(SIGMA) and washed 3 times and incubated with affinity-purified antibody peroxidase-labeled goat anti-mouse IgG + IgM(H + L) human serum absorbed peroxidase (2000×) (KPL, Gaithersburg, MD). Finally, the membranes were visualized with ECL™ Western blotting Detection Reagent (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's protocol.

In vitro induction of HPV16 E6 and E7-specific T cells with oligomannose-coated liposome-encased pCAGGS/E6E7

Peripheral blood mononuclear cells (PBMCs) from HPV16-positive cervical carcinoma patients were separated by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). PBMCs were incubated in AIM-V medium (Life Technologies, Inc.) containing 10% pooled human AB serum. On day 1, 1×10^7 PBMCs/well was pulsed with oligomannose-coated liposome-encased pCAGGS/E6E7 (10 μg/mL). On day 3, IL-2 was added to each well at a final concentration of 100 IU/mL. During CTL induction, cells were fed with fresh AIM-V medium containing 10% pooled human AB serum supplemented with IL-2 (100 IU/mL) every 3–4 days. On day 10, the cytotoxic activity of T cells was assessed by an enzyme-linked immunospot (ELISPOT) assay. The time schedule is summarized in Fig. 3A.

Enzyme-linked immunospot assay (ELISPOT) assay

The specificity of CTLs for HPV16 E6 and E7 peptides was evaluated by IFN-γ ELISPOT assay as described previously (Inoda et al., 2009). Multiscreen 96-well plates (Millipore, Bedford, MA) were coated with 100 μL/well of 5 μg/mL anti-IFN-γ capture antibody (PharMingen, San Diego, CA) in PBS at 4 °C overnight. The plates were washed once with 200 μL/well complete RPMI 1640 and blocked with 200 μL/well complete RPMI 1640 at room temperature for 2 h. Then 2×10^4 CTLs were incubated with 5×10^4 of T2-A24 cells pulsed with the HPV-specific peptide or control peptide (5 μg/mL) or K562 cells. After incubation in a 5% CO_2 humidified chamber at 37 °C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-gamma antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Germany).

Results

Oncoprotein E6E7 expression with pCAGGS and OML-HPV

To generate the OML-HPV DNA vaccine, an HPV16 E6E7 fused gene was constructed as described above. To confirm the expression of the pCAGGS-E6E7 construct, 293T cells, human embryonic kidney cells, were transduced with FuGENE HD reagent and analyzed by Western blotting. FLAG-tag fused E6E7 oncoproteins were detectable by anti-FLAG antibody (Fig. 1A). To confirm the transduction of OML-HPV DNA into PBMCs, we transduced OML-HPV DNA into PBMCs of a healthy volunteer at serial concentrations as described in Materials and methods. FLAG-tag fused E6E7 protein expression was detectable with several concentrations ranging from 0.1 μg/mL to 2.0 μg/mL (Fig. 1B). We could detect E6E7 protein stably in serial experiments at the OML-HPV concentration of 1.0 μg/mL, and we used this condition for the following CTL induction protocol.

*Analysis of the binding of synthetic peptides to HLA-A*2402 molecules*

Since HPV16 E6 and E7 are expressed in HPV16-positive cervical epithelium cells but not in normal cells, they might be suitable targets for tumor immunotherapy. Amino acid sequences of HPV16 E6 and E7 were screened for a possible HLA-A24-bound 9- to 12-mer peptide, based on the HLA-A24-specific anchor motif (Kondo et al., 1995). The motif consists of Y, F, M or W at position 2 and L, I, F or M at the COOH-terminal residue. As shown in Table 1, 6 of the E6 peptides, E6 8–19 (MFQDPQERPRKL), E6 49–57 (VYDFAFRDL), E6 66–74 (PYAVCDKCL), E6 82–90 (EYRHYCYSL), E6 87–95 (CYSLYGTTL) and E6 98–106 (QYNKPLCDL), and 3 of the E7 peptides, E7 10–20 (IVLHLEPQNEI), E7 51–60 (HYNIVTFCCK) and E7 83–93 (LMGTLGIVCPI), were selected as potential HLA-A24-binding peptides. Then HLA-A24 binding ability was evaluated using T2-A*2402 cells. HLA-A24 level on the cell surface of T2-A*2402 cells is up-regulated in the presence of two positive control peptides, HLA-A24-restricted EBV (LMP2) epitope and HIV(env) peptide HLA-A24-binding peptides, and negative controls (SL-8 peptide and HLA-A68-restricted EBV epitopes) did not show any HLA-A24 binding (Fig. 2). Synthetic peptides E6 49–57, 66–74, 82–90, 87–95, 98–106 and E7 83–93 were capable of up-regulating the HLA level to levels almost the same as those in the case of EBV-LMP2 and HIV peptides, suggesting that these five E6 synthetic peptides and one E7 peptide might have relatively high binding affinity to HLA-A24 molecules (Fig. 2).

Efficient induction of tumor peptide-specific CTL by immunizing OML-HPV complex

In this study, to generate HPV16-specific CTLs in vitro, we compared OML and other standard liposomes. PBMCs of Case # 18 HPV16-positive and HLA-A24-positive cervical carcinoma patient were stimulated once with HPV16 E6E7 DNA and OML or standard liposome (Lipofectamine 2000, Invitrogen) mixture in vitro. Then immunological reactivity was evaluated with the ELISPOT assay. We could detect anti-E6 66–74 peptide-specific reactivity with PBMCs stimulated by OML-HPV but not with PBMCs stimulated with Lipofectamine 2000 (Fig. 3B), suggesting that OML is more efficient than

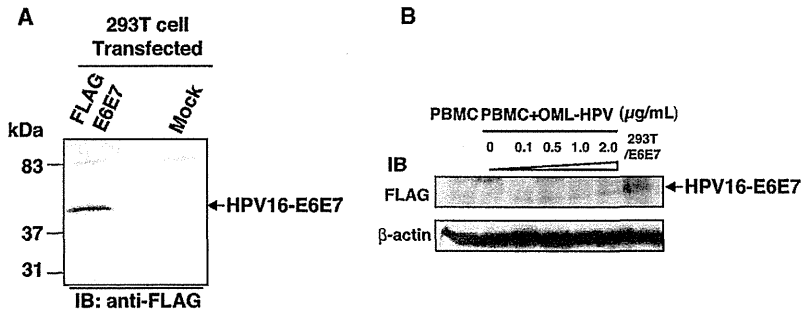

Fig. 1. A. Expression of E6E7 protein by 293T cells transduced with pCAGGS-E6E7. Lane 1, 293T cells transduced with pCAGGS-E6E7: lane 2, 293T cells alone as a negative control. E6E7 fused protein-specific band was detected by an anti-FLAG monoclonal antibody. B. Expression of E6E7 protein in human PBMCs. PBMCs from a healthy volunteer were transduced with OML-HPV at serial concentrations (0.1–2.0 μg/mL). pCAGGS-E6E7-transfected 293T cells were used as a positive control. E6E7 protein was detected with an anti-FLAG monoclonal antibody by Western blot analysis. β-actin was detected as an internal control.

