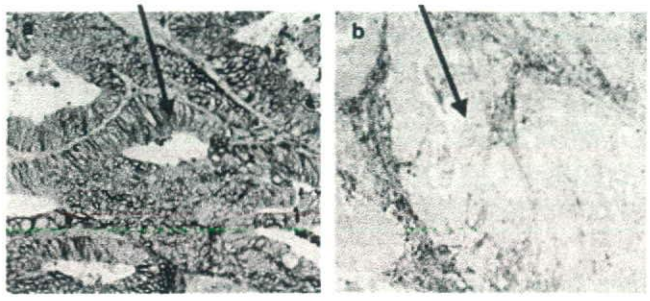

Figure 9 Representative view of immunohistochemistry colon cancers as assessed on the anti-pan HLA class I mAb EMR8-5. (**a**) Positive and (**b**) negative staining of colon cancer cells. (**b**) Positive staining of stromal area, which includes leukocytes, vessels and connective tissue portions. Of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

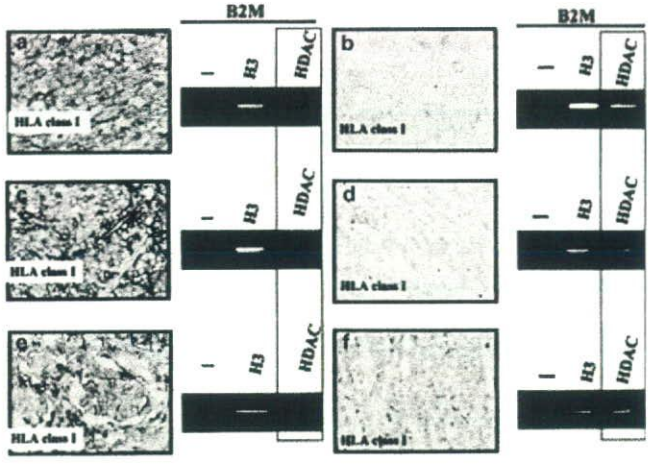

Figure 10 Chromatin immunoprecipitation assays of histone acetylation and deacetylation status in B2M genome of primary breast cancers. (**a**,**c**,**e**) Representative HLA class I heavy chain/B2M positive (patients 1–3) and (**b**,**d**,**f**) negative (patients 5–7) cases are shown. H3 and HDAC,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

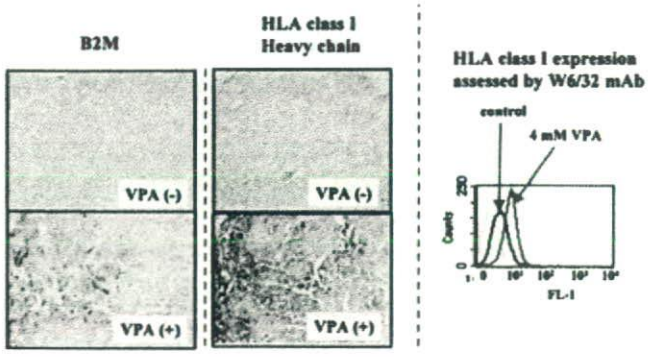

Figure 11 (**a**) *In vivo* and (**b**) *in vitro* restoration of the HLA class I/B2M expression in MCF human breast tumor by the HDAC inhibitor valproic acid (VPA). (**a**) Severe combined immunodeficiency (SCID) mice transplanted with MCF7 human breast cancer were treated by feeding in water with 0.4% 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.

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ORIGINAL PAPER

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

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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 immunosuppressive 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.

Keywords HSP70 · Deoxyspergualin · Dendritic cells · Immunosuppressant

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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

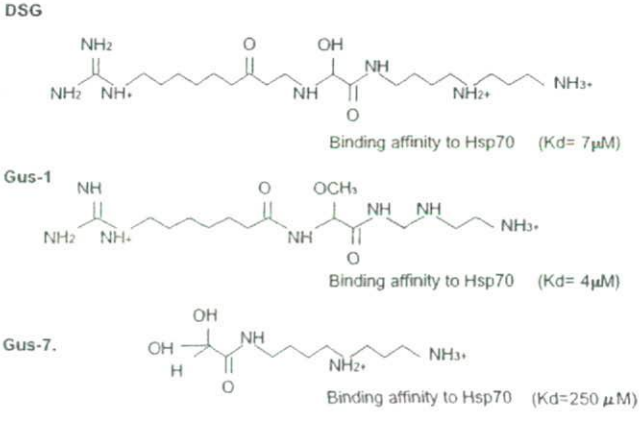

Fig. 1 Structure of DSG and two distinct DSG analogs, Gus-1 and Gus-7. Gus-1 (methoxyDSG) has high binding affinity for HSP70 (K_d=4 μM), whereas Gus-7 has reduced binding affinity for HSP70 (Kd=250 μM) as compared to DSG and Gus-1

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

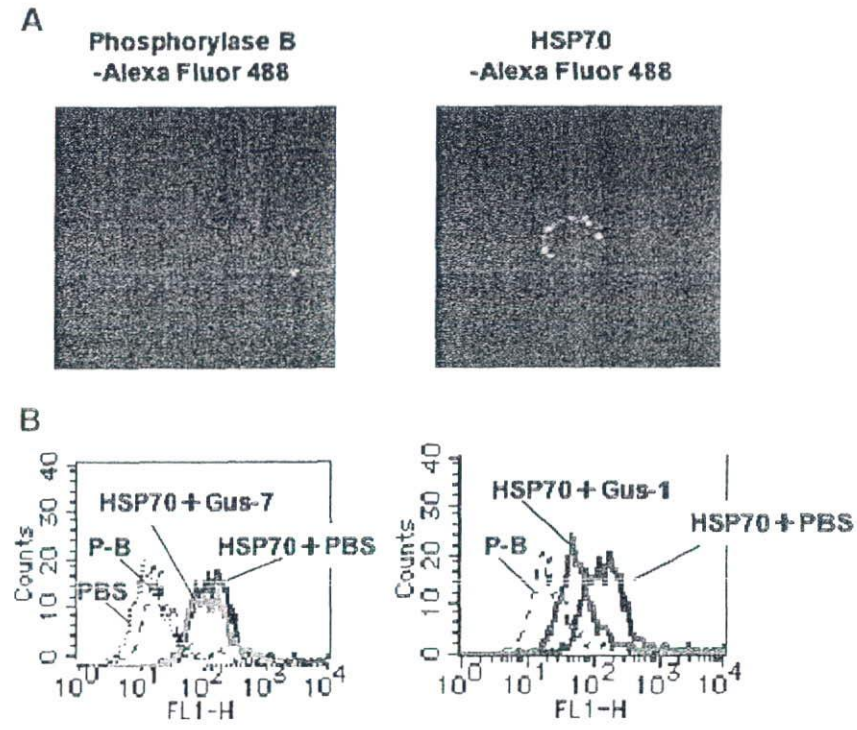

Fig. 2 DSG inhibits the binding of HSP70 to the cell surface of immature DCs. Purified HSP70 and phosphorylase B were labeled with Alexa Fluor 488 and then incubated with immature DCs for 30 min on ice. After washing twice with ice-cold PBS, cells were fixed in 1% formaldehyde PBS and subjected to laser 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 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).

