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

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.

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]

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 immuno-regulatory 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
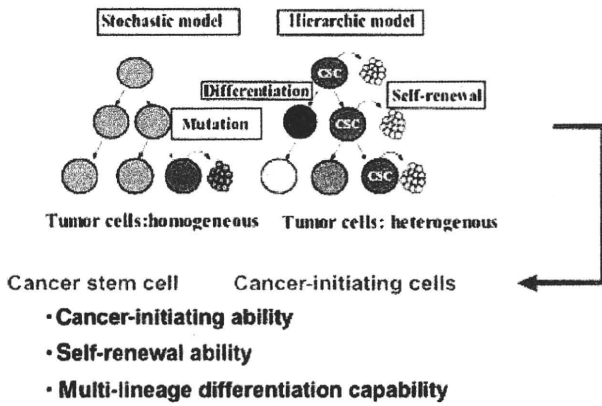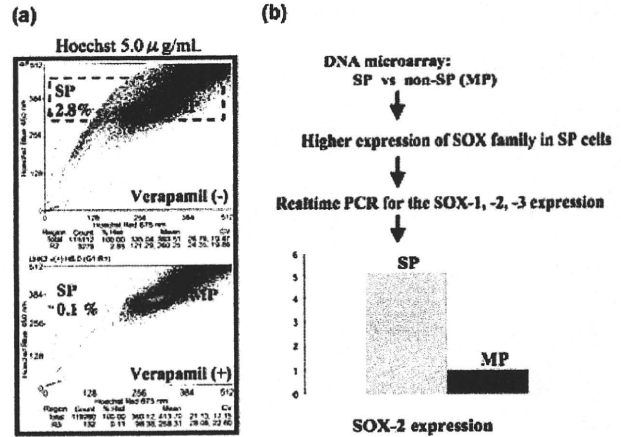
Figure 1 Models of origins for cancer stem cells and cancer-initiating cells.
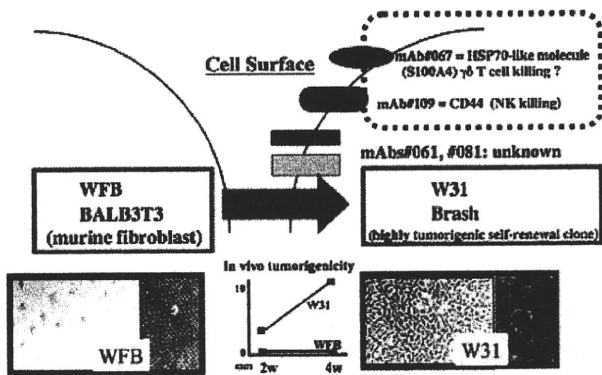
Figure 2 W31 and Brash cells as cancer stem cell/cancer-initiating cell (CSC/CIC) counterparts and the expression of cell surface antigens associated with activated H-ras-induced cell transformation. HSP, heat shock protein; NK, natural killer; WFB, WKA-H rat fetal fibroblast.

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).

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
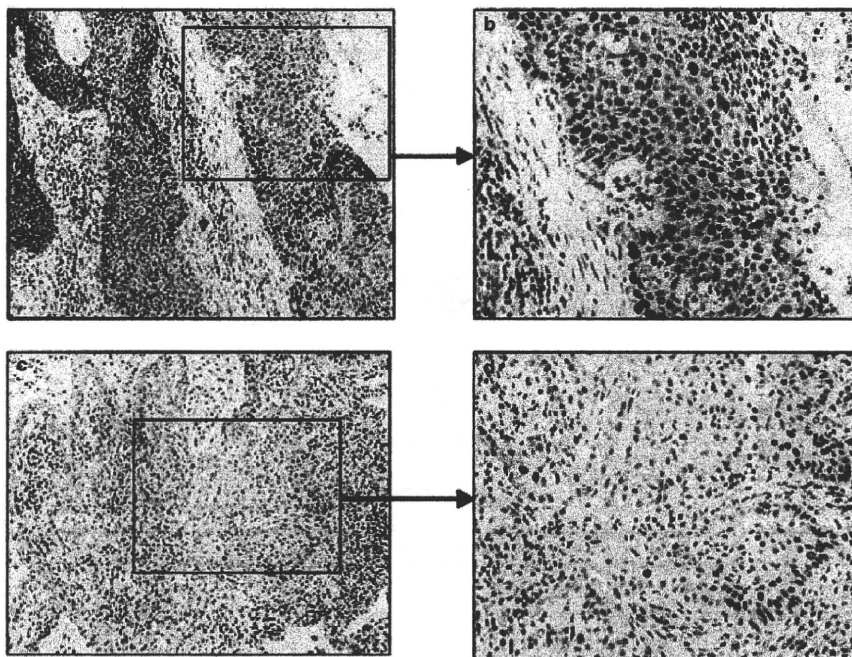
tumorigenic in vivo, because inoculation of syngeneic rats or nude mice with even as few as 100 cells results in rapid tumor development, but they also show very high anchorage-independent growth, implying that the cells exhibit the most critical characteristics of CSC/CIC. Subsequent molecular cloning 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]
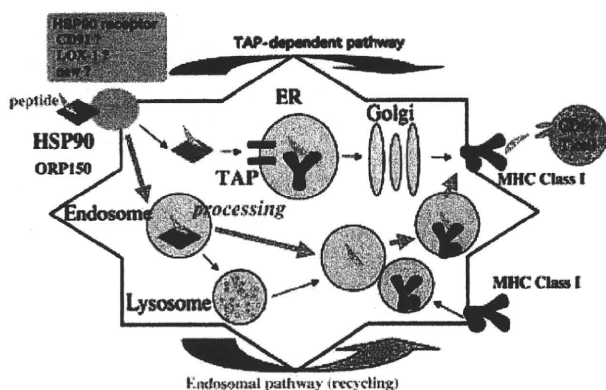
Although these cell-surface markers are useful to determine the biological nature of CSC/CIC, it is difficult to use immunological targets because their expression is distributed widely even in normal tissues. It is well known that CD44 and CD133 are both expressed in normal tissues and cells, including 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

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.

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.