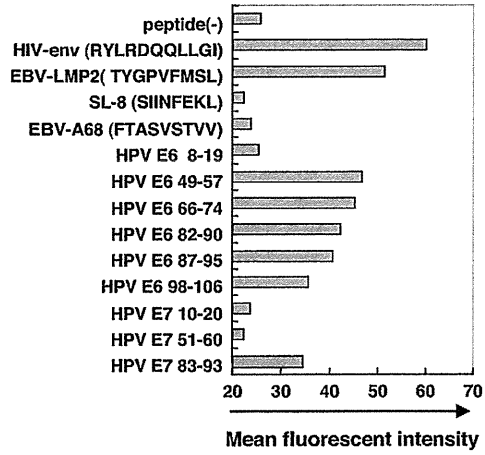

Fig. 2. HLA-A24 binding assay with HPV16 E6 and E7-derived peptides carrying the HLA-A24-binding motif. Nine peptides carrying the HLA-A24-binding motif were synthesized. Binding affinity of HPV16 E6 and E7-derived peptides to HLA-A24 molecules was evaluated by MFI of cell surface HLA-A24 molecules on T2-A*2402 cells that were pulsed with each peptide. HLA-A24-bound EBV LMP2-derived peptide (TYGPVFMSL) and HIVenv-derived peptide (RYLRDQQLLGI) were used as positive controls. SL-8 peptide (SIINFEKL) and EBV A68-derived peptide (FTASVSTVV) were used as negative controls. Histograms of MFI are displayed for each peptide.

Lipofectamin 2000 for generating CTLs. We therefore chose OML-HPV to generate CTLs in the following experiments.

To confirm the CTL induction ability of OML-HPV DNA vaccine and to identify the most immunogenic HLA-A24-restricted peptide, we performed CTL generation with PBMCs from CIN3 or cervical carcinoma patients. PBMCs collected from four of the HLA-A24-positive HPV16-positive patients and four of the HLA-A*2402-positive, HPV16-negative patients were tested for induction of CTLs in vitro (Patients' profiles are summarized in Table 2.). PBMCs were pulsed only once with OML-HPV and the peptide reactivities were evaluated with the ELISPOT assay (Fig. 3A). CTLs specific for HPV16 E6 66–74 peptide were induced from PBMCs with Cases # 1, # 6, # 16 and # 18, that were completely identical to HPV 16-positive cases, whereas E6 66–74 peptide-specific reactivity was not detectable with HPV 16-negative cases (Fig. 3C), suggesting that E6 66–74 peptide-specific CTLs might be expanded in vivo. Case # 1 also showed reactivity for E6 82–90. Case # 6 showed reactivity for all HLA-A24-binding E6 and E7-derived peptides. Case # 16 showed reactivity for E6 49–57 and E7 83–93. Case # 18 also showed reactivity for E6 98–106. On the other hand, none of the HPV 16-negative cases showed any HPV 16 peptide reactivity, suggesting that peptide relativities are related to HPV 16 viral infection. These results indicate that these epitopes might be useful for eliciting an anti-tumor immune response in HLA-A24-positive and HPV16-positive patients and that E6 66–74 is the most immunogenic HLA-A24 peptide coded by HPV16 E6 and E7 proteins.

Discussion

Since, viral proteins are expressed in virus-infected tumor cells but not in normal cells, these proteins are thought to be promising targets for immunotherapy. HPV16 E6 and E7 are widely and homogenously expressed in cervical lesions that have persistent infection with high-risk HPV and have been identified as target antigens against cervical cancer. HPV16 is the most common serotype and is found in over 45% of cases in Japanese patients. A small number of HLA-A24-restricted HPV16 E6 coded epitopes including, E6 49–57, E6 82–90 and E6 98–106 have already been reported (Hara et al., 2005; Morishima et al., 2007). However, it has not been determined which HLA-A24-restricted epitope is the most immunogenic and suitable for immunotherapy. In this study, we identified several novel HLA-A24-restricted E6 or E7 coded epitopes. Our CTL induction method is based on the fused-whole gene of HPV16 E6 and E7, and we can

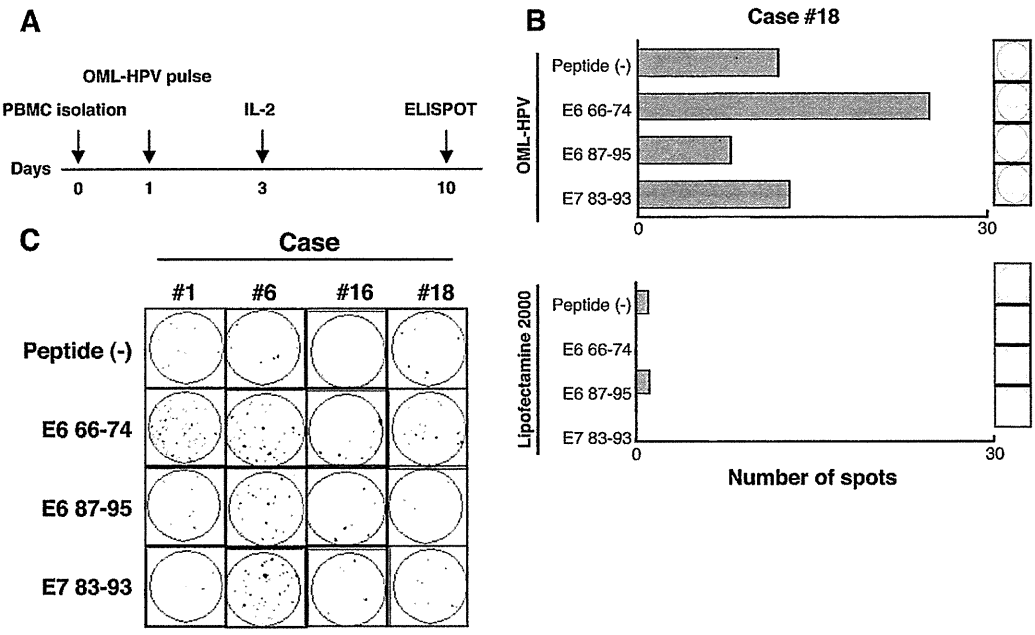

Fig. 3. A. Time course of CTL induction from PBMCs. B. Time course of CTL induction using OML-HPV. B. Comparison of CTL induction efficiency with OML-HPV and Lipofectamine 2000. PBMCs from an HPV16-positive and HLA-A24-positive cervical carcinoma patient (Case # 18)' were stimulated with OML-HPV and Lipofectamine 2000 pCAGGS-E6E7 complex. On day 10, HPV16-specific CTLs were detected with the ELISPOT assay. T2-A24 cells were used as antigen-presenting cells. E6-derived peptides E6 66–74 and E6 87–95 and E7-derived peptide E7 83–93 were used. The number of ELISPOTs produced by 6×10^4 CTLs in response to 5×10^4 T2-A*2402 cells is shown. C. CTL induction with PBMCs from HPV16-positive and HLA-A24-positive patients. PBMCs from HPV16-positive cervical cancer patients (Cases # 1, # 6, # 16 and # 18) were stimulated with OML-HPV in vitro. Responding CTLs were tested for IFN-gannma ELISPOTs in response to T2-A*2402 cells with or without a peptide.