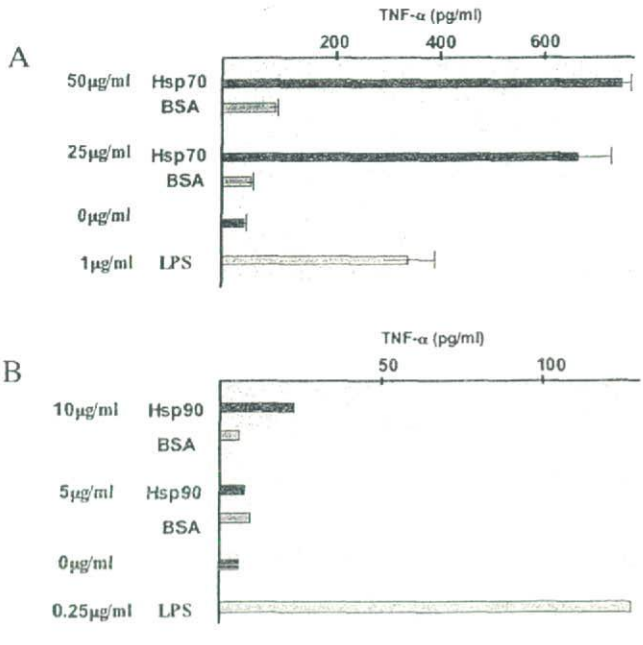

Fig. 3 HSP70 stimulation induces TNF-α release of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (50,000 cells/well), and TNF-α concentration of culture supernatant was quantified after 12 hours (A). The indicated concentrations of HSP90 or BSA were pulsed 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

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.

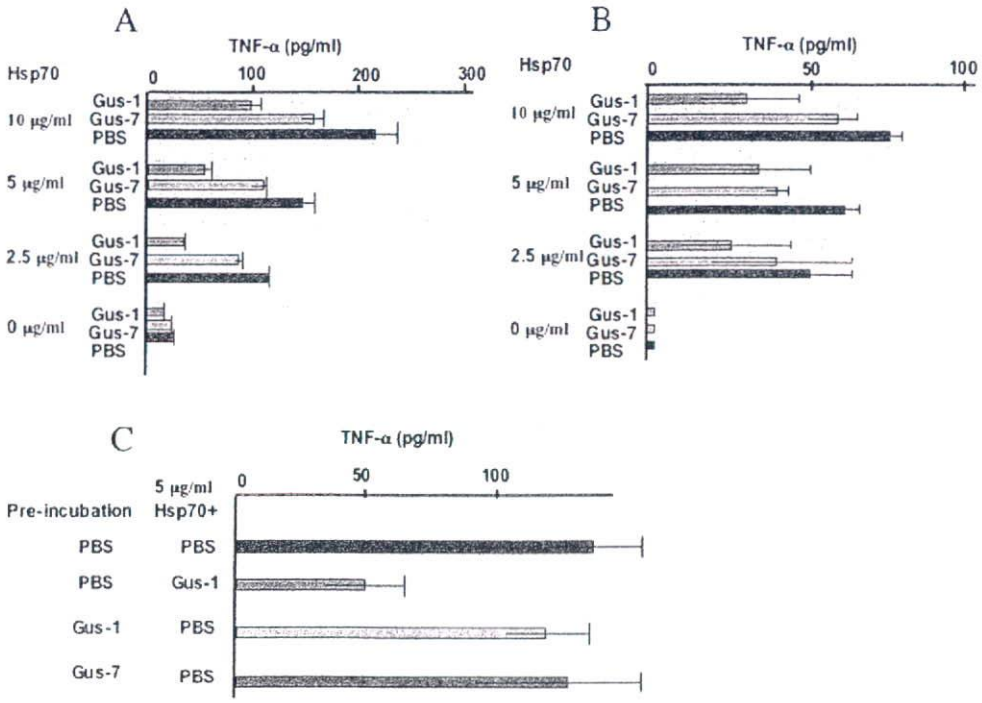

Fig. 4 DSG inhibits the HSP70-induced TNF-α release of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (5×10^4 cells/well) in the presence or absence of Gus-1 or Gus-7 (10 μg/ml) for 12 hours. TNF-α concentration of culture supernatant was quantified (A). 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.

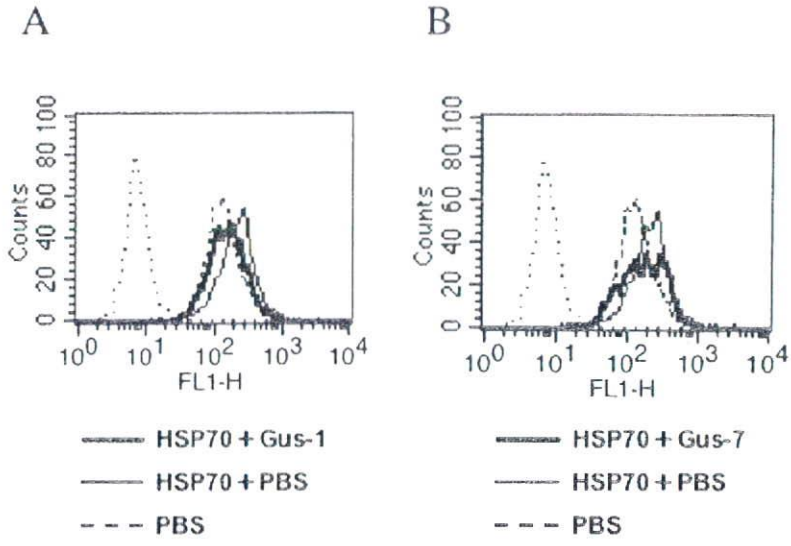

Fig. 5 HSP70 stimulation induces up-regulation CD80 level of immature DCs. Immature mouse DC were stimulated with 10 μg/ml of HSP70 or BSA, cultured for 12 hours and examined for the cell surface level of CD80 by flow cytometry. In A and B, Gus-1 or Gus-7 (10 μg/ml) was included in the culture, respectively

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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Solicited Review Article

The functioning antigens; beyond just as the immunological targets

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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)

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 $p185^{HER2/meu}$ for breast carcinoma, CD20 for B-cell lymphoma, vascular endothelial growth factor (VEGF) for renal cell carcinoma, epidermal growth factor receptor (EGF-R) for colorectal carcinoma and 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 ERRAP.[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 molecular aspects of oncogenesis and also cancer immunity.