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
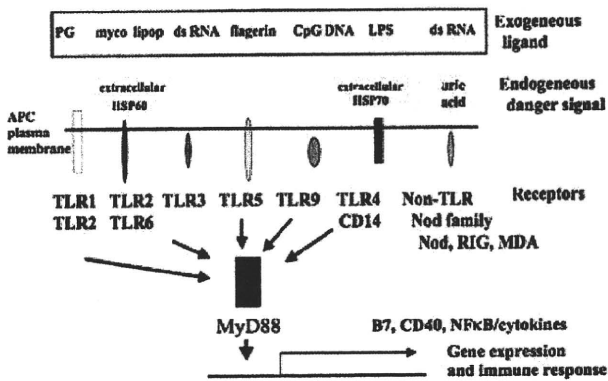
blocks ABC transporters, particularly ABCG2, contributed to CSC/CIC isolation as an SP. We demonstrated that we could isolate SP cells from malignant fibrous histiocytoma (MFH2003), lung cancer (LHK2), breast cancer (MCF7), and colon cancers (SW480, KM12LM, HCT15, Colo206 and HT29). *In vivo* tumorigenicity 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

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, myco-plasma 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]
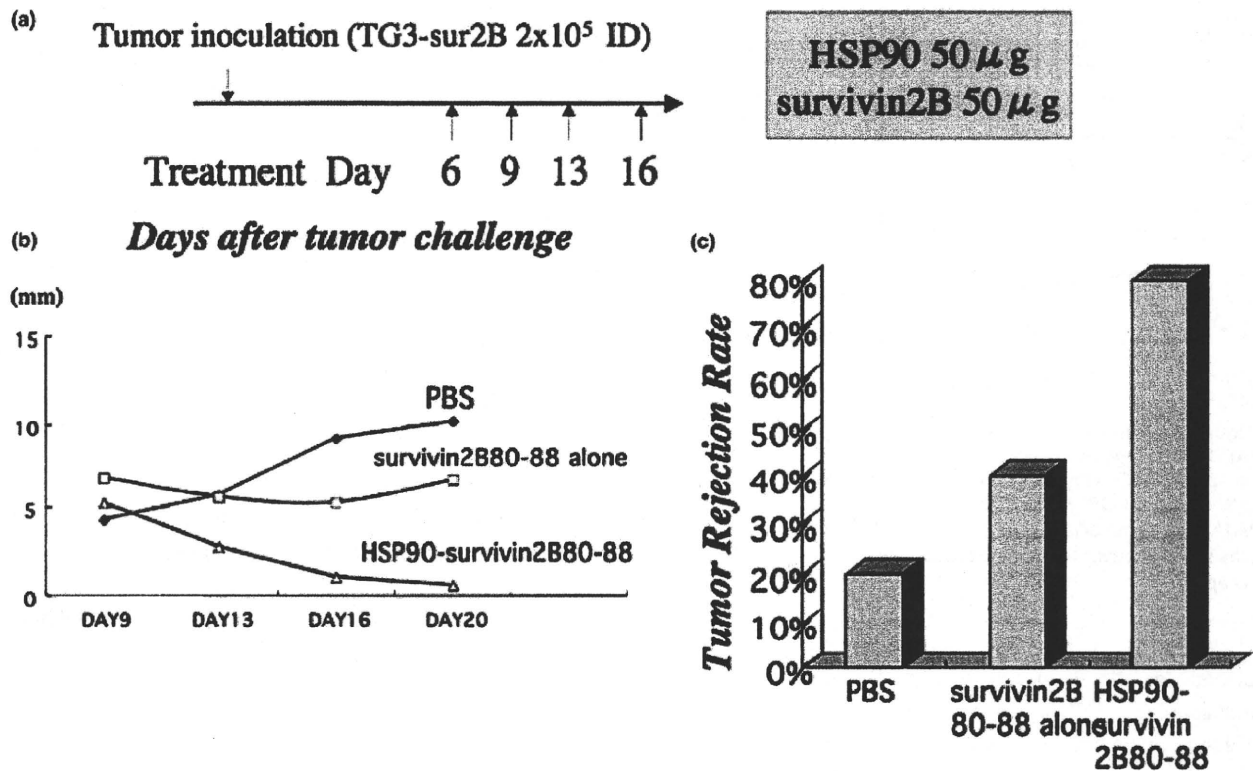
Meanwhile, for the past decade, the whole mechanism of APC activation was largely clarified by the findings on Toll-like receptors (TLR) and non-TLR such as NOD family proteins as well as non-NOD proteins.[63–65] The ligands of TLR were also clarified as illustrated in Fig. 5. In addition to exogenous 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.

(a)

Tumor inoculation (TG3-sur2B 2x10⁵ ID)

Treatment Day   6   9   13   16

HSP90 50 μg
survivin2B 50 μg

(b) *Days after tumor challenge*

(mm)

PBS

survivin2B80-88 alone

HSP90-survivin2B80-88

DAY9   DAY13   DAY16   DAY20

(c)

Tumor Rejection Rate

PBS   survivin2B
80-88 alone   HSP90-
survivin
2B80-88

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/Kᵇ 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

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.

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

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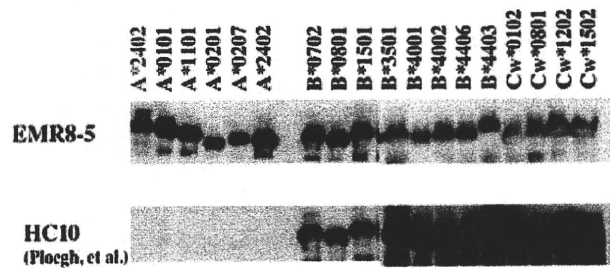
Figure 8 Anti-human leukocyte antigen (HLA) class I heavy chain mAb EMR8-5 can detect all recombinant proteins of HLA-A, B and C alleles on western blot. HC10 mAb reacts with HLA-B and C, but scarcely with HLA-A alleles.