Table 2
Summary of the patients' reactivities to HPV16 peptides.

Case no.	Age	HPV genotype	HLA-[a]A2402	Diagnosis[b]	Peptide specificity[a]					
					E6 peptide					E7 peptide
					49–57	66–84	82–90	87–95	98–106	83–93
1	41	16	+	Cx.Ca Ib1	−	+	+	−	−	−
2	35	66	+	Cx.Ca Ia1	−	−	−	−	−	−
3	33	18	+	Cx.Ca Ib1	−	−	−	−	−	−
6	37	16	+	CIN3	+	+	+	+	+	+
9	29	18	+	Cx.Ca Ib1	−	−	−	−	−	−
13	37	58	+	CIN3	−	−	−	−	−	−
14	44	52	+	Cx.Ca 0	−	−	−	−	−	−
16	39	16	+	Cx.Ca Ib1	+	+	−	−	−	+
17	27	51	+	CIN3	−	−	−	−	−	−
18[c]	46	16	+	Cx.Ca Ib2	−	+	−	−	+	−
Peptide positivities in HPV16 (+) patients					50% (2/4)	100% (4/4)	50% (2/4)	25% (1/4)	50% (2/4)	50% (2/4)

[a] Peptide specificity was evaluated by ELISPOT assay using peptide pulsed T2-A24 cells. More than twice IFN-γ spots than peptide (−) T2-A24 cells were classified as 'positive (+)'.
[b] CxCa, cervical cancer; CIN, cervical intra-epithelial neoplasia.
[c] Case no 18: post radiation therapy.

thus simply compare the immunogenicities of the peptides examined, and we concluded that the novel HLA-A24-restricted peptide E6 66–74 is the most potent. We could detect HPV16 E6 or E7-specific relativities only for HPV 16-positive patients, suggesting that HPV16 E6 or E7-specific CTLs might be stimulated and expanded clonally in vivo. This also supports the immunogenicity and efficiency of HPV16 E6 or E7-derived antigenic peptides.

To immunize immune cells with whole E6 and E7 molecules, we used OML as a DNA transducer into PBMCs. Several methods for inducing CTLs with whole E6 or E7 genes or proteins have already been reported (Murakami et al., 1999). Usually, it is very difficult to transduce a gene into human PBMCs, and thus few human CTL induction methods using HPV genes have been reported. However, as described above, gene introduction efficiency has been dramatically improved using OML. Furthermore, we could detect CTL relativities with stimulation of PBMCs using OML-HPV only one time, suggesting that OML-HPV stimulation induces immune reaction very efficiently.

In a clinical trial using a vaccinia virus vector expressing HPV 16 and 18 E6 and E7, 5 of 12 cervical cancer patients showed more than 50% regression tumor (Baldwin et al., 2003). The results of that trial indicate the efficiency of immunization with HPV-derived viral genes for tumor regression. However, a virus-based vector sometimes induces a neutralizing antibody in cancer patients which attenuates the efficiency of the viral vector. Thus, the use of viral vectors is sometimes restrictive. On the other hand, OML is a complex of lipid and mannose, and it must therefore be less immunogenic for humoral immunity. Hence, frequent administration of OML might be more tolerable for cancer patients.

OML is an effective gene transducer into human PBMCs, and OML is also well known to enhance the activation of antigen-presenting cells (APCs), especially macrophages and dendritic cells (DCs). Thus, OML might also work as an adjuvant. Furthermore, it has been revealed that administration of OMLs leads to a T-helper type I (Th1) immune response specific for the antigen encased in the OMLs in BALB/c mice (Shimizu et al., 2007). Recently, signaling mechanisms of this response such as activation of the PI3k/Akt pathway by OMLs through phosphorylation of Src family kinases to induce ERK activation have been reported (Kato et al., 2008). These reports indicate that OML works not only as a DNA transducer but also as an adjuvant.

In summary, we have described a novel HPV16 E6-derived HLA-A24 restricted peptide E6 66–74, which is the most potent epitope of HLA-A24-restricted HPV 16 E6 and E7 proteins. We also suggest that OML-HPV16 E6E7 DNA complex might be an efficient CTL induction tool in vitro. Further investigations, especially in vivo, will provide more information about the efficiency of OML-HPV.

Conflict of interest statement

The authors have no financial conflict of interest.

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Efficiency of G2/M-related tumor-associated antigen-targeting cancer immunotherapy depends on antigen expression in the cancer stem-like population

Takashi Mori [a,b], Satoshi Nishizawa [a,b], Yoshihiko Hirohashi [a,*], Toshihiko Torigoe [a,*], Yasuaki Tamura [a], Akari Takahashi [a], Vitaly Kochin [a], Reona Fujii [b], Toru Kondo [c], Mark I. Greene [d], Isao Hara [b], Noriyuki Sato [a]

[a] Department of Pathology, Sapporo Medical University, School of Medicine, South-1 West-17, Chuo-Ku, Sapporo 060-8556, Japan
[b] Department of Urology, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-0012, Japan
[c] Department of Stem Cell Biology, Ehime University Proteo-Medicine Research Center, To-on 701-0295, Ehime, Japan
[d] Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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ABSTRACT

The aim of this study was to establish a novel efficient cancer DNA vaccine approach. Many tumor-associated antigens (TAAs) have been reported; however, there is little information of the efficiency of each TAA. Normal cells barely undergo mitosis, whereas cancer cells divide frequently and grow well. Thus, G2/M-related antigens are cancer cell-specific and are regarded to be suitable candidates as targets of cancer immunotherapy. In this study, we compared the efficiencies of G2/M-related antigens including Birc5, Aurka, Nke2 and Plk1 by using a DNA vaccination model. Mice that had been immunized with G2/M-related antigens coding plasmid were challenged with CT26 colon cancer cells. Interestingly, *Birc5*- and *Aurka*-immunized mice showed an anti-tumor effect, whereas *Nek2*- and *Plk1*-immunized mice did not show any anti-tumor effect. We investigated the expression of G2/M-related antigens in cancer stem-like cell (CSC)/cancer-initiating cell (CIC) population to verify the difference in the anti-tumor effect. CSCs/CICs were isolated as side population (SP) cells using Hoechst 33342 dye from CT 26 cells. It was found that *Birc5* and *Aurka* are expressed in both CSCs/CICs and non-CSCs/CICs (shared antigens), whereas *Nek2* and *Plk1* are expressed preferentially in non-CSCs/CICs (non-CSC antigens). Therefore, antigen expression in the CSC/CIC population might be related to the anti-tumor efficiency of cancer immunotherapy. Furthermore, we established a heat shock protein (*Hsp90*)-fused *Birc5* plasmid to improve anti-cancer immunity. *Birc5* fused to the N-terminal region of *Hsp90* showed a stronger anti-tumor effect, whereas *Birc5* fused to the C-terminal region of *Hsp90* did not show enhancement compared with Birc5. These observations indicate that expression in the CSC/CIC population is essential to achieve tumor regression and that fusing antigens to the N-terminal region of *Hsp90* enhances the anti-tumor effect.