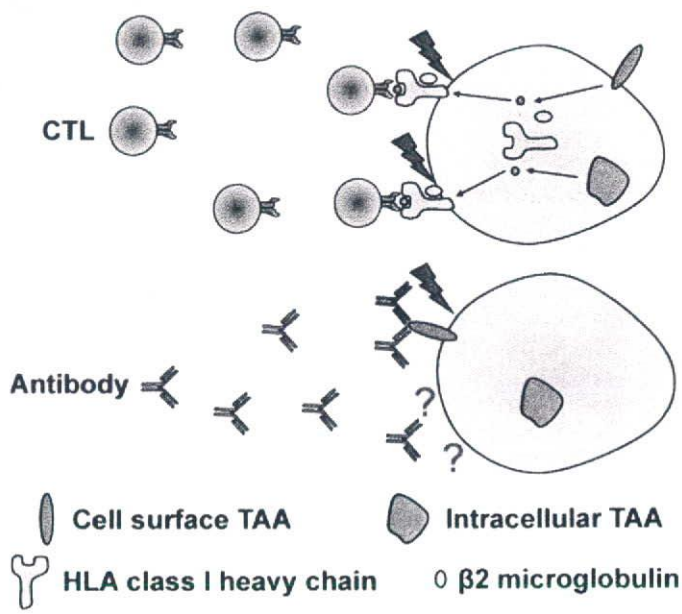

Fig. 1. Cancer-specific immunotherapy based on cytotoxic T lymphocytes (CTLs) and antibodies. In a variety of immuno systems, CTLs and B cell (antibody) show antigen-specific reactions through their antigen-specific receptors. CTL recognizes endogenously processed antigenic peptides presented with HLA 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.

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The abbreviations used are:
TAA, tumor associated antigen; CTL, cytotoxic T lymphocyte.

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Table 1. Exploitation of cancer vaccine and related companies (October 2008)

Designation	Content	Company	Country	Organs	Approved
Oncophage	Protein/peptide	Antigenics	Russia	Kidney	April 2008
DCVax	Cell	Northwest Biotherpeutics	Switzerland	Brain	July 2007
BiovaxID	Protein	Biovest International	USA	B cell lymphoma	Phase III
MAGE-A3ASCI	Protein	GlaxoSmithKline	Belgium	Lung	Phase III
GV1001	Peptide	Pharmexa	UK	Pancreas	Phase III
GVAX	Cell	Cell Genesys	USA	Pancreas	Phase II
Stimuvax	Peptide	Oncothyreon	Several	Lung	Phase III
TroVax	DNA	Oxford Biomedica	UK	Kidney	Phase III

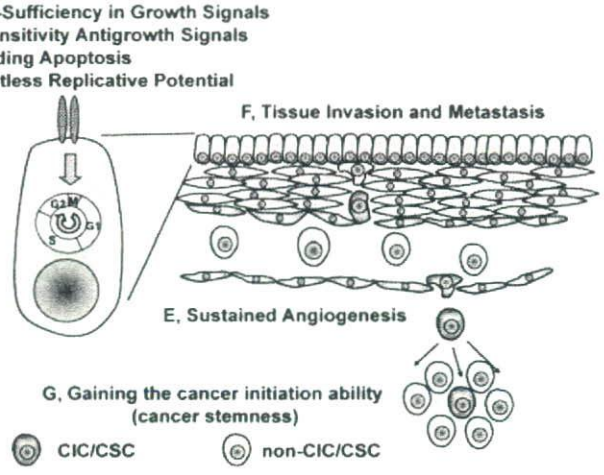

Fig. 2. The 'Functioning antigens'. The 'Functioning antigens' play several roles in carcinogenesis, tumor initiation, invasion and metastasis.

This enabled van der Bruggen and colleagues to publish their milestone work on the identification of melanoma antigen (MAGE) gene family as the first human TAA.[3] Initially, TAAs were screened with cDNA expression cloning using CTLs specific for melanomas. Later, serological analysis of recombinantly expressed clones (SEREX) and the reverse-immunogenetical approach were developed to identify novel TAAs and CTL epitopes. To summarize these TAAs, Boon *et al.* proposed categorizing TAAs based on the expression profiles of malignant and normal cell TAAs.[4] This categorization includes four groups: (A) tumor antigens resulting from mutations; (B) shared tumor-specific antigens; (C) differentiation antigens; and (D) antigens overexpressed in tumors (Cancer Immunity web site http://www.cancerimmunity.org/index.htm.). This provides us with very important information to establish cancer immunotherapy protocols. Malignant cells commonly have genomic instability and are genetically unstable, and often lose the expression of immunogenic antigens after cancer vaccine therapy, suggesting that non-functioning antigens might not be suitable for cancer vaccine therapies. The functioning antigens are usually non-mutated cancer-related antigens, and belong to group (D) antigens overexpressed in tumors. To discriminate functioning antigens from non-functioning antigens, several features of malignant phenotypes as follows are essential:[5] (A) self-sufficiency in growth signals; (B) insensitivity antigrowth signals; (C) evading apoptosis; (D) limitless replicative potential; (E) sustained angiogenesis; and (F) tissue invasion and metastasis. Furthermore, to understand the tumor initiation ability *in vivo*, we need to mention about the 'cancer stem cell' theory. Cancer initiating cells/cancer stem cells (CICs/CSCs) are described as small populations that have (i) high tumorigenic potency, (ii) self-renewal and (iii) differentiation ability. This concept is very important and intriguing, since CICs/CSCc have very high tumor generating ability resistance to treatment and high metastatic ability.[6] Therefore, we propose that (G) gaining cancer initiation ability (cancer stemness) should also be included as a seventh malignant phenotype (Fig. 2). In this review, we re-categorize the functioning antigens into the seven new categories (summarized in Table 2).