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
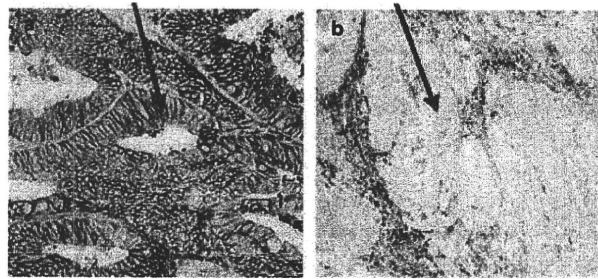
Figure 9   Representative view of immunohistochemistry colon cancers as assessed on the anti-pan HLA class I mAb EMR8-5. **(a)** Positive and **(b)** negative staining of colon cancer cells. **(b)** Positive staining of stromal area, which includes leukocytes, vessels and connective tissue portions. Of 15 colon cancers studied, 11 were HLA-class I positive (73%), and four were negative (27%).
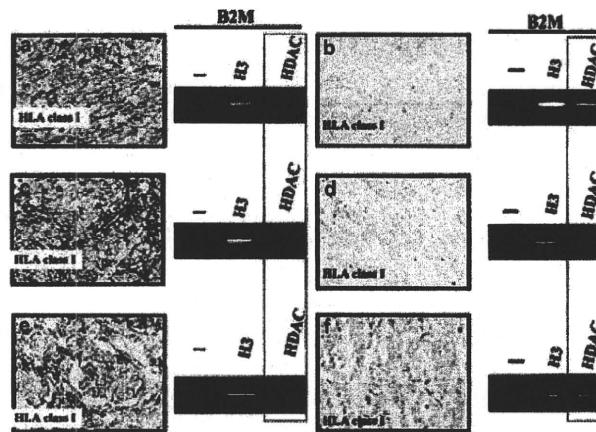

Figure 10   Chromatin immunoprecipitation assays of histone acetylation and deacetylation status in B2M genome of primary breast cancers. **(a,c,e)** Representative HLA class I heavy chain/B2M positive (patients 1–3) and **(b,d,f)** negative (patients 5–7) cases are shown. H3 and HDAC, mAbs that can make immunoprecipitates with acetylated and deacetylated histone, respectively.

tissues. The data indicated that in these breast cancer materials there was reduced methylation. Consequently, a chromatin immunoprecipitation experiment clearly showed that in many such cases deacetylated histone in the B2M genome was present (Fig. 10). Furthermore, treatment of breast cancer cells *in vitro* with a histone deacetylation inhibitor (HDACI) such as tricostatin-A and valproic acid restored and increased the expression of B2M at the mRNA and protein levels.[78,79] This phenomenon was also seen *in vivo* when an MCF7 human breast tumor developed in the SCID mouse was treated with HDACI valproic acid (Fig. 11). Thus, we will be able to restore and increase the cell surface expression of HLA class I molecules in primary breast tumor cells by regulating the acetylation and deacetylation status of histone.[75–77] Intriguingly, this is obviously applicable for clinical trials in conjunction with tumor vaccines.

PERSPECTIVES

Human tumor immunology and immunotherapeutic clinical approaches have advanced to a substantial extent over the
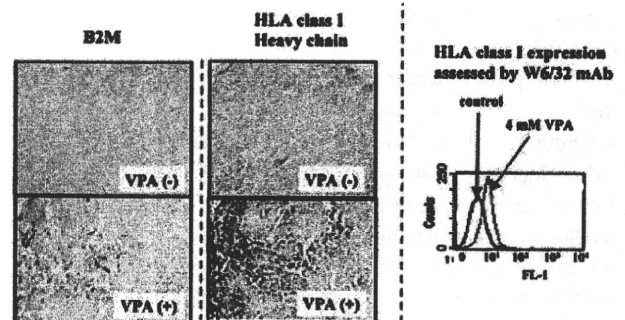
Figure 11   **(a)** *In vivo* and **(b)** *in vitro* restoration of the HLA class I/B2M expression in MCF human breast tumor by the HDAC inhibitor valproic acid (VPA). **(a)** Severe combined immunodeficiency (SCID) mice transplanted with MCF7 human breast cancer were treated by feeding in water with 0.4% VPA for 10 days, and the tumor was analyzed immunohistochemically for the expression of HLA class I heavy chain and B2M molecules. The expression of these molecules was detected with the anti-HLA class I heavy chain mAb EMR8-5 and anti-B2M mAb EMR6, respectively. **(b)** MCF7 cells were treated *in vitro* with 4 mmol/L VPA for 48 h, and were analyzed for the cell surface expression of HLA class I expression with mAb W6/32 using a fluorescence-activated cell sorter.

past 10 years. Many human tumor antigens were identified and, using these antigens and antigenic peptides, clinical trials have been conducted. In fact, immunotherapeutic phase III clinical trials with MAGE-A3, a tumor antigen against lung cancer, are now under way in Europe and Japan. If they are successful, the world's first T-cell-based cancer vaccine will be routinely utilized in hospitals. A DC-based tumor vaccine has already been commercially approved in Switzerland. The vast majority of cancer vaccine clinical trials, however, did not exhibit dramatic clinical effectiveness, even though at least some patients exhibited clinical and immunological effects.

Thus, several points are likely to be important for the next decade. There is still no question that further determination of highly antigenic tumor peptides is pivotal. It may not currently be scientifically interesting, but practical characterization and assessment of these highly antigenic tumor peptides still remains highly important clinically. The intracellular generation mechanism of natural tumor antigenic peptides in tumor cells that bind to HLA is one critical point to be analyzed. The efficient and safe activation of innate immunity and concomitant activation of specific immunity against tumors is necessary for future development of effective immunotherapy.[80,81]

Meanwhile, there is no doubt that basic research into immunological tumor escape mechanisms is increasingly important, and methods to overcome these escape mechanisms need to be created more aggressively.[82-84] Without such research progress we cannot develop substantially effective tumor immunotherapy. It has also been suggested that tumor cells interacts at different levels with various stromal cell components. These features may possibly affect various immunological characteristics of tumor cells. Finally, detailed molecular pathological analysis will be of great importance. *In vivo* imaging to examine recruitment of CTL after vaccinations may be possible in cancer patients if a tracer reagent for human trials is available, providing us with ultimate information about immunological and clinical responsiveness to tumor immunotherapy.