Introduction

Since the identification of human tumor-associated antigens (TAAs) (van der Bruggen et al., 1991), great progress has been achieved in this field, and many cancer immunotherapy clinical trials based on TAAs have been launched (Rosenberg, 1999). Some patients who underwent cancer immunotherapy using TAAs showed a favorable response (Marchand et al., 1999; Schwartzentruber et al., 2011), indicating that cancer immunotherapy using TAAs is a promising approach. A large number of such TAAs have been reported, but there is little information about which TAAs are suitable to achieve a good response in cancer patients.

In cell cycle G2/M phase, cells go through mitosis and show dramatic morphological change, and many proteins work together for cells to go through mitosis. Normal somatic cells barely undergo mitosis, while transformed cancer cells undergo mitosis frequently because of the loss of cell growth controls by several mechanisms. Therefore, mitosis is regarded as one of the distinct characters of cancer cells, and G2/M-related proteins can be suitable targets to treat cancers (Hanahan and Weinberg, 2011). Several approaches to target G2/M phase-related gene products have been developed. Molecular targeting therapies for BIRC5 (SURVIVIN), AURKA (AURORA-A kinase), NEK2 and PLK1 have been established, and clinical trials using those reagents are underway (Degenhardt and Lampkin, 2010; Kokuryo et al., 2007; Ochi et al., 2009b; Ryan et al., 2009).

Since most of the G2/M related-proteins show cancer-specific expression, they can be tumor rejection antigens. BIRC5-derived

Abbreviations: TAA, tumor-associated antigen; CSC, cancer stem-like cell; CIC, cancer-initiating cell; SP, side population; MP, main population.

* Corresponding authors. Fax: +81 11 643 2310.
E-mail addresses: hirohash@sapmed.ac.jp (Y. Hirohashi), torigoe@sapmed.ac.jp (T. Torigoe).

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antigenic peptides can induce antigenic peptide-specific cytotoxic T lymphocytes (CTLs) and BIRC5 peptide specific CTLs can recognize BIRC5-positive cancer cells (Andersen et al., 2007; Hirohashi et al., 2002). Results of cancer immunotherapies using BIRC5-derived antigenic peptides have already been reported (Honma et al., 2009; Kameshima et al., 2011; Miyazaki et al., 2011; Tsuruma et al., 2004; Tsuruma et al., 2008). AURKA and PLK1 have been proven to be targets of CLTs (Ochi et al., 2009a; Park et al., 2011). NEK2 is recognized by the humoral immune system in neuroblastoma patients (Kohler et al., 2010). Therefore, all of these G2/M-related antigens are surely immunogenic.

Cancer tissues show heterogeneity with regard to morphology and genotypically. A small population of cancer cells show (a) high tumor-initiating ability, (b) self-renewal ability and (c) differentiation ability (cancer stem cell theory) (Reya et al., 2001). Therefore, targeting cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) is thought to be essential to cure cancer (Park et al., 2009). CSCs/CICs show resistance to cancer treatments including chemotherapy and radiotherapy; however, immune systems including CTLs can recognize CSCs/CICs efficiently (Hirohashi et al., 2010; Inoda et al., 2011). Therefore, cancer immunotherapy is a promising approach to eradicate CSCs/CICs. Furthermore, we recently proposed that TAAs can be classified into 3 groups according to their expression in CSCs/CICs and non-CSCs/CICs: these are (i) CSC/CIC antigens, which are expressed in CSCs/CICs but not in non-CSCs/CICs; (ii) shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs; and (iii) non-CSC/CIC antigens, which are expressed in non-CSCs/CICs but not in CSCs/CICs (Hirohashi et al., in press).

HSP family proteins work as molecular chaperones and bind to their client proteins. Antigenic peptide-bound HSP 90 family proteins are known to work as immune modulators that enhance the cross-priming pathway and enhance anti-tumor immunity (Kurotaki et al., 2007; Oura et al., 2011).

In this study, we compared the immunogenicity of G2/M-related antigens using DNA vaccine model. *Birc5*- and *Aurka*-immunized mice showed an anti-tumor effect, whereas *Nek2*- and *Plk1*-immunized mice did not show any anti-tumor effect. Since CSCs/CICs play a major role in tumor initiation, we investigated the expression of *Birc5*, *Aurka*, *Nek2* and *Plk1* in both CSCs/CICs and non-CSCs/CICs. *Birc5* and *Aurka* are expressed in both CSCs/CICs at the same levels. *Nek2* and *Plk1*, however, are expressed preferentially in non-CSCs/CICs. Therefore, Birc5 and Aurka are CSC antigens, whereas Nek2 and Plk1 are non-CSC antigens, and expression in CSCs/CICs might be a major reason for the efficiency of anti-tumor effects. Furthermore, to improve the efficacy of a DNA vaccine, we fused the *Birc5* gene to the *Hsp90* gene and found that Burc5 fused to the N-terminal of the *Hsp90* gene showed significant enhancement of the anti-tumor effect. These observations indicate that expression of TAAs in CSCs/CICs is essential for effective cancer immunotherapy and that Hsp90 can enhance anti-tumor immunity.

Materials and methods

Cell line

The mouse colon carcinoma cell line CT26 of BALB/c mouse origin was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS). The cells were kept in a 37 °C incubator with humidified air and 5% CO2. The culture medium was changed two times in a week.

Mice

All mouse procedures were performed in accordance with institutional protocol guidelines at Sapporo Medical University School of Medicine. BALB/c female mice were purchased from Clea Japan (Tokyo, Japan) at the age of 6–8 weeks.

Plasmid construction

Birc5, *Aurka*, *Nek2* and *Plk1* cDNAs were amplified by RT-PCR from CT26 cells and subcloned into a restriction enzyme-digested FLAG-tag inserted pcDNA3.1(+) vector (Invitrogen). The DNA sequences were confirmed by direct sequencing, and the expression was confirmed by transfection into 293T cells and Western blotting (data not shown). Then *Birc5*, *Aurka*, *Nek2* and *Plk1* cDNAs were subcloned into an enzyme-digested pCAGGS expression vector (a kind gift from Prof. J Miyazaki, Osaka, Japan) (Niwa et al., 1991).

Hsp90α cDNA was amplified from CT26 cDNA and cloned into the pcDNA3.1(+) vector, and the sequence was confirmed. *Hsp90-N–Birc5* and *HSP90-C–Birc5* plasmids were constructed by inserting *Birc5* cDNA into N-terminus of *Hsp90* and C-terminus, respectively. The protein expression was confirmed by transfection into 293T cells and Western blotting (data not shown). The sense sequences and anti-sense sequences of the inserted oligo DNA are summarized in Table 1. The plasmid DNA (pDNA) was amplified in the BL21 strain of *Escherichia coli* and purified using a Qiagen Endofree Plasmid Giga Kit (Qiagen). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide staining.