Self-Sufficiency in Growth Signals

One of the features of cancer cells that distinguish them from normal cells is their uncontrolled cell division. Usually, normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind several signaling molecules. Cancer cells are often overexpressed in the genes related to cell growth to mimic normal growth signaling. In consequence, cancer cells are overexpressed in cell-cycle-related molecules like normal cells. Thus, antigens of this category contain two subgroups: (i) antigens that code for receptors of growth factors, including $p185^{HER2/neu}$ and EGFR, and (ii) cell cycle-related antigens, including Cyclin B1, Cep55/c10orf3, survivin and Aurora-A kinase. These overexpressed molecules are also expressed in normal cells, whereas CTLs can be generated from cancer patients' lymphocytes, suggesting that CTLs specific for this category of antigens are not tolerated in cancer patients.

p185HER2/neu and EGFR. $p185^{HER2/neu}$ belongs to the ErbB family, is one of the receptor tyrosine kinases (RTKs) and is overexpressed in several types of cancer cells playing several essential roles in oncogenesis, cancer progression and metastasis.[7] Amplification and overexpression of $p185^{HER-2/neu}$ have been reported in 20–40% of primary breast cancers and also in ovarian (20–25%), colorectal and pancreatic adenocarcinomas (80–85%). However, $p185^{HER2/neu}$ does express in several normal tissues at very low levels, so the CTLs might be tolerated in cancer patients. Fisk *et al.* reported the identification of a $p185^{HER2/neu}$-coded HLA-A2-restricted antigenic peptide.[8] The authors found that 9-mer peptide E75 (HER-2, 369–377:KIFGSLAFL)-specific cytotoxic activity could be detected in malignant ascites of ovarian carcinoma. This report indicated that the $p185^{HER2/neu}$ derived antigenic peptide could be presented, and CTLs might be activated and clonally expanded *in vivo*. Thereafter, several reports on identification of HLA-class I restricted $p185^{HER2/neu}$ peptides have suggested that the CTLs specific for $p185^{HER2/neu}$ are not eliminated in the thymus and exist in the periphery.

Epidermal growth factor receptor is also in the ErbB family and is overexpressed in several types of malignancies. EGF-R signaling is essential in some malignancies, thus targeting EGF-R might be a reasonable treatment. Although EGF-R is also expressed ubiquitously in normal tissues at a very low level, Shomura *et al.* have shown that EGF-R can also be a target

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Table 2. Summary of the tumor-associated antigens (TAAs) and antigenic peptides

Groups	Antigen	HLA restriction	Peptide sequence	Position	Reference
A, Self-sufficiency in growth signals	$p185^{HER2/neu}$	A2	ALCRWGLLL	5–13	(87)
		A2	HLYQGCQVV	48–56	(88)
		A2	KIFGSLAFL	369–377	(8)
		A2	PLQPEQLQV	391–399	(89)
		A2	TLEEITGYL	402–410	(89)
		A2	ILHNGAYSL	435–443	(87)
		A2	ALIHHNTHL	466–474	(89)
		A2	PLTSIISAV	650–658	(89)
		A2	IISAVVGIL	654–662	(90)
		A2	VVLGVVFGI	665–673	(91)
		A2	RLLQETELV	689–697	(91)
		A2	YMIMVKCWMI	952–961	(91)
		A2	YLVPQQGFFC	1023–1032	(88)
		A3	VLRENTSPK	754–762	(92)
		A24	TYLPTNASL	63–71	(93)
	EGF-R	A2	KLFGTSGQKT	479–488	(9)
		A2	YLNTVQPTCV	1138–1147	(9)
		A24	MFNNCEVVL	54–62	(10)
		A24	NYDANKTGL	124–132	(10)
		A24	DYVREHKDNI	800–809	(10)
	Cyclin B1	A2	AGYLMELCC*	323–341	(11)
	Aurora-A	A2, A24	YLILEYAPL	207–215	(16)
	Cep55/c10orf3	A24	VYVKGLLAKI	193–202	(14)
B, Insensitivity antigrowth signals	MDM2	A2	VLFYLGQYI	53–61	(18)
C, Evading apoptosis	Survivin	A1	QFEELTLGEF	92–101	(94)
		A2	TLPPAWQPFL	5–14	(95)
		A2	RISTFKNWPFL	18–28	(94)
		A2	ELTLGEFLKL	95–104	(95,96)
		A2	LTLGEFLKL	96–104	(96)
		A11	DLAQCFFCFK	53–62	(94)
		A24	STFKNWPFL	20–28	(31)
		A24	FFCFKELEGW	58–67	(33)
		B35	CPTENEPDL	46–54	(97)
		B35	EPDLAQCFF	51–59	(97)
	Survivin-2B	A24	AYACNTSTL	80–88	(28)
	ML-IAP/Livin	A2	SLGSPVLGL	34–42	(47)
		A2	RLASFYDWPL	90–99	(47)
		A2	RLQEERTCKV	245–254	(49)
		A2	QLCPICRAPV	280–289	(49)
		A3	RLQEERTCK	245–253	(48)
		A24	KWFPSCQFLL	146–155	(44)
	Bcl-2	A2	PLFDFSWLSL	208–217	(51)
	Bcl-xL	A2	YLNDHLEPWI	173–182	(53)
	Mcl-1	A1	RLLFFAPTR	95–103	(54)
		A1	QSLEIISRY	177–185	(55)
		A1	RTKRDWLVK	300–309	(55)
D, Limitless replicative potential	hTERT	A2	ILAKFLHWL	540–548	(58)
		A2	RLFFYRKSV	572–580	(62)
		A2	RLVDDFLLV	865–873	(63)
		A3	KLFGVLRLK	973–981	(64)
		A24	VYAETKHFL	324–332	(65)
		A24	VYHFVRACL	461–469	(65)
		B*0702	RPAEEATSL	277–285	(66)
		B*0702	RPSFLLSSL	342–350	(66)
		B*0702	RPSLTGARRL	351–360	(66)
E, Sustained angiogenesis	VEGF	B*2705	SRFGGAVVR	5′UTR	(75)
	VEGF-R1	A2	TLFWLLLTL	770–778	(74)
		A2	VLLWEIFSL	1087–1095	(74)
	VEGF-R2	A2	YMISYAGMV	190–198	(73)
		A2	VIAMFFWLL	773–781	(73)
F, Tissue invasion and metastasis	MMP2	A2	GLPPDVQRV	484–492	(79)
G, Gaining the cancer initiation ability (cancer stemness)	SOX2	A2	TLMKKDKYTL	118–127	(84)
	SOX10	A2	AWISKPPGV	332–340	(86)

*wild type sequence is AKYLMELTM; EGF-R, epidermal growth factor receptor; ML-IAP, melanoma inhibitor apoptosis protein; hTERT, human telomerase reverse transcriptaseVEGF-R, vascular endothelial growth facotr-receptor; MMP2 matrix metalloprotease 2; SOX2, SRY (sex determining region Y)-box 2.

of CTLs, indicating that cellular immunity for EGF-R is not tolerated.[9,10] The authors identified HLA-A2- and HLA-A24-restricted CTL epitopes.