ACKNOWLEDGMENTS

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Polyamine compound deoxyspergualin inhibits heat shock protein-induced activation of immature dendritic cells

Atsushi Sugawara · Toshihiko Torigoe ·
Yasuaki Tamura · Kenjiro Kamiguchi ·
Kyuichi Nemoto · Hiroshi Oguro · Noriyuki Sato

Abstract Polyamine compound deoxyspergualin (DSG) is a potent immunosuppressive agent that has been applied clinically for protecting graft rejection and treatment of Wegener's granulomatosis. Though DSG can bind to heat-shock proteins (HSPs) in cells, its mechanism of immuno-suppressive action remains unknown. It is widely accepted that extracellular HSPs are capable of stimulating dendritic cells (DC) through cell surface receptors, leading to DC activation and cytokine release. In this study, we examined if DSG analogs could inhibit HSP70-induced DC activation. Bone marrow derived immature mouse DCs and peripheral blood mononuclear cell-derived immature human DCs were generated and incubated with Alexa 488-labeled Hsp70 in the presence of methoxyDSG (Gus-1) that had comparable HSP70-binding affinity to DSG or DSG analog GUS-7, which had much more reduced binding affinity for HSP70. The binding of HSP70 to immature DCs was analyzed by laser microscopy and flow cytometry. HSP70-induced DC activation was assessed by TNF-α release by enzyme-linked immunosorbent assay. Binding of Hsp70 to the cell surface of immature DCs was inhibited under the presence of Gus-1, but not under the presence of Gus-7. Immature DCs were activated and released TNF-α by the stimulation with HSP70 for 12 hours; however, the HSP70-induced TNF-α release was suppressed under the presence of Gus-1, and partially suppressed under the presence of Gus-7. Similar results were observed when immature human DCs were stimulated under the same conditions. Immunosuppressive mechanism of DSG may be explained, at least in part, by the inhibition of extracellular HSP70-DC interaction and HSP70-induced activation of immature DCs.

A. Sugawara · T. Torigoe (✉) · Y. Tamura ·
K. Kamiguchi · N. Sato
Department of Pathology (Section 1),
Sapporo Medical University School of Medicine,
S-1, W-17, Chuo-ku,
Sapporo 060-8556, Japan
e-mail: torigoe@sapmed.ac.jp

A. Sugawara · H. Oguro
Department of Ophthalmology,
Sapporo Medical University School of Medicine,
Sapporo, Japan

K. Nemoto
Nippon Kayaku, Co. Ltd.,
Tokyo, Japan

Introduction

Heat-shock proteins (HSPs) are proteins whose expression is increased when the cells are exposed to elevated temperatures or other stress. They are known to play an important role in intracellular protein quality control since they serve to assist folding, sorting, and degradation of cellular proteins, thus preventing intracellular accumulation of degenerated proteins. Recently, it was revealed that extracellular HSPs could serve as a danger signal, which activate inflammatory response and natural immunity in response to cellular injury (Manjili et al. 2005; Asea 2006;

Calderwood et al. 2007). 70 kD HSP (HSP70) and 96 kD HSP (gp96) have been well documented for the mechanism of activation (Doody et al. 2004; Massa et al. 2005; Li et al. 2006; Bendz et al. 2007). HSPs are released into extracellular fluid during cell death or injury, and then bind to the surface of immune cells through cell surface receptors, such as toll-like receptor (TLR) 2, TLR4, CD91, CD40, CCR5, LOX-1, and Scavenger receptor A (Arnold-Schild et al. 1999; Delneste et al. 2002; Roelofs et al. 2006; Warger et al. 2006; Facciponte et al. 2007; Pido-Lopez et al. 2007). The extracellular HSPs can elicit cytokine releases from dendritic cells, macrophages and lymphocytes, leading to activation of innate immunity (Todryk et al. 1999; Asea et al. 2000; Asea 2006). In addition, HSPs are capable of facilitating antigen cross-presentation in dendritic cells, i.e., presentation of extracellular antigens to major histocompatibility complex (MHC) class I pathway, therefore leading to activation of adaptive immunity as well (SenGupta et al. 2004; Ueda et al. 2004; Facciponte et al. 2005; Enomoto et al. 2006; Bendz et al. 2007; Kurotaki et al. 2007). Such an unique immunopotentiative character of HSPs are now applied to vaccine adjuvants, especially in the field of cancer immunotherapy (Srivastava and Udono 1994; Tamura et al. 1997; Noessner et al. 2002; Hauser et al. 2004; Ueda et al. 2004; Wang et al. 2005).

On the other hand, immunosuppressive therapy is required in the field of organ transplantation and autoimmune diseases. Deoxyspergualine (DSG) is one of such immunosuppressive agents, which has been used after renal transplantation and for the treatment of glomerulonephritis (Amemiya 1996; Hotta et al. 1999; Kozaki et al. 1999; Amada et al. 2005; Lorenz et al. 2005). Recently, it was revealed that DSG was effective in the treatment of refractory Wegener's granulomatosis (Schmitt et al. 2005; Erickson and Hwang 2007). Disease improvement during treatment with DSG was achieved in 70% of cases (Birck et al. 2003). In spite of the clinical efficacy of DSG, molecular mechanism of the immunosuppressive action has been still enigmatic. Nadler et al. demonstrated previously that DSG could bind to HSP70 and HSP90 (Nadler et al. 1992, 1998; Nadeau et al. 1994). They showed that DSG can suppress NF-kB signal indirectly by binding to intracellular HSP70 (Nadler et al. 1995). We have found that DSG inhibited the association of HSP70 to TAP, thus leading to inhibition of MHC class I antigen presentation (Kamiguchi et al. 2008). In the present study, we focused on the effect of DSG to immunopotentiative action of extracellular HSPs. Binding of HSP70 to the cell surface and subsequent cytokine release from dendritic cells were assessed in the presence or absence of DSG analogs that have a distinct binding affinity to HSP70. We demonstrate that immunosuppressive action of DSG is mediated, at least in part, through blocking of danger signals of HSP70.

## Materials and methods

Reagents

Two distinct DSG analogs, Gus-1 (methoxyDSG) and Gus-7 (Fig. 1), were provided by Nippon Kayaku (Tokyo, Japan). DSG and Gus-1 have a high binding affinity for HSP70 (K_d=7 μM and 4 μM, respectively), whereas Gus-7 has much reduced affinity for HSP70 (K_d=250 μM) (Nadeau et al. 1994). Purified human Hsp70 and recombinant HSP90 were purchased from StressGen Biotech (Ann Arbor, MI). Bovine serum albumin (BSA) and phosphorylase B were purchased from Sigma-Aldrich (St. Louis, MO).