DNA vaccination and tumor cell challenge

One hundred micrograms of plasmid was injected into both sides of BALB/c mouse footpads twice weekly. One week after the last plasmid injection, CT26 cells were injected into the subcutaneous space of the back of syngeneic BALB/c mice. Tumor growth was monitored weekly, and tumor volume was calculated by $XY^2/2$ (X = long axis, Y = short axis).

SP analysis

Side population (SP) cells and main population (MP) cells were isolated as described previously using Hoechst 33342 dye (Lonza, Basel, Switzerland) with some modifications (Goodell et al., 1996). Briefly, cells were resuspended at 1×10^6/mL in pre-warmed DMEM supplemented with 5% FBS. Hoechst 33342 dye was added at a final concentration of 1.25 μg/mL in the presence or absence of verapamil (50 μM; Sigma-Aldrich) and the cells were incubated at 37 °C for 90 min with intermittent shaking. Analyses and sorting were performed with a FACSAria II cell sorter (Becton Dickinson).

RT-PCR analysis

RT-PCR analysis was performed as described previously (Nakatsugawa et al., 2009). Total RNA (tRNA) was isolated from CT26 SP cells and MP cells by an RNeasy Mini Kit (Qiagen, Valencia, CA) using DNase (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using Superscript III and oligo (dT) primer (Invitrogen) according to the manufacturer's protocol. The PCR procedure was carried out using Taq DNA polymerase (Qiagen) and 12 pmol of primers. The mixture was initially incubated at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. Primers used in the experiments are summarized in Table 1.

Results

Immunogenicity of G2/M-related antigens

Cancer cells undergo mitosis more frequently than do normal cells, and thus mitosis is one of the cancer cell specific features. Several G2/M phase-related proteins are essential for cancer cell

Table 1
List of primers.

Number	Name	Sequence[a]	Target gene	Purpose	Enzyme site	Product (bp)
1	mSurvivin_fw1	CGCGGATCCATGGGAGCTCCGGCGCTGCCC	*Birc5*	Gene cloning	BamHI	441
2	mSurvivin_rv1	CCGCTCGAGGGCAGCCAGCTGCTCAATTGA	*Birc5*	Gene cloning	XhoI	
3	mAuroraA_fw1	CGCGGATCCATGGCTGTTGAGGGCGAGCC	*Aurka*	Gene cloning	BamHI	1271
4	mAuroraA_rv1	CCGCTCGAGAGATGATTTGCTGGTTGGCTC	*Aurka*	Gene cloning	XhoI	
5	mNek2_fw1	CGCGGATCCATGCCGTCCCGGGTGGAGGAC	*Nek2*	Gene cloning	BamHI	1350
6	mNek2_rv1	CCGCTCGAGGCGCATGCCCAGGATCTGCCT	*Nek2*	Gene cloning	XhoI	
7	mPlk1_fw1	CCCAAGCTTATGAATGCAGTGGCCAAAGCT	*Plk1*	Gene cloning	HindIII	1830
8	mPlka_rv1	TGCTCTAGAGGAGGCCTTGAGGCGGTTGCT	*Plk1*	Gene cloning	XbaI	
9	mSurvivin_fw2	TGGCAGCTGTACCTCAAGAA	*Birc5*	Expression check	N/A	220
10	mSurvivin_rv2	CAGGGGAGTGCTTTCTATGC	*Birc5*	Expression check	N/A	
11	mAuroraA_fw2	GCCCACTAGGAAAAGGGAAG	*Aurka*	Expression check	N/A	553
12	mAuroraA_rv2	AGGCATCCCCACTAGGAACT	*Aurka*	Expression check	N/A	
13	mNek2_fw2	TGCCTCCTGTATGAGCTGTG	*Nek2*	Expression check	N/A	472
14	mNek2_rv2	GCGGTGTTCTCTTTGCTTTC	*Nek2*	Expression check	N/A	
15	mPlk1_fw2	CCCTATTACCTGCCTCACCA	*Plk1*	Expression check	N/A	445
16	mPlk1_rv2	ATAGGACTCCGTGCCATCAC	*Plk1*	Expression check	N/A	
17	G3PDH_fw2	ACCACAGTCCATGCCATCAC	*Gapdh*	Expression check	N/A	452
18	G3PDH_rv2	TCCACCACCCTGTTGCTGTA	*Gapdh*	Expression check	N/A	
19	HSP90_fw1	GCTCTAGACAATTGCCACCATGCCTGAGGAAACCCAGACC	*Hsp90*	Hsp90-N construct	XbaI,MfeI	2235
20	HSP90_rv1	GGGGTACCGCTAGCGTCTACTTCTTCCATGCGTGA	*Hsp90*	Hsp90-N construct	NheI,KpnI	
21	HSP90_fw4	CCGCTCGAGATGCCTGAGGAAACCCAGACC	*Hsp90*	Hsp90-C construct	XhoI	2220
22	HSP90_rv4	GGCCAATTGTTAGTCTACTTCTTCCATGCG	*Hsp90*	Hsp90-C construct	MfeI	

[a] Underline indicates restriction enzyme sequence.

viability, and G2/M-related proteins can therefore be suitable candidates for cancer immunotherapy. Some G2/M-related proteins have already been proved to be targets of immunity (Hirohashi et al., 2002; Kohler et al., 2010; Ochi et al., 2009a; Park et al., 2011). However, there has been no report showing which antigen is potent for cancer immunotherapy. In this study, we compared the potencies of G2/M-related antigens. BALB/c mice were immunized with plasmids coding *Birc5*, *Aurka*, *Nek2*, and *Plk1* twice weekly. One week after the last immunization, 1×10^6 CT26 mouse colon cancer cells were injected into the back of BALB/c mice (Fig. 1A). *Birc5*- and *Aurka*-plasmid immunized mice showed significant anti-tumor effects compared with the effects in Mock plasmid-immunized mice and PBS-immunized mice, whereas *Nek2*- and *Plk1*-plasmid immunized mice did not show any anti-tumor effect (Fig. 1B).