Cyclin B1. Cyclin B1 is expressed predominantly in the G2/M phase of cell division and is essential for the initiation of chromosome condensation, destruction of the nuclear membrane, and assembly of the mitotic spindle. Kao *et al.* eluted antigenic peptides from an HLA-A2.1-positive breast carcinoma cell line, and isolated several peptides highly homologous to the Cyclin B1 amino acid sequence.[11] Interestingly, these 9-mer peptide sequences were changed at the second position lysine to glycine. In the C-terminal methionine was substituted methionine to valine, phenylalanine or cysteine. They confirmed that peptide4 (AGYLMRLCV) was immunogenic with an HLA-A2.1-positive cancer patient's blood. The natural sequence expected from the gene sequence was actually AKYLMELTM. The precise mechanisms of the amino-acid substitutions remain elusive. Several reports showed that overexpression of Cyclin B1 protein was related to poor prognosis and radiotherapy resistance, suggesting that Cyclin B1 had some role in cancer progression and resistance to therapy.

Cep55/c10orf3. Cep55/c10orf3 is one of the proteins localized to centrosomes and the midbody, and has an essential role in cytokinesis.[12] The centrosome is the principal microtubule organizing center of the mammalian cell, consisting of a pair of barrel-shaped microtubule assemblies that are non-identical and are referred to as the mother and daughter centrioles.[13] Defects in the number, structure or function of centrosomes can generate mono- or multipolar mitotic spindles and cytokinesis defects, resulting in aneuploidy and chromosome instability, which are common characteristics of tumor cells. Thus, abnormal centrosome constituents may be exploited as therapeutic targets for malignantly transformed or dysplastic cells. Survivin, Aurora-A kinase and part of Cyclin B1 are also centrosome-related antigens.

Recently, we found that Cep55/c10orf3 could be a target of CTLs from HLA-A24-positive breast cancer patients.[14] As Cep55/c10orf3 is one of the mitosis-related molecules, low level expression of Cep55/c10or3 mRNA can be detected in some normal tissues, including thymus and testis. On the other hand, we could not detect the Cep55/c10orf3 protein in normal tissues adjacent to Cep55/c10orf3-positive cancerous tissues. Furthermore, the Cep55/c10orf3 protein expression can be detected not only in mitotic cells but also in the cytosol of interphase cells. The accumulation of Cep55/c10orf3 protein might evoke immuno-reactivity.

Aurora-A kinase. Aurora-A kinase is a member of the serine/threonine kinase family, and the Aurora-A gene is located at chromosome 20q13, a region frequently amplified in breast cancer. Aurora-A kinase is mainly expressed in the G2/M phase of the cell cycle and regulates mitotic cell division in normal cells. Aurora-A kinase is overexpressed in several types of malignancies, and its overexpression causes transformation of rodent fibroblasts.[15] Recently, Ochi *et al.* reported that an HLA-A2- and A24-restricted Aurora-A kinase derived peptide could induce CTL.[16] The authors showed that an Aurora-A peptide-specific CTL clone could recognize Aurora-A-positive acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) cell lines in the context of HLA-A2, suggesting that the peptide was presented by HLA-A2 endogenously. As Aurora-A is expressed in several kinds of malignancies, this antigenic peptide might also be suitable for other types of malignancies.

Insensitivity Antigrowth Signals

In normal tissues, several anti-proliferative signals maintain the normal cell growth. The representative way to suppress growth signals is cyclin-dependent kinase inhibitors (CDKIs). CDKIs, including p15, p16, p21 and p27 directly bind a cyclin-cyclin-dependent kinase (CDK) complex and suppress the kinase activity. Tumor growth factor (TGF)-beta signals suppress tumor growth by up-regulating CDKIs. p53 suppresses tumor growth by up-regulating p21. One of the p53 regulators, MDM2, was identified as a target of CTLs.

MDM2. MDM2 is overexpressed in several types of cancer cells[17] and has an essential role in oncogenesis by down-regulating the p53 tumor suppressor protein level via degradation. Thus MDM2 might be a reasonable target for cancer therapy; however, MDM2 is also expressed in normal tissues at a low level, thus MDM2-specific CTLs might be tolerated.

Asai *et al.* found that HLA-A2-restricted HDM2 (human MDM2 homolog) peptide-specific CTLs could be generated in human systems.[18] However, HLA-A2-restricted HDM2-specific CTLs could be established only from HLA-A2-positive healthy volunteers, not from HLA-A2-positive cancer patients. Stanislawski *et al.* and Ramirez *et al.* also showed that cellular immunity for MDM2 was tolerated, whereas high-affinity T-cell receptor (TCR) gene-transfer T-cells or multiple peptide vaccination could break the tolerance.[19,20]

Evading Apoptosis

In physiological conditions, the gross cell number is well controlled by programmed cell death, that is, apoptosis. Growth signal stimulation converts cells from the quiescent state to the proliferative state to recover and maintain the tissue. Then the excess cells will be eliminated by apoptosis. Malignant transformed cells growing in an uncontrolled fashion use several mechanisms to evade apoptosis and survive. One subgroup of this gene is inhibitor apoptosis proteins (IAPs), and the other is Bcl-2 family proteins. IAP family proteins inhibit the lower effector enzymes termed caspases, including caspase 9, caspase 8 and caspase 3. IAP family protein like survivin and Melanoma-IAP (ML-IAP)/Livin, are reported to be the targets of CTLs. Bcl-2 family proteins mainly inhibit the secretion of cytochrome *c* from mitochondria following apoptosis. This group contains Bcl-2, Bcl-$_{XL}$ and Mcl-1, which are already proved to be targets of CTLs. The functions of this group of proteins are well characterized and related to poor prognosis; thus this group of proteins is a reasonable target for cancer immunotherapy.