### Generation of bone marrow derived dendritic cells

Bone marrow derived immature mouse DCs were generated from the femurs and tibia of 5- to 6-week-old C57BL/6 mice (CLEA Japan, Tokyo, Japan). Bone marrow cells (1×10^5/well in a 24-well plate) were incubated in a complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (GIBCO/Invitrogen, Carlsbad, CA) and 20 ng/ml GM-CSF (Endogen, Woburn, MA) for 5 days. GM-CSF-containing medium was gently replaced on day 2 and day 4.

Immature human DCs were generated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers after obtaining informed consent. Briefly, PBMCs were isolated by using Lymphoprep (Nycomed, Oslo, Norway), and then separated into CD14$^+$ cells and CD14$^-$ cells by using MACS separation system and anti-CD14 monoclonal antibody-coupled magnetic microbeads (Miltenyi Biotech, Bergish Blabach, Germany) according to the manufacturer's
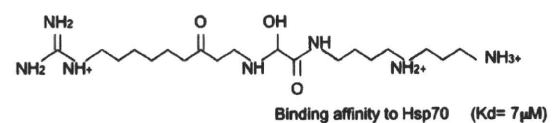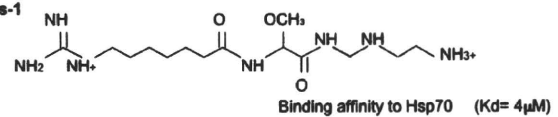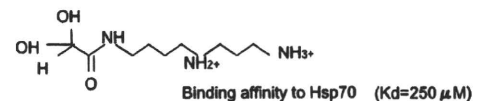

Fig. 1 Structure of DSG and two distinct DSG analogs, Gus-1 and Gus-7. Gus-1 (methoxyDSG) has high binding affinity for HSP70 (K_d=4 μM), whereas Gus-7 has reduced binding affinity for HSP70 (Kd=250 μM) as compared to DSG and Gus-1

instruction. Immature DCs were generated from CD14^{+} cells in the plastic flask by culturing in AIM-V medium (GIBCO-Invitrogen Japan) supplemented with 10% human serum, HEPES (10 mmol/L), 2-mercaptoethanol (50 µmol/L), granulocyte macrophage colony-stimulating factor (100 ng/mL), and IL-4 (1,000 units/mL) for 7 days.

Detection of fluorescence-labeled Hsp70 on the cell surface of DCs

HSP70 and phosphorylase B were labeled with Alexa Fluor 488 by using Alexa Fluor 488 Protein Labeling Kit (Invitrogen, Japan) according to the manufacturer's instruction (Kurotaki et al. 2007). DCs were incubated with 10 µg/ml of Alexa-labeled Hsp70 or Alexa-labeled phosphorylase B in the presence of Gus-1 or Gus-7 (10 µg/ml). DCs were then washed twice with ice-cold phosphate-buffered saline (PBS), fixed with ice-cold 1% formaldehyde PBS, and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) or laser microscopy (Olympus, Japan).

TNF-α release assay

Dendritic cells were pulsed with the indicated concentrations of Hsp70 in the presence of Gus-1 or Gus-7 (10 µg/ml). After incubation for 12 hours, the concentration of TNF-α released into the culture supernatant was quantified by using mouse TNF ELISA kit (Endogen, Woburn, fMA).

Results

DSG inhibits the binding of HSP70 to the cell surface of immature DCs

Purified HSP70 and phosphorylase B were labeled with Alexa Fluor 488 and then incubated with immature mouse DCs for 30 min on ice. Laser microscopic analysis revealed that HSP70 was bound to the cell surface of DCs with a dot-like pattern, whereas phosphorylase B was not (Fig. 2A). Then, the fluorescence intensity was analyzed by flow cytometer in the presence or absence of Gus-1 or Gus-7 (10 µg/mL). As shown in Fig. 2B, the fluorescence intensity of HSP70-pulsed DCs was not changed between PBS and Gus-7 (left panel), whereas it was decreased in the presence of Gus-1 (right panel). These data indicate that Gus-1, but not Gus-7 could inhibit the binding of HSP70 to the cell surface of DCs.

HSP70 stimulation induces TNF-α release of immature DCs

Next, we examined if HSP70 can activate immature DCs. As an activation index, the level of TNF-α release was assessed by ELISA. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (1×10^5 cells/well), and TNF-α concentration of culture supernatant was quantified after 12-hour incubation. Stimulation with


Fig. 2 DSG inhibits the binding of HSP70 to the cell surface of immature DCs. Purified HSP70 and phosphorylase B were labeled with Alexa Fluor 488 and then incubated with immature DCs for 30 min on ice. After washing twice with ice-cold PBS, cells were fixed in 1% formaldehyde PBS and subjected to laser microscopic examination (A) or flow cytometry (B). In B, fluorescence intensity was analyzed after incubation with or without Gus-1 (*right panel*) or Gus-7 (*left panel*). P-B indicates fluorescence intensity after incubation with Alexa 488-labeled phosphorylase B
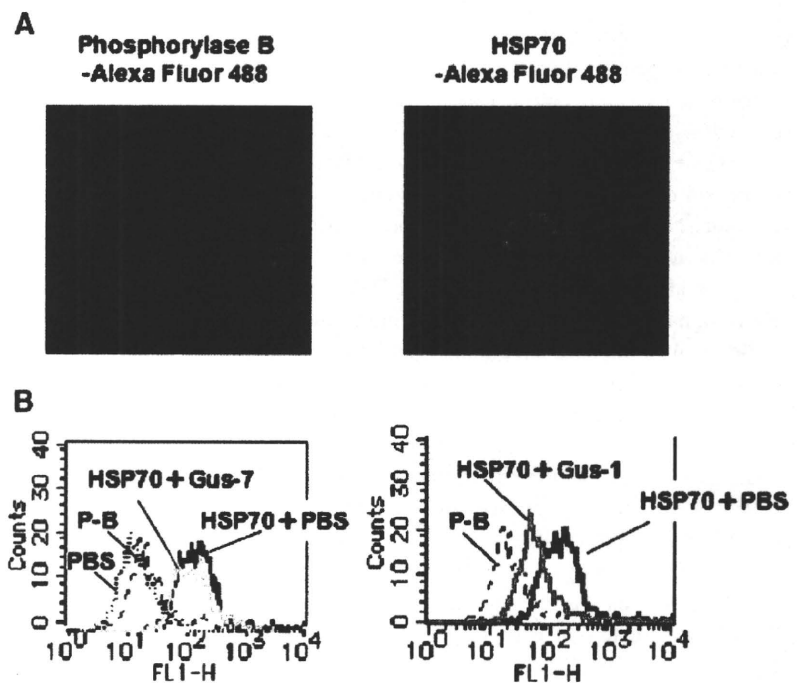
HSP70 clearly induced the release of TNF-α from DCs, while BSA failed under the same condition (Fig. 3A). Time course of TNF-α release indicated that it reached to almost the peak level after 12 hours incubation (supplemental figure). We also examined if HSP90 was capable of stimulating human DCs. As shown in Fig. 3B, HSP90 has just minimal effect on the immature human DCs. As a positive control, the same numbers of DCs were stimulated with the indicated concentration of lipopolysaccharide (LPS). Examination of the LPS concentration in the HSP70 used in this study revealed approximately 25 pg/ml (Mitsubishi Chemical Medience, Japan). These data indicate that HSP70 may have higher stimulatory activity to immature DCs as compared with HSP90, as far as TNF-α release is assessed as an activation index. IL-12 release assay resulted in the similar results (data not shown).