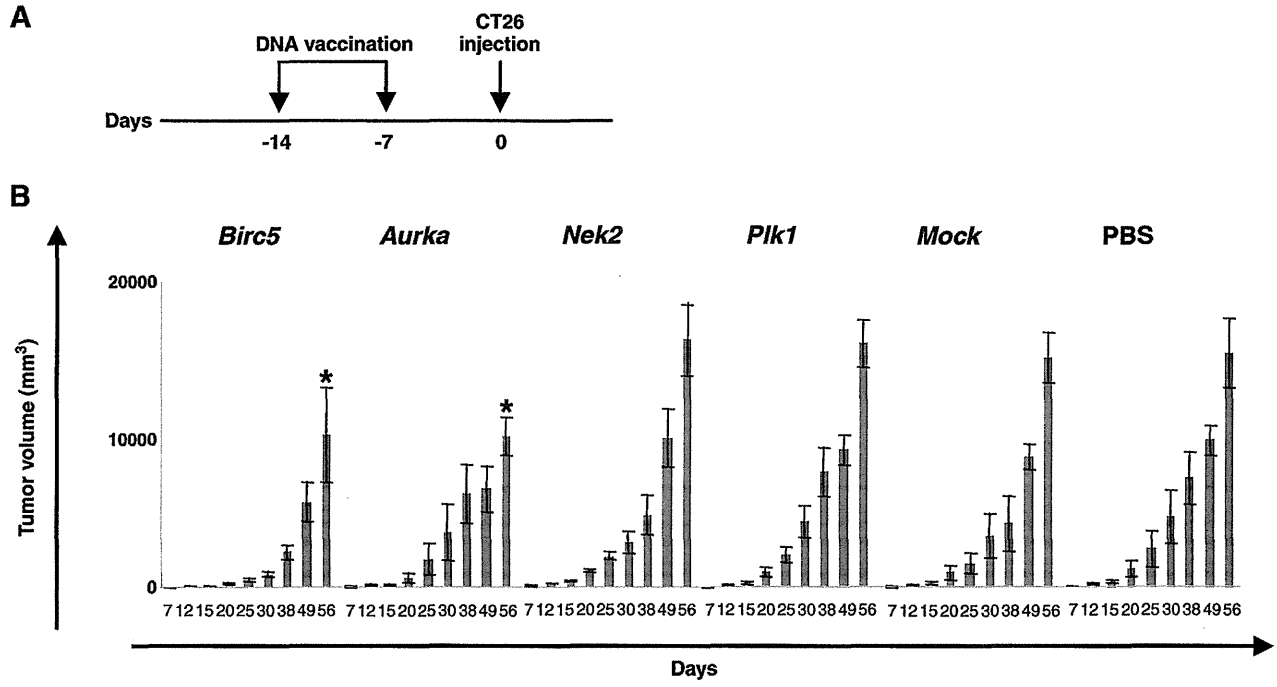

Fig. 1. DNA vaccination of G2/M-related antigens. A. Time schedule of DNA vaccination. BALB/c mice were immunized with G2/M-related antigens coding plasmids 2 times weekly by injecting 100 μg of plasmid into foodpads. One week after the last immunization, 1×10^6 of CT26 cells were injected into the back of mice subcutaneously. B. Tumor growth of DNA-vaccinated mice. Time courses of tumor growth in mice immunized with *Birc5*, *Aurka*, *Nek2*, *Plk1* and Mock plasmids and with PBS are shown. Data are means ± SD. N = 5. Asterisk represents statistically significant difference compared with Mock-plasmid immunized mice.

Expression of G2/M-related antigens in CSCs/CICs

The anti-tumor efficiency of TAAs depends on several factors, and recently one study in which 75 TAAs were evaluated by several parameter, showed that expression in cancer stem cells is one of the parameters for evaluation of TAAs (Cheever et al., 2009). Thus, we evaluated the expression of G2/M-related antigens in both CSCs/CICs and non-CSCs/CICs. CSCs/CICs were isolated from CT26 cells as SP cells, and the ratio of SP cells was 0.387% (Fig. 2A). Since some SP cells do not represent CSCs/CICs (Burkert et al., 2008), we confirmed the tumorigenicity of SP cells and that of MP cells. SP cells could initiate a tumor with 10^2 cells, whereas MP cells needed 10^4 cells to initiate a tumor (Fig. 2B). The volume of a tumor derived from SP cells was greater than that of a tumor derived from MP cells (Fig. 2B). Therefore, SP cells derived from CT26 cells were enriched with CSCs/CICs.

The expression of G2/M-related antigens in SP cells and MP cells was evaluated by RT-PCR (Fig. 2C). *Birc5* and *Aurka* are expressed in SP cells and MP cells at the same levels, whereas *Nek2* and *Plk1* are expressed preferentially in MP cells.

Enhancement of anti-tumor effect of Birc5 DNA vaccine by Hsp90

An immune potentiator is needed to enhance cancer-specific immune reactions. We previously reported that HSP90 protein associated with an antigenic peptide and enhanced antigenic peptide-specific immune reaction through cross-presentation (Kurotaki et al., 2007). Thus, we hypothesized that *Hsp90* gene fusion to an antigen gene would enhance the anti-tumor effect. We constructed two plasmids containing the *Birc5* gene fused to the N-terminal and C-terminal regions of the *Hsp90* gene (Fig. 3A). Immunization with the *Hsp90 N–Birc5* plasmid showed a significantly grater anti-tumor effect than did *Birc5* and *Hsp90* plasmids (Fig. 3B). Interestingly, *Hsp90 C–Birc5* plasmid did not show any anti-tumor effect.

Discussion

In this study, we compared the anti-tumor effects of G2/M-related antigens, including *Birc5*, *Aurka*, *Nek2* and *Plk1*, by DNA vaccination. All of these genes are expressed in only the G2/M phase in normal cells; however, they are ectopically overexpressed in cancer cells (Altieri, 2003; Li and Li, 2006). They have an essential role in cancer cell growth and are therefore attractive targets of cancer immunotherapy, because functional essential antigens are barely downregulated under pressure of antigen-specific immunity (Hirohashi et al., 2009). Therefore, we investigated the difference in immunigenicity of G2/M-related antigens and found that only *Birc5* and *Aurka* show an anti-tumor effect in a DNA vaccination model. *Birc5* and *Aurka* are expressed in both CSCs/CICs and non-CSCs/CICs at similar levels; however, *Nek2* and *Plk1* are expressed preferentially in non-CSCs/CICs. In a previous study, it was shown that a strong anti-tumor effect can be obtained by targeting CSCs/CICs specifically in a rat glioma model (Xu et al., 2009). Therefore, these observations indicate that antigen