Survivin. Originally, survivin was isolated as one of the IAP family.[21] As described above, it was proved to have a critical role in cell cycle progression, especially mitosis. Survivin expression is up-regulated in a large proportion of malignancies, and is related to resistance to chemotherapy or radiotherapy, and its overexpression is linked to poor prognosis. Thus, survivin is thought to be promising target molecule.[22]

As survivin is overexpressed in several types of malignancies, it is thought to be one of the universal and ideal antigens.[23] From this point of view, several HLA-class I-restricted survivin peptides have been reported (summarized in Fig. 3). There are several splicing variants with different functions and subcellular localizations.[24] Survivin, survivin-del Ex3 and survivin-3B have anti-apoptotic potential. On the other hand, survivin-2alpha have pro-apoptotic potential. Survivin-2B is quite complicated, with three different kinds of reports. First, Ling *et al.* reported that high expression of survivin-2B was related to good prognosis and no relapse in non-small-cell lung cancer and that its overexpression caused apoptosis.[25] Second, Nakano *et al.* reported that Survivin-2B expression showed no relation to tumor progress.[26] Finally, in contrast, Wagner *et al.* reported that low-expression of survivin-2B was related to good prognosis in adult AML.[27] Since these three reports focused on different types of malignancies, the functions of survivin-2B are still elusive. Above all, survivin-2B is expressed in several types

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Table 3. Phase I clinical trials of survivin-2B peptide vaccines with three protocols in colon cancers (Sept. 2008)

Protocol	Adverse effects	Tumor marker (not increased)	CT (SD, PR)	CTL detection (tetramer)
(1) Peptide alone	Anemia, fever	20%	40%	20%
	General malaise	(2/10)	(4/10)	(2/10)
(2) Peptide + IFA	Induration	0%	20%	0%
	Itching, fever	(0/5)	(1/5)	(0/5)
(3) Peptide + IFA + IFNα	Induration	67%	67%	50%
	Leucopenia	(4/6)	(4/6)	(3/6)
	Itching, fever			

CTL, cytotoxic T lymphocyte; IFN, interferon.

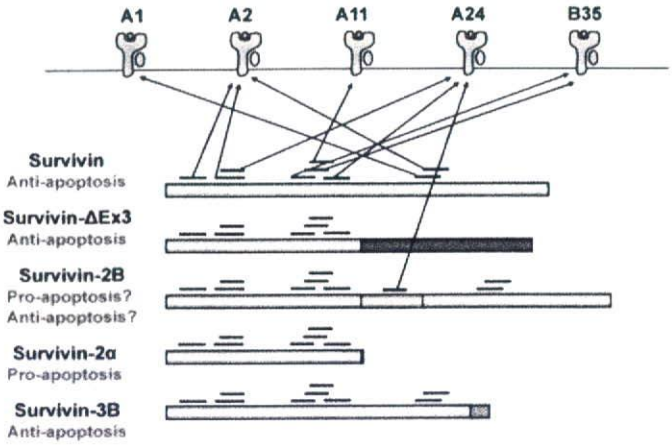

Fig. 3. Several antigenic peptides from survivin and variants. There are five survivin transcripts already reported. Wild-type survivin, survivin-ΔEx3 and survivin-3B have anti-apoptotic function. Survivin-2α has pro-apoptotic function. Survivin consists of four exons. Survivin-ΔEx3 skips exon 3 causing frame shift and code 64 different amino acids with exon 4 (red bar). Survivin-2B contains an additional exon 2B between exon 2 and exon 3. Exon 2B codes an additional 23 amino acids (yellow bar). Survivin-2α contains exon 2α following exon 2. Exon 2α codes only one amino acid (light blue) and ends with the stop codon. Survivin-3B contains an additional exon 3B between exon 3 and exon 4. Exon 3B codes seven additional amino acids (green bar) and ends with the stop codon. There are several antigenic peptides derived from survivin and its variants. Survivin-2B contains the HLA-A24-restricted splicing variant-specific antigenic peptide.

of malignancies in very high proportion,[28–30] thus survivin-2B can also be an ideal target for cancer immunotherapy. HLA-A1-, A2-, A11- and B35-restricted survivin peptides are derived from wild type survivin and several splicing variants, including survivin delEx3, survivin-2B, survivin-2alpha and survivin-3B. These peptides target anti-apoptotic molecules and also pro-apoptotic molecules. One of the HLA-A24 restricted peptides is derived only from survivin-2B (survivin-$2B_{80-88}$).[28] Andersen *et al.* reported that different HLA-A24 restricted peptides from survivin and splicing variants shared the sequence ($survivin_{20-28}$).[31] However, the authors did not show $survivin_{20-28}$ peptide-specific CTLs could recognize HLA-A24-positive and survivin-positive cancer cells. Thus, it is unclear whether the $survivin_{20-28}$ peptide is presented by HLA-A24 molecules endogenously. Survivin-$2B_{80-88}$ peptide shows high immunogenic potency from HLA-A24 cancer patients,[32] and survivin-$2B_{80-88}$ peptide-specific CTLs do recognize HLA-A24-positive cancer cells, suggesting that survivin-$2B_{80-88}$ is presented endogenously with the HLA-A24 molecule. And, survivin-$2B_{80-88}$-specific CTL precursors were ~10-fold more frequent than wild-type HLA-A24 survivin-derived peptide-specific CTL precursors in HLA-A24-positive cancer patients in our recent study.[33] At this moment, we do not know why the survivin-2B-derived peptide is more immunogenic than the survivin-derived peptide. One of the reasons might be the different expression levels of these molecules. As survivin is expressed in several normal tissues at very low levels, cellular immunity for survivin might be partially tolerated. However, survivin-2B expression is several-fold less than that of survivin,[28,34] thus survivin-2B-specific immunity might not be tolerated and it retains high CTL cytotoxic activity. To summarize, we cannot conclude whether survivin or survivin-2B is the most suitable target for immunotherapy, but we can conclude that both of them have CTL-inducing potential and might be ideal targets.

CTLs kill target cells through secretion of perforin and Granzyme B, causing apoptosis on target cells. However, survivin-overexpressing cells are sensitive to CTLs, suggesting that survivin has no or little role in apoptosis caused by Granzyme B. Caldas *et al.* showed that fusion of the survivin gene promoter to the coding sequence of active Granzyme B led to increased expression of Granzyme B in tumor cells, resulting in a higher rate of apoptotic cell death.[35] This also supports the idea that survivin-overexpressing cells are sensitive to CTLs. Survivin is also immunogenic for humoral immunity, supporting its high immunogenicity in the immune system.[36–39] The significant survivin immunogenicity for the cellular and humoral immune systems suggests fascinating possibilies for its use as a molecular target of immunotherapy.