DSG inhibits the HSP70-induced TNF-α release of immature DCs

We then examined if DSG analogs could inhibit the HSP70-induced activation of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (5×10^4 cells/well) in the presence or absence of
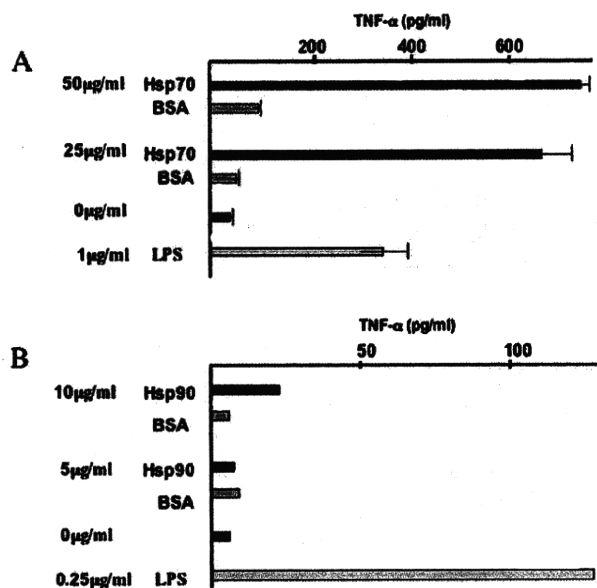
Fig. 3 HSP70 stimulation induces TNF-α release of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (50,000 cells/well), and TNF-α concentration of culture supernatant was quantified after 12 hours (A). The indicated concentrations of HSP90 or BSA were pulsed to immature human DCs (1×10^5 cells/well), and TNF-α concentration of culture supernatant was quantified after 12 hours (B). The data represent the mean values of triplicated samples and standard deviations. As a positive control, the indicated concentration of LPS was pulsed to the same numbers of immature mouse DCs

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Gus-1 or Gus-7 (10 μg/ml). Following incubation for 12 hours, TNF-α concentration of culture supernatant was quantified. It was shown that TNF-α release was inhibited to almost the half level of PBS control in the presence of Gus-1, whereas it was partially inhibited in the presence of Gus-7 (Fig. 4A). Similar results were obtained when immature human DCs (1×10^5 cell /well) were pulsed with HSP70 in the presence or absence of Gus-1 or Gus-7 (10 μg/ml), though the significant difference was observed only in the case of 10 μg/ml pulsation of HSP70 (Fig. 4B).

In order to rule out the possibility that the suppressive action of DSG might be mediated through intracellular molecules, such as intracellular HSP70 and HSP90, immature mouse DCs were pre-incubated for 2 hours with Gus-1 or Gus-7 (10 μg/ml), then washed twice and pulsed with 5 μg/ml of HSP70 in the presence or absence of Gus-1 (10 μg/ml). The data show that pre-incubation did not have any effect on the HSP70-induced TNF-α release, indicating that the suppressive action of DGS might be resulted from the binding of DSG to extracellular HSP70 (Fig. 4C).

DSG inhibits the HSP70-induced maturation of DCs

We next examined if DSG analogs could affect the HSP70-induced maturation of immature DCs. Immature mouse DCs were incubated for 12 hours in the presence or absence of 10 μg/ml of HSP70. In some cases, Gus-1 or Gus-7 (10 μg/ml) was added into the culture. Following incubation for 12 hours, cell surface level of CD80 was analyzed by flow cytometry as a DC maturation index. It was shown that incubation of immature DCs with HSP70 increased the CD80 levels, indicating that HSP70 induced DC maturation. The HSP70-induced DC maturation was suppressed in the presence of Gus-1 (Fig. 5A), but not in the presence of Gus-7 (Fig. 5B). The other maturation markers such as CD86 and CD40 were not changed after the stimulation with HSP70. The data imply that HSP70-induced partial maturation of immature DCs detected by CD80 expression was inhibited in the presence of DSG.

Discussion

In the present study, we demonstrated for the first time that DSG analogs had suppressive activity to the so-called "danger signals." A variety of cell injuries damage the cell membrane, leading to the release of intracellular HSPs into extracellular fluids. The released HSPs are bound to immunostimulatory molecules, such as TLRs (TLR2 and 4), CD40 and CCR5, or captured by scavenger receptor families, such as CD91, LOX-1 and SR-A (Arnold-Schild et al. 1999; Delneste et al. 2002; Lipsker et al. 2002; Roelofs et al. 2006; Warger et al. 2006; Facciponte et al.