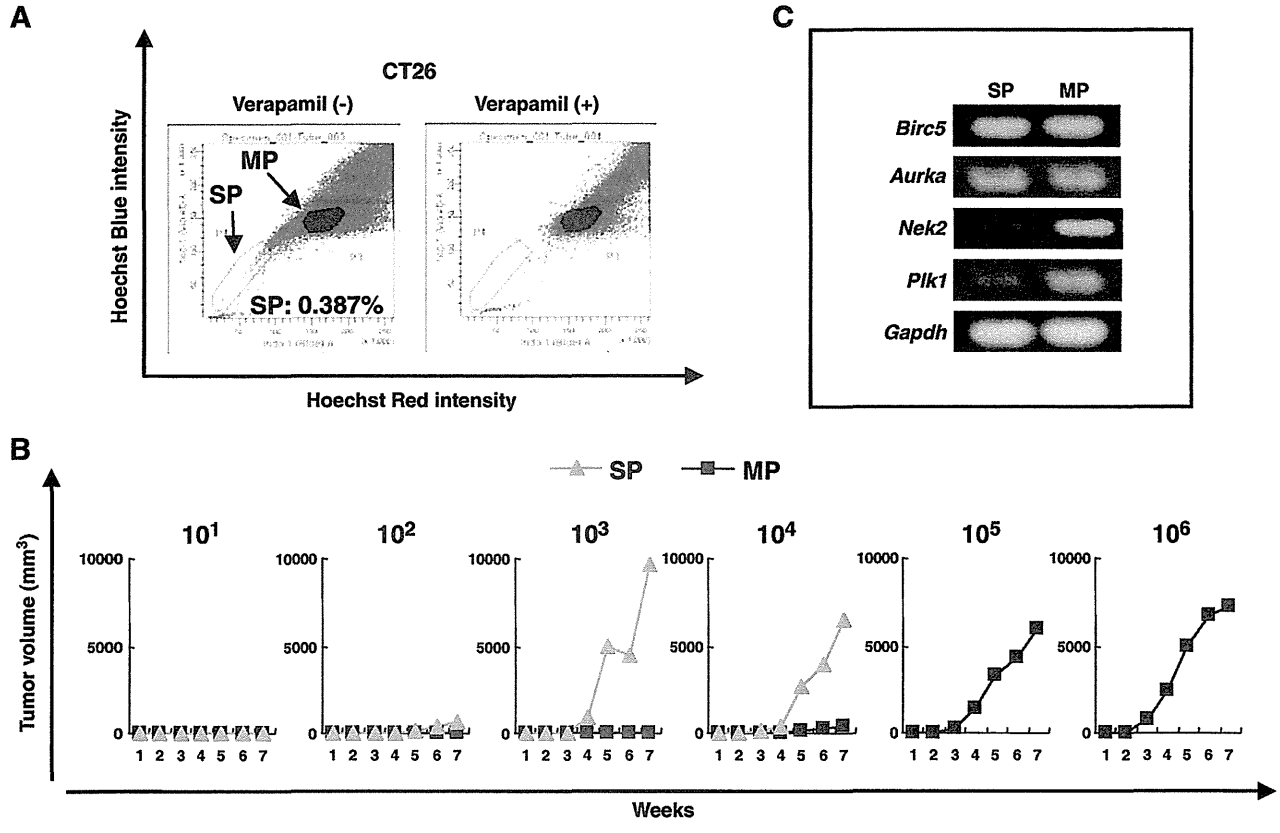

Fig. 2. Isolation of CSCs/CICs from CT26 cells and expression of G2/M-related antigens in CSCs/CICs. A. Isolation of SP cells from CT26 mouse colon cancer cells. CT26 cells were stained with Hoechst 33342 dye as described in Materials and methods with or without Verapamil. Stained cells were analyzed using a FACS Aria II cell sorter. Ratio of SP cells was 0.387%. B. Tumor growth of SP cells and MP cells derived from CT26 cells. Serially diluted SP cells and MP cells (10^1–10^4 SP cells and 10^1–10^6 MP cells) were injected into BALB/c mice and tumor growth was observed. Data are means. N = 2. C. Expression of G2/M-related antigens in SP cells and MP cells. Expression of G2/M-related antigens was evaluated by RT-PCR.

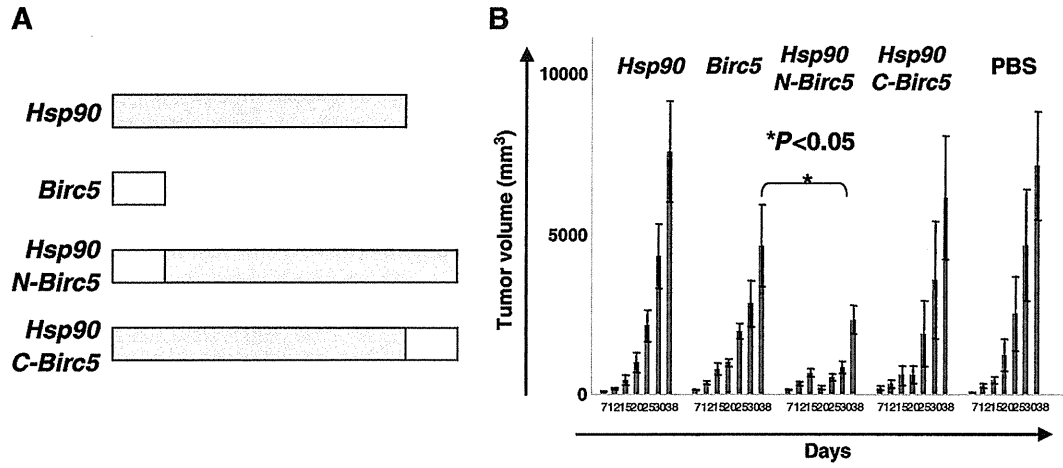

Fig. 3. Augmentation of anti-tumor effect by fusing to Hsp90. A. Schema of constructs. *Hsp90 N–Birc5* plasmid was constructed by fusing *Birc5* to the N-terminal region of *Hsp90*, and *Hsp90 C–Birc5* plasmid was constructed by fusing *Birc5* to the C-terminal region of *Hsp90*. B. Tumor growth of DNA-vaccinated mice. Time course of tumor growth in mice immunized with *Hsp90*, *Birc5*, *Hsp90 N–Birc5* and *Hsp90 C–Birc5* plasmids and PBS. Data are means ± SD. N = 5. Asterisk represents statistically significant difference between *Birc5* plasmid-immunized mice and *Hsp90 N–Birc5* plasmid-immunized mice.

expression in the CSC/CIC population is a significant factor for achieving an anti-tumor effect.

We isolated mouse CSCs/CICs as SP cells. CSC/CIC populations have been reported to be enriched in SP cells in several types of cancers (Kondo et al., 2004; Murase et al., 2009; Nakatsugawa et al., 2011). On the other hand, it has been shown that some SP cells are not to be enriched with CSCs/CICs (Burkert et al., 2008). Therefore, SP cells need confirmation by further analysis to be used as a source of CSCs/CICs. We confirmed the high tumor-initiating ability of SP cells derived from CT 26 cells for the first time, and thus these SP cells can be a suitable material for CSC/CIC study.

Antigen fusion to the N-terminal region of *Hsp90* enhanced the anti-tumor effect; whereas fusion to the C-terminal region abrogated its anti-tumor effect. There is an ATP-binding domain in the N-terminal region of HSP90, which is responsible for association with client proteins, and there is a dimerization domain in the C-terminal region of HSP90, which is responsible for formation of an HSP90 homodimer (Whitesell and Lindquist, 2005). Both domains are essential for HSP90 functions. We fused *Birc5* covalently to *Hsp90*, and thus the N-terminal ATP-binding domain may not be needed in this model. However, *Birc5* fusion to the C-terminal region might abrogate the dimerization domain and formation of an HSP90–BIRC5 protein homodimer and thus the anti-tumor effect.

In summary, we showed that the potency of G2/M-related antigens as targets of cancer immunotherapy depends on their expression in CSCs/CICs and that anti-tumor effects can be enhanced by fusion of an antigen to the N-terminus of *Hsp90*.

Declaration of financial disclosure

The authors have no financial conflict of interest.