On the basis of the immunogenicity of survivin antigenic peptides, some clinical trials have already been launched and reported, using survivin-$2B_{80-88}$[40,41] and $survivin_{96-104}$.[42] Each report showed some clinical response with survivin-2B and survivin-derived peptides, indicating that survivin/survivin-2B immunotherapy is a promising modality for cancer therapy. In our recent studies, survivin-$2B_{80-88}$ peptide and interferon-α (IFN-α) vaccination therapy is under Phase I study (Table 3). With peptide alone, we could detect CTL precursor (CTLp) frequency elevation in only 20% of patients, and the clinical responses were also relatively low (tumor marker 20%, imaging 40%). However, peptide plus IFN-α vaccination improved the detection of CTLp up to 50%. And also we could observe 67% tumor marker response and tumor mass stabilization or regression in clinical imaging. These observations suggest that immunological response to survivin-$2B_{80-88}$ peptide in the periphery is associated with the clinical response of peptide vaccine therapy, and IFN-α improves the efficacy of peptide vaccination therapy.

ML-IAP/Livin. Melanoma-IAP (ML-IAP)/Livin was identified as a novel IAP family protein overexpressed in melanoma.[43] ML-IAP/Livin contains a single BIR and RING domain, and inhibits caspase 3 and caspase 9 activities, causing the inhibition of apoptosis. In the following studies, ML-IAP was proved to be overexpressed in lung cancer,[44] renal cell carcinoma[45] and childhood AML.[46] High-level expression of ML-IAP/Livin is related to poor prognosis.[46] These reports suggest that ML-IAP/Livin can be the target of immunotherapy for such malignancies.

ML-IAP/Livin is also immunogenic for cellular and humoral immune systems.[44,47–49]

Bcl-2, Bcl-xL and Mcl-1. Bcl-2 was initially identified as the t(8;14) and t(14;18) translocations those are related to hematological malignancies. In subsequent reports, Bcl-2 protein was proved to be overexpressed in several types of malignancies including carcinomas. Bcl-2 is located in the inner membrane of the mitochondrion and regulates all major types of cell death, including apoptosis, necrosis, and autophagy; hence Bcl-2 is suitable for molecular targeting therapy, including cancer immunotherapy.[50] Andersen *et al.* found an HLA-A2 restricted low affinity Bcl-2 peptide (bcl208) could be a target of CTLs.[51] Interestingly, Bcl-2 peptides with high HLA-A2 affinity (bcl85, bcl124, bcl218, bcl220, bcl222 and bcl224) did not show any CTL response. These observations might indicate that CTLs specific for Bcl-2 peptides with high affinity to HLA-A2 are tolerated.

Bcl-x_L is one of the Bcl-2 protein families and has anti-apoptotic activity. Increased expression of Bcl-xL has been reported in a variety of different malignancies, including acute myeloid leukemia and multiple myeloma as well as solid cancers like bladder cancer, breast cancer, pancreatic cancer, and melanoma.[52] Andersen *et al.* detected immunological responses for the Bcl-$x_{L173-182}$ peptide in 9/18 breast cancer patients and 2/6 melanoma patients' PBLs with ELISpot assays after stimulation *in vitro*.[53]

Mcl-1 is also a Bcl-2 family protein with anti-apoptotic potential. Andersen *et al.* reported that immune reactivity for Mcl-1-derived peptides could be detected in cancer patients' lymphocytes.[54,55] However, they did not find that Mcl-1 peptide-specific CTLs could recognize Mcl-1-positive cancer cells; thus it remains elusive whether Mcl-1-derived peptides are presented by HLA-A1 molecules endogenously.

Limitless Replicative Potential

Growth signal autonomy, insensitivity to antigrowth signals and evading apoptosis lead to cell growth independent from its environment. However, the resulting deregulated proliferation program does not guarantee the growth of a tumor. Mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. One representative intrinsic mechanism that limits cell growth is the telomere, the cap of the chromosomal end. Each replication of the genomes shorten the telomere by 50–100 base pairs (bps), finally causing chromosomal instability and cell growth arrest termed 'crisis'.[56] Telomerase, a eukaryotic ribonucleoprotein (RNP) complex, helps to stabilize telomere length in human stem cells, reproductive cells and cancer cells by adding TTAGGG repeats onto the telomeres using its intrinsic RNA as a template for reverse transcription. The human telomerase reverse transcriptase catalytic subunit (hTERT) is overexpressed in 85–90% of several malignancies. This makes telomerase a target not only for cancer diagnosis but also for the development of novel anti-cancer therapeutic agents.[57]

hTERT. On the way to find universal TAAs that are expressed in various malignancies, Vonderhide *et al.* identified the HLA-A2-restricted hTERT-derived peptide (hTERT540).[58] The authors reported that hTERT peptide-specific CTLs could recognize HLA-A2-positive and hTERT-positive cancer cells in an HLA-restricted and peptide-specific fashion. Several clinical studies using hTERT-derived peptides have already been launched. hTERT-specific T lymphocytes were induced in four of seven patients with advanced breast or prostate carcinoma after vaccination with dendritic cells pulsed with hTERT peptides, resulting in partial tumor regression in one patient.[59] Another clinical trial used hTERT messenger RNA (mRNA)-transfected dendritic cells in patients with metastatic prostate cancer.[60] A trial investigating vaccination with hTERT peptides in patients with non-small-cell lung cancer showed immune responses in 12 of 24 evaluable patients during the primary regimen, with a complete tumor response observed in one patient.[61] These studies justify further clinical testing to evaluate the efficacy of hTERT-based vaccinations, and several hTERT-based clinical vaccination trials are currently ongoing. Several HLA-class I-restricted hTERT-derived peptides make the hTERT peptide more universal: HLA-A2,[62,63] HLA-A3,[64] A24[65] and B*0702.[66]

In contrast to the above studies, several groups reported that the hTERT540 peptide is not presented on the surface of tumor cells in the context of HLA-A2.[67–69] Above all, Wenandy *et al.* confirmed that hTERT540-specific CTL clones could recognize HLA-A2-positive and hTERT-positive cancer cell lines.[70] The discrepancies among these groups might depend on the conditions, including cell culture conditions, T-cell avidity, target cell condition and so on.

Sustained Angiogenesis

Oxygen and nutrients are essential for cell survival. After organogenesis, angiogenesis can be observed in only strictly limited situations such as wound healing and tumorigenesis. Targeting angiogenesis is believed to be an attractive approach for cancer therapy. This method has several merits compared to others. First, angiogenesis is one of the physiological reactions of the host, therefore therapy resistance caused by genomic instability will be unlikely to occur. Second, each capillary has the potential to feed hundreds of cancer cells; thus, targeting angiogenesis may have amplified effects.[71] Angiogenesis can be the target of immunotherapy, and several studies have demonstrated the effects of anti-angiogenesis immunotherapy *in vitro* and *in vivo*.

