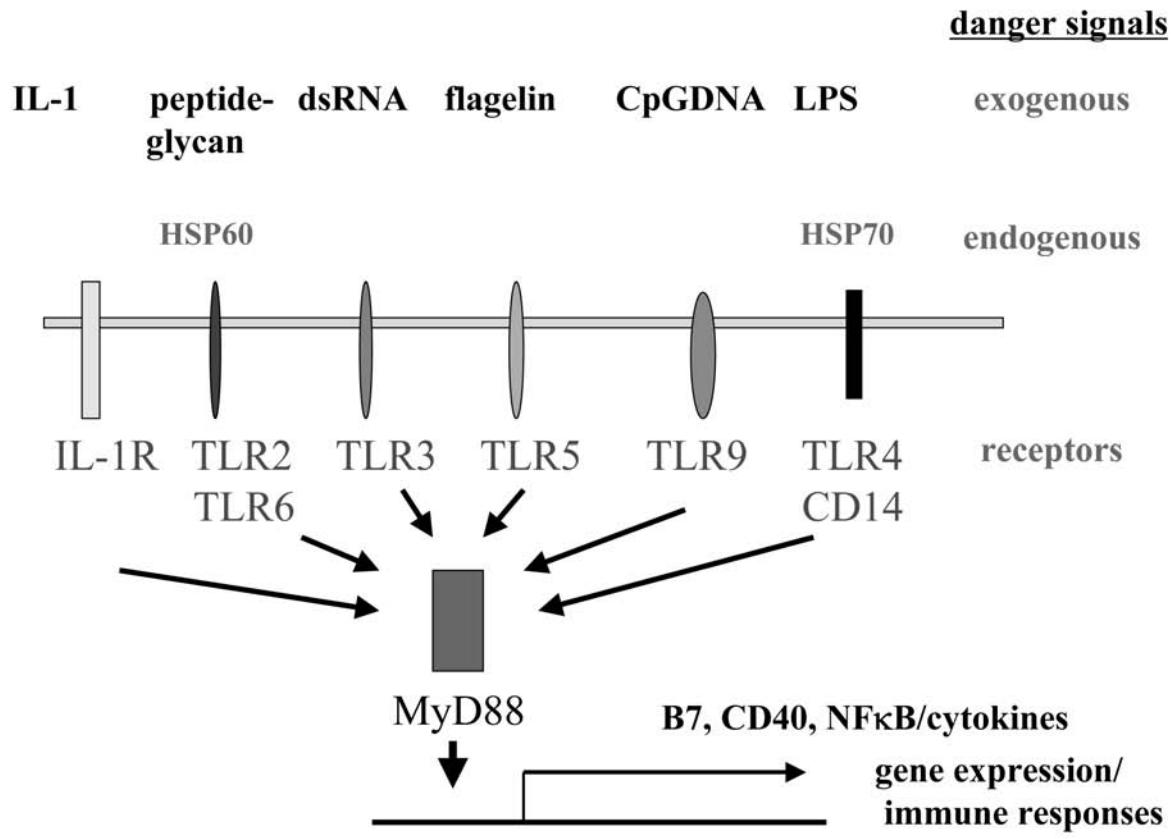

Fig.5 An illustration of APC signal transduction of exogenous and endogenous danger signals and their receptors.

cells by regulating the epigenetic mechanism[26-28].

Potentiation of immunogenic peptides by manipulating innate immunity

It can be easily hypothesized that peptide vaccines alone are not enough to induce tumor regression since, as shown in Fig. 4, the activation of APC such as dendritic cells is pivotal for the induction and proliferation of activated functional CTL [29]. For the past decade the whole mechanism of APC activation was largely clarified by the findings on Toll-like receptors (TLRs) [30]. The ligands of TLRs were also clarified as shown in Fig. 5, and Matzinger et al. proposed these ligands via TLRs as danger signals[31]. HSP are considered to be strong endogenous ligands for certain TLRs. In fact exogenously-added HSP70 can induce inflammatory cytokine production in dendritic cells such as TNFa and IL-6.