A

Hsp70

TNF-α (pg/ml)

B

Hsp70

TNF-α (pg/ml)

C

TNF-α (pg/ml)

Pre-incubation   5 μg/ml Hsp70+
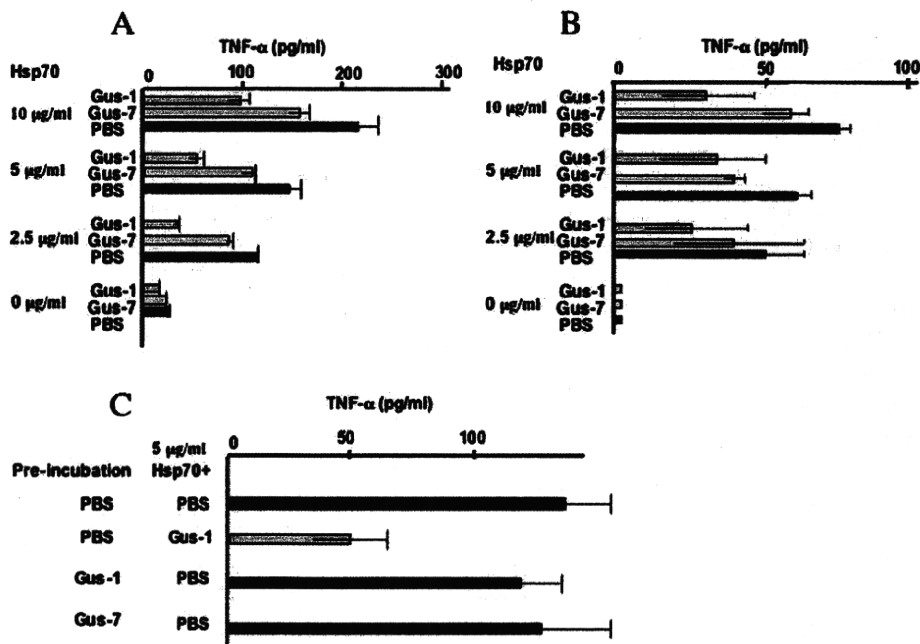
Fig. 4 DSG inhibits the HSP70-induced TNF-α release of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (5×10^4 cells/well) in the presence or absence of Gus-1 or Gus-7 (10 μg/ml) for 12 hours. TNF-α concentration of culture supernatant was quantified (A). The indicated concentrations of HSP70 or BSA were pulsed to immature human DCs (1×10^5 cells/well) in the presence or absence of Gus-1 or Gus-7 (10 μg/ml) for 12 hours. TNF-α concentration of culture supernatant was quantified (B). Immature mouse DCs (50,000 cells/well) were pre-incubated for 2 hours with Gus-1 or Gus-7 (10 μg/ml), then washed twice and pulsed with 5 μg/ml of HSP70 in the presence or absence of Gus-1 (10 μg/ml), followed by TNF-α quantification (C). The data represent the mean values of triplicated samples and standard deviations

2007; Pido-Lopez et al. 2007). The former stimulation induces release of inflammatory cytokines, thus activating innate immune responses (Asea et al. 2000). On the other hand, the latter stimulation induces endocytosis of HSPs with HSP-bound antigenic proteins or peptides, then facilitating the cross-presentation of the HSP-bound antigens and acquired immunity (Noessner et al. 2002; SenGupta et al. 2004). Though the mechanism of the

HSP-mediated efficient cross-presentation remains largely unknown, we have reported previously that HSP70 and HSP90 could facilitate the transfer of endosomal antigens to recycled endosomes that contain empty MHC class I molecules (Ueda et al. 2004; Kurotaki et al. 2007). In addition, HSP90 could also assist the transfer of endosomal antigens into cytosol by unknown mechanism, and facilitate the proteasomal degradation of the antigens (Kurotaki et al.

Fig. 5 HSP70 stimulation induces up-regulation CD80 level of immature DCs. Immature mouse DC were stimulated with 10 μg/ml of HSP70 or BSA, cultured for 12 hours and examined for the cell surface level of CD80 by flow cytometry. In A and B, Gus-1 or Gus-7 (10 μg/ml) was included in the culture, respectively

A

—— HSP70 + Gus-1
—— HSP70 + PBS
--- PBS

B

—— HSP70 + Gus-7
—— HSP70 + PBS
--- PBS
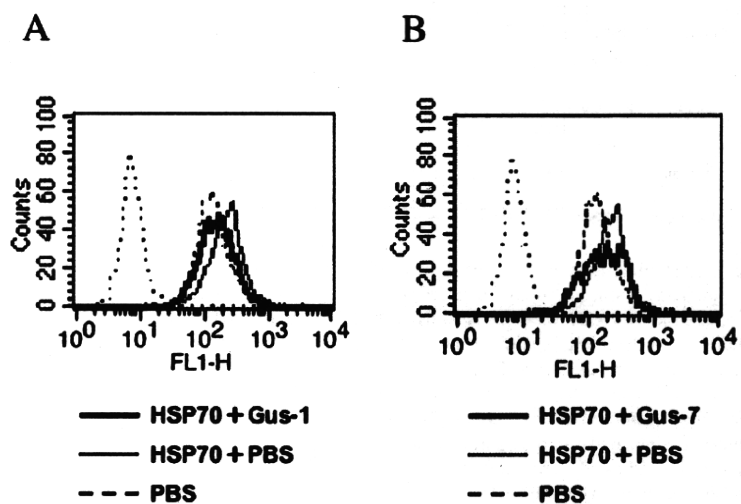
2007). In the present study, we clearly demonstrate that DSG was capable of inhibiting the binding of extracellular HSP70 to the cell surface of DCs. Therefore, it is highly likely that DSG inhibits not only innate immune responses but also acquired immune responses.

Deoxyspergualin has been used clinically as an immunosuppressive agent after organ transplantation (Nomura et al. 1998). Moreover, most recent clinical trials in Germany demonstrated that it is effective to refractory autoimmune vasculitis including Wegener's granulomatosis (Schmitt et al. 2005; Erickson and Hwang 2007). Concerning the mechanism of immunosuppressive and anti-inflammatory actions of DSG, there are several reports that explain the molecular mechanism (Amemiya 1996). Nadler et al. demonstrated that the intracellular targets of DSG were HSP70 and HSP90 and suggested that suppression of NF-kB signal might be involved in the anti-inflammatory reaction (Nadler et al. 1995). However, there are some controversial data as to the hypothesis. Kawada et al. showed that DSG inhibits AKT signals and phosphatidylcholine synthesis (Kawada et al. 2002). Waaga et al. reported that DSG could down-regulate the expression of MHC class II molecules, though the precise mechanism remained unknown (Waaga et al. 1996). Our group showed that intracellular HSP70 might be a target of DSG, and the binding of DSG to HSP70 disrupted the association of cytosolic antigen-bound HSP70 with TAP, leading to inhibition of TAP-mediated peptide transfer into the endoplasmic reticulum and down-regulation of cell surface MHC class I levels (Kamiguchi et al. 2008). In the present study, we demonstrated that the target molecules of DSG might be not only intracellular HSPs but also extracellular HSPs, which mediate the so-called "danger signals". It is expected that DSG might be effective in the treatment of other refractory inflammatory or autoimmune diseases, such as Behcet disease, inflammatory bowel diseases, glomerulonephritis, and systemic inflammatory response syndrome, which are considered to be broken out by dysregulated activation of danger signals.