VEGF-R1, VEGF-R2, VEGF. Since, vascular endothelial growth factor (VEGF) is not expressed in normal blood vessels, but is expressed in pathologic angiogenic vessels, including tumor angiogenesis, and VEGF signaling plays a major role in angiogenesis, VEGF-receptors (VEGF-Rs) might be a major target for anti-angiogenesis immunotherapy. Niethammer *et al.* showed the efficacy of targeting VEGF-R2 with DNA vaccination in a mouse tumor model in which a significant anti-tumor effect was observed.[72] The authors reported that vaccination with VEGF-R2 DNA caused inhibition of tumor growth, prolongation of survival and a decrease of vessels in tumor tissues. They further showed that DNA vaccination did not affect wound healing, so this strategy might be safe and feasible for a clinical trial. HLA-A2-restricted VEGF-R2 and VEGF-R1 antigenic peptides have also been reported.[73,74] Weinzierl reported an HLA-B27-restricted antigenic peptide that is coded in the cryptic translated region of VEGF using differential mass spectrometry in primary renal cell carcinomas (RCCs), suggesting that this peptide is endogenously presented *in vivo*.[75] This antigenic peptide might be useful for RCCs.

Survivin. Survivin is related to angiogenesis and anti-sense targeting of survivin inhibits capillary formation by endothelial cells, suggesting that targeting survivin does target angiogenesis.[76] Xiang demonstrated the anti-angiogenesis effect of therapy targeting survivin with DNA vaccination.[77] These findings suggest that targeting survivin equals therapy targeting both cancer cells and angiogenesis as well. This makes survivin a unique and attractive antigen.

Tissue Invasion and Metastasis

In the process of malignant tumor cell growth, the cells need to invade the normal connective tissue barrier. Tissue invasion and metastasis are landmarks of malignant diseases that distinguish

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them from benign diseases. Several classes of proteins are involved in this mechanism. One classic mechanism for gaining invasive ability is the overexpression of several enzymes to break the connective tissue barrier. Matrix metalloproteases (MMPs) are representative molecules of tissue invasion. MMPs are known to be overexpressed in cancerous tissues, and further, to be related to poor prognosis, cancer progression, advanced stages and high risk of metastasis.[78] MMPs play an essential role in carcinogenesis and might be targets for immunotherapy. However, MMPs are also expressed in normal tissues so that immunity for MMPs might be tolerated. Cellular immunity for MMP-2 has been demonstrated in a very unique fashion, as described below.

MMP-2. Matrix metalloproteinase-2 plays an essential role in tumor progression; however it is expressed ubiquitously, and the peptides derived from MMP-2 might be tolerated. Godefroy *et al.* showed how an MMP-2-derived peptide could be presented by tumor cells specifically and recognized by CTLs.[79] They reported that an HLA-A*0201-restricted peptide from the MMP-2 (GLPPDVQRV) was cross-presented by αvβ3-expressing tumor cells following clathrin-coated pit-mediated uptake of secreted extracellular MMP-2 and proteasome activity. The classic endogenous cytosolic pathway did not present this peptide. Hence, an MMP-2-derived peptide vaccine might be useful for MMP-2 secreting and αvβ3-expressing melanoma cells.

Gaining Cancer Initiation Ability (Cancer Stemness)

The cancer-initiating cell (CIC)/cancer stem cells (CSC) hypothesis is both an old and a new concept. In the former studies it was discovered that a small population of cancer cells had growth potential in soft agar and were called 'tumor stem cells'. Recently, CICs/CSCs are described as small populations that have (i) tumor initiation ability, (ii) differentiation ability and (iii) self-renewal. Since CICs/CSCc have very high tumor-generating ability, resistance to treatment and high metastatic ability, the CICs/CSCs concept is very important.[6] The resistances to several types of treatments will through us the major question that CICs/CSCs are also resistant to CTLs. Kawasaki *et al.* reviewed CD200, one of the immunosuppressive factors expressed in normal and cancer stem cells, and CD200-expressing cancer cells suppressed secretion of type 1 helper T-cell cytokines (IFN-gamma and interleukin [IL]-2),[80] suggesting that CICs/CSCs might suppress immunity against cancers. At this moment, CIC/CSC-related molecules SRY (sex determining region Y)-box 2 (SOX2) and SOX10 are known to be targets of CTLs as described below. Since these reports did not show cytotoxic activity for CICs/CSCs, we cannot conclude whether CICs/CSCs are susceptible to CTLs. The concept targeting CICs/CSCs with CTLs is intriguing and further reports are eagerly awaited.

SOX2. SRY (sex determining region Y)-box 2 is a transcription factor that is essential to maintain self-renewal of undifferentiated embryonic stem cells. SOX2 is one of the red-hot genes. In 2006, Takahashi and Yamanaka reported that induced pluripotent stem (iPS) cells could be generated from mouse fibroblasts by retrovirus-mediated introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, in an epoch-making work of regenerative medicine.[81] This reports suggest that SOX2 is essential in the maintenance of ES cells and neuronal progenitor cells. It is also reported to be expressed in several malignancies.[82–84] Furthermore, Schmits *et al.* reported HLA-A2-restricted SOX2 peptides, and SOX2 could be the target of CTLs in glioma cells.[84] In addition, Ben-Porath *et al.* reported that an embryonic stem cell-like gene expression signature including Nanog, Oct3/4, Sox2 and c-Myc was preferentially overexpressed in poorly differentiated aggressive human tumors.[85] To summarize these reports, targeting ES phenotypic genes seems to be a reasonable strategy and SOX2 is representative of this approach.

SOX10. Khong *et al.* showed that SOX10 was recognized by tumor-infiltrating lymphocytes obtained from a patient who experienced a dramatic clinical response to immunotherapy.[86] It acts as a critical transactivator of tyrosinase-related protein-2 during melanoblast development and as a potent transactivator of micropthalmia-associated transcription factor (MITF), which is considered to be a master gene that controls the development and postnatal survival of melanocytes. Thus, SOX10 is a potential target for melanoma CICs/CSCs.

Conclusion

As described above, considerable numbers of TAAs have essential roles in carcinogenesis. Since, TAAs are defined as antigens that are expressed in cancer cells specifically, it is reasonable that various overexpressed carcinogenesis-related genes can also be TAAs. Thus, the functioning antigen concept is tightly connected to carcinogenesis and essential for both cancer immunotherapy and cancer biology. Finally, to accomplish effective cancer immunotherapy, we need to improve recent therapies. There are several possibilities: combination of several peptides, combination with another molecular targeting therapy, and combination with radiation therapy and combination with chemotherapy. The concept of functioning antigens is the blueprint of cancer therapy, including immunotherapy and points us in the direction to move ahead.

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