In our laboratory, we investigated whether

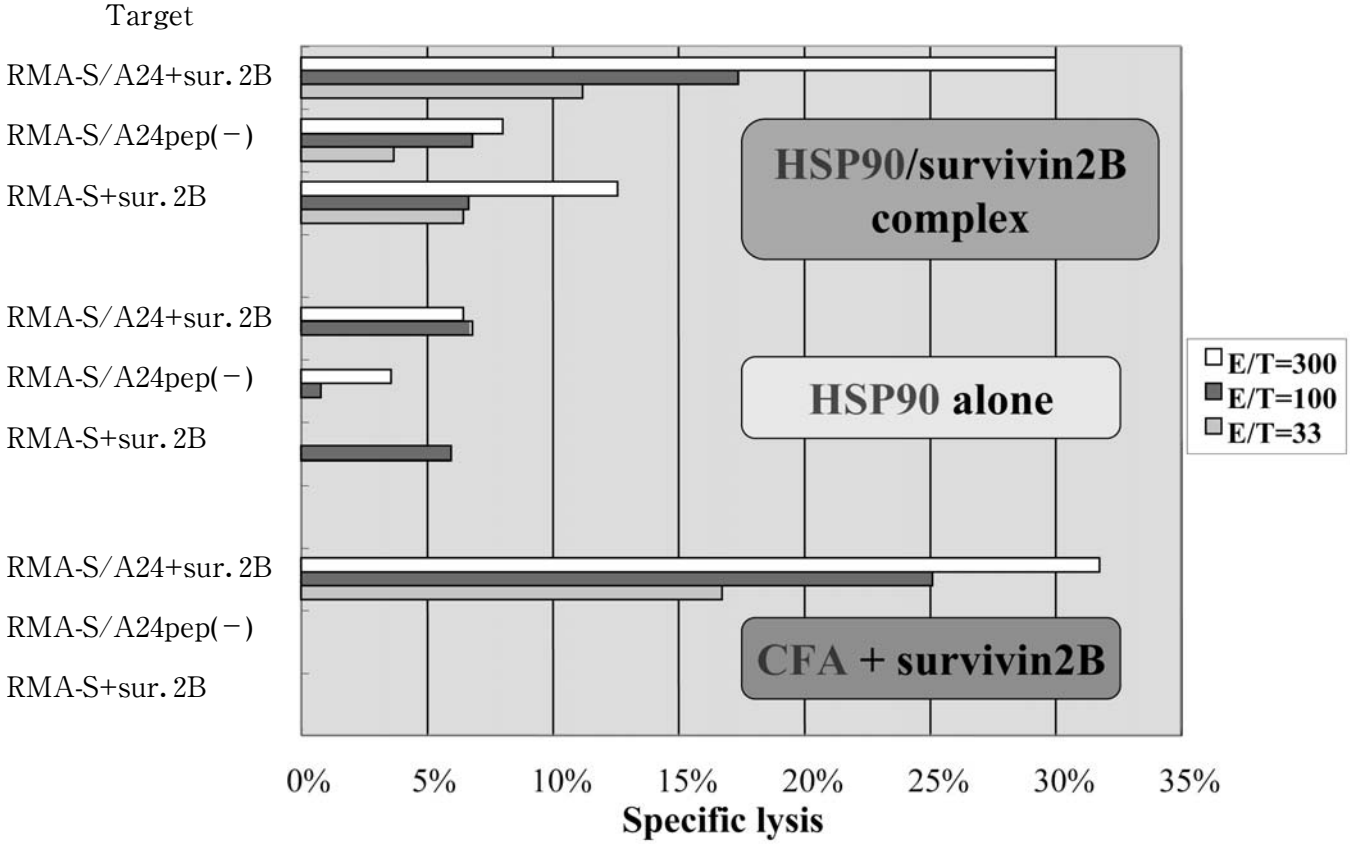

Fig.6 Potentiation of survivin 2B cancer peptide vaccine in vivo immunogenicity by complexing peptide vaccine with an HSP90 molecular chaperone. HSP90 and the survivin 2B peptide were complexed (HSP90/survivin 2B complex) in vitro at 45℃ for 30 min, and the complex was administered 4 times at weekly intervals subcutaneously to HLA-A*2402/K^b transgenic mice. At the 5th week after immunization, splenic CTL cytotoxic activity was assessed against TAP-deficient RMA-S/A24 cells pulsed with and without the survivin 2B (sur.2B) peptide. In this experiment, a mixed solution of CFA and survivin 2B peptide was also administered to transgenic mice, and their capability to induce CTL was compared with those with the HSP90/survivin 2B complex or HSP90 alone.

HSP could induce APC activation and maturation and consequent effective CTL activation[17, 18]. We assessed immunogenic potentiation by using HSP70 and HSP90. As a result, certain forms of the HSP-peptide complex could strongly induce peptide-specific CTL activation.

Particularly, such activation was done with an HSP90-peptide complex. As shown in Fig. 6, immunization with the HSP90-survivin 2B peptide complex, which was formed for 30min at 45 ℃ with purified HSP90 and HLA-restricted survivin 2B peptide, into the HLA A*2402 transgenic mouse resulted in highly efficient in vitro induction of HLA A24-restricted survivin 2B specific CTL. This was not possible with the immunization using control HSP90 alone. Very importantly, the immunogenic potentiation by the survivin 2B cancer peptide vaccine was almost the same as with the immunization of a solution composed of a mixture of CFA and survivin 2B peptide.

These observations strongly suggest that the HSP90-survivin 2B peptide complex is worth employing for in vivo immunotherapy. To this end we established a methylcholanthrene-induced fibrosarcoma line derived from the HLA-A*2402 transgenic mouse. Subsequently, the survivin 2B gene was transfected to this fibrosarcoma line, and the immunization of TG3-sur2B bearing HLA-A*2402 transgenic mice with the HSP90-survivin 2B peptide complex resulted in reduced TG3-sur2B tumor growth.

In addition to the determination of tumor antigenic peptide, 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.

Perspective

As we discussed in this article, human tumor immunology and immunotherapy have advanced to a certain extent over the past ten years. Many human tumor antigens were identified and, using these antigens and antigenic peptides, clinical trials have been conducted. However, clinical outcomes in these trials did not exhibit dramatic clinical effectiveness. Thus the following points are likely to be important for the next decade: (1) More determination of antigenic tumor peptides is pivotal. It may not be scientifically interesting these days, but assessment of highly antigenic tumor peptides is still clinically important. (2) Basic research into immunological tumor escape mechanisms and maneuvers to overcome these mechanisms needs to be done more aggressively [32, 33]. Without these research developments we cannot develop clinically effective tumor immunotherapy. (3) Finally, the linkage between innate and acquired immunity for efficient CTL induction should be analyzed extensively since the immunogenic potential of peptide vaccines is not strong enough. With such progress human tumor immunotherapy and tumor immunoprophylaxis will come to be practically used in the clinical setting.

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