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Review Article

The functioning antigens: beyond just as the immunological targets

Yoshihiko Hirohashi,[1] Toshihiko Torigoe,[1] Satoko Inoda,[1] Jun–ichi Kobayasi,[1] Munehide Nakatsugawa,[1] Takashi Mori,[2] Isao Hara[2] and Noriyuki Sato[1,3]

[1]Department of Pathology, Sapporo Medical University School of Medicine, South-1 West 17, Chuo-ko, Sapporo, 060-8556 Japan; [2]Department of Urology, Wakayama Medical University, Kimiidera 811-1, Wakayama, 641-0012 Japan

Antigenic peptides derived from tumor-associated antigens (TAAs) facilitate peptide cancer vaccine therapies. With the recent progress in cancer immunity research, huge amounts of antigenic peptides have already been reported. Clinical trials using such peptides are underway now all over the world. Some reports have shown the efficacy of peptide vaccine therapies. However, others ended with unfavorable results, suggesting fundamental underlying problems. One major mechanism that negates the peptide vaccine therapy is tumor escape from immunological systems caused by loss of antigens. TAAs that are used in cancer vaccine therapies may be divided into two major groups: functioning antigens and non-functioning antigens. A 'functioning antigen' could be defined as a TAA that is essential for tumor growth, is expressed in several kinds of malignancies and shows homogenous expression in cancerous tissues. It is not difficult to imagine that antigen loss will occur easily with non-functioning antigens as a target of cancer vaccine therapy. Thus, it is essential to use functioning antigens for successful cancer vaccine therapy. In this review, we discuss the functioning antigens and their categorization in detail. (*Cancer Sci* 2009; 100: 798–806)

Immunotherapy is a very old concept that stems from the vaccination therapy established by Edward Jenner for treating smallpox. That novel therapeutic strategy has had a great impact, enabling complete elimination of the disease. This glittering triumph also raised a simple and significant question: 'Are malignant diseases treatable with vaccination?' Since then, a vast body of work on cancer immunity has been reported, and tumor immunity research have already reached the bedside.

There are two major approaches of tumor immunity: (i) tumor immunotherapy based on tumor-specific cytotoxic T lymphocytes (CTLs); and (ii) tumor immunotherapy based on tumor-specific antibodies (Fig. 1). In 1997, anti-CD20 monoclonal antibody (rituximab) has been approved by the US Food and Drug Administration (FDA) for treating CD20-positive B-cell malignancies, and antibody-based immunotherapy has become one of the standard therapies in several malignancies. However, antibody-based immunotherapy can target only cell surface proteins or secreted proteins like p185[HER2/neu] for breast carcinoma, CD20 for B-cell lymphoma, vascular endothelial growth factor (VEGF) for renal cell carcinoma, epidermal growth factor receptor (EGFR) for colorectal carcinoma and chemokine (C-C motif) receptor 4 (CCR4) for T cell lymphoma. So, antibody-based immunotherapy is very restricted for further application. On the other hand, CTLs recognize 9- to 14-mer antigenic peptides that are derived from endogenously expressed proteins digested by several proteases, including proteasomes and the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP).[1,2] Thus, CTLs can recognize

potentially all tumor-specific antigens (Fig. 1). Very recently, some lines of CTL-based immunotherapy reagents have been approved (Table 1). Heat shock proteins (HSPs) purified from cancer cells have the potency to induce CTL reactivity, and HSP-based reagent (Oncophage) was approved in Russia in April 2008. Further, other CTL-based immunotherapy reagents are now under Phase III studies, and part of these will be approved in a few years. The wave of CTL-based immunotherapy is coming to the bedside.

The progress of molecular biological techniques in the past several decades has brought us enormous knowledge about

Cell surface TAA   **Intracellular TAA**

HLA class I heavy chain   **β2 microglobulin**
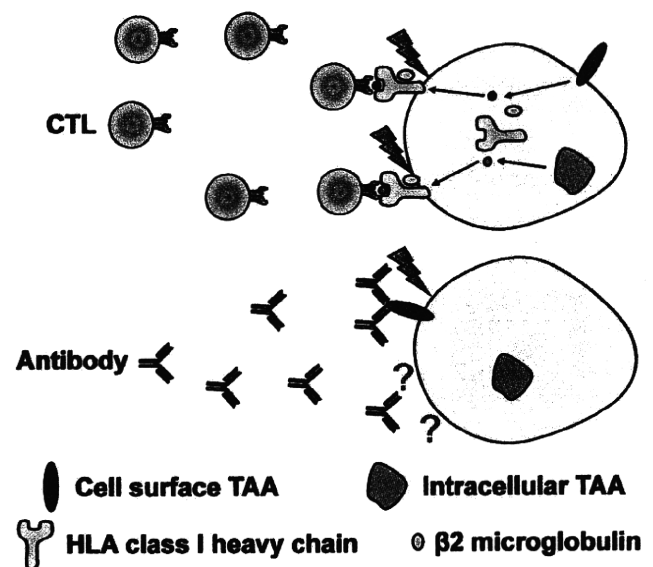
Fig. 1. Cancer-specific immunotherapy based on cytotoxic T lymphocytes (CTLs) and antibodies. In a variety of immuno systems, CTLs and B cell (antibody) show antigen-specific reactions through their antigen-specific receptors. CTL recognizes endogenously processed antigenic peptides presented with human leukocyte antigen (HLA) molecule on the cell surface. Thus, CTL can recognize all cell-distributed antigens. Antibody recognizes cell surface antigen with direct binding to antigen molecule with its fragment antigen binding (Fab) region. Antibody only recognizes cell surface antigens, but does not recognize intracellular antigens.