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Depletion of Tregs *in vivo*: a promising approach to enhance antitumor immunity without autoimmunity

Evaluation of: Rech AJ, Mick R, Martin S *et al.* CD25 Blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patents. *Sci. Transl. Med.* 4(134), 134ra62 (2012). Tregs are involved in the maintenance of immunological self-tolerance. Recent studies have revealed that Tregs suppress anti-tumor immunity and that they are major obstacle for cancer immunotherapy. Various approaches have been carried out to cancel immunological suppression by Tregs in clinical settings; however, side effects such as autoimmunity occurred and expected anti-tumor effects were not achieved. In a recent study, Rech *et al.* evaluated daclizumab, a US FDA-approved humanized anti-CD25 antibody, for regulation of Treg cells in a peptide vaccination trial of breast cancer patients. Daclizumab caused long-lasting depletion of CD25$^+$ Tregs, reprogramming of CD25$^+$ Tregs, and enhancement of antipeptide immune response. Of note, major autoimmune responses were not observed in daclizumab-treated patients. This study provides a possible safe and promising approach to regulate Tregs in cancer vaccine therapy.

KEYWORDS: anti-CD25 antibody daclizumab immunotherapy Treg

Rena Morita, Yoshihiko Hirohashi & Yoshihiro Oka

Regulatory T cells (Tregs) are suppressive lymphocytes capable of preventing runaway immune responses. Although Tregs control autoimmunity, they have been shown to inhibit anti-tumor immune responses one of the obstacles for cancer immunotherapy [1–4]. Tregs have been reported to increase in number in peripheral blood or regional lymph nodes of tumor-bearing patients [5–7], and it has been shown that higher Treg accumulation in tumor tissues are correlated with poor prognosis [8,9]. These findings indicate that Tregs are a major obstacle to achieving effective anti-tumor immunity. In fact, it has been shown that depletion or inhibition of Tregs enhances anti-tumor immunity in a mouse model [10,11]. These reports suggest that Tregs promote the immune escape of cancers. Various approaches to deplete Tregs have been carried out in clinical settings (e.g., by administration of anti-CTLA-4 antibodies, denileukin diftitox and low-dose cyclophosphamide); however, in many previous studies, side effects such as autoimmunity occurred and the expected anti-tumor effects were not achieved [12–14].

It has been reported that CD4$^+$ Tregs are characterized by cell surface expression of the high-affinity IL-2 receptor α-chain, CD25, and that Tregs can be efficiently depleted by antibodies targeting CD25 [4]. Daclizumab is one of the human anti-CD25 monoclonal antibodies and is a US FDA-approved agent for immunosuppression in organ transplantation. Daclizumab blocks IL-2 binding to CD25, which is also a critical signal for the maintenance of Tregs. However, it might attenuate the effect of immunotherapy because of injury to other CD25-expressing effector T cells.

Rech *et al.* evaluated the hypothesis that long-term CD25 blockage accomplished by daclizumab would have a negative impact human Tregs but spare or enhance effector T-cell function in a trial of peptide vaccine therapy for breast cancer, and they verified that CD25 blockage by daclizumab depleted and selectively reprogrammed Tregs in concert with active immune therapy [15]. This study indicated that inhibition of Tregs by daclizumab can improve peptide vaccine therapy for cancer.

Summary of methods & results

The authors started their study with evaluation of CD4$^+$CD25^{high}FoxP3$^+$CD45RA$^+$ Tregs (CD45RA$^+$ Tregs) and CD4$^+$CD25^{high}FoxP3^{+-} CD45RA^{neg} Tregs (CD45RA^{neg} Tregs), two different subpopulations of Tregs. Daclizumab did not show any antibody-dependent or complement-mediated cytotoxicity for either CD45RA$^+$ Tregs or CD45RA^{neg} Tregs *in vitro*. Daclizumab suppressed the expression of FOXP3 in CD45RA^{neg} Tregs but not in CD45RA$^+$ Tregs. Surprisingly, daclizumab-treated CD45RA^{neg} Tregs lost suppressive function and regained the

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ability to produce IFN-γ, indicating reprogramming of CD45RA^{neg} Tregs. On the other hand, CD45RA$^+$ Tregs were unaffected by daclizumab. These results indicate that CD25 blockade by daclizumab results in selective suppression and reprogramming of CD45RA^{neg} Tregs.

The successful suppression of CD45RA^{neg} Tregs *in vitro* encouraged the authors to perform a clinical trial. They treated ten metastatic breast cancer patients with daclizumab and cancer vaccines using HLA-A2$^+$-restricted antigenic peptides, to examine the impact of daclizumab on Tregs *in vivo*. The patients received a single intravenous infusion of daclizumab (1 mg/kg) 1 week before receiving the cancer peptide vaccine until toxicity or disease progression. The peptide vaccine included five peptides, three peptides from human telomerase reverse transcriptase (hTERT), one peptide from survivin and a control peptide from pp65 of *Cytomegalovirus* (100 μg/ml per injection), that were emulsified in the adjuvant Montanide ISA 51 VG (SEPPIC, Paris, France). Patients also received the seven-valent CRM197-containing pneumococcal conjugate vaccine (0.5 ml per injection) at the time of the first, third and fifth vaccine injections. Daclizumab administration led to a marked and prolonged decrease in CD4$^+$CD25$^+$FoxP3$^+$ Tregs in patients *in vivo*. This decrease occurred within 1 week and lasted at least 7 weeks in all patients with mean reductions ranging from 56 to 77% at weeks 1, 2, 5 and 7. Recovery of Tregs was observed between 7 and 11 weeks after daclizumab administration. They observed that CD4$^+$CD25$^-$ T cells, CD8$^+$CD25$^-$ T cells and CD56^{dull}CD16^{bright}CD25^{dull} NK cells were not significantly affected by daclizumab.

Finally, they evaluated the impact of daclizumab on peptide vaccine-specific immune responses. In all patients, robust peptide-specific CD8$^+$ T cells against at least one tumor vaccine epitope were detected after daclizumab and vaccine administration, which was confirmed by peptide/MHC tetramer reactivity. *Cytomegalovirus* peptide-specific CD8$^+$ T cells and CRM197 antigen-specific CD8$^+$ and CD4$^+$ T cells were also induced after daclizumab and vaccine administration. These responses were observed after daclizumab-mediated Treg depletion.

Of note, dose-limiting toxicities and autoimmune diseases were not observed despite of the suppression of Tregs *in vivo*. Progression-free survival was 4.8 months (95% CI: 3.0–6.5 months) and median overall survival was 27.8 months (95% CI: 19.5–36.1 months) in a median follow-up period of 22.3 months. Moreover, 65.5 ± 17.3% of the patients were alive at the 24 months. Compared with their past clinical trial without daclizumab, median overall survival in this study was improved (previous study without daclizumab: 20.9 months; 95% CI: 12.2–29.6 months) and immune responses to the hTERT epitope were better.

They conclude that CD25 blockade depletes and selectively reprograms Tregs in concert with active immune therapy in cancer patients. Their results suggest a mechanism to target cancer-associated Tregs while avoiding autoimmunity.

Future perspective

Since the purpose of this study was to determine the effects of daclizumab on Treg, the number of patients was limited. However, they concluded that there is very efficient cytotoxic T lymphocyte (CTL) induction of CTLs by administration of daclizumab. There have been previous studies on daclizumab administration with dendritic cell (DC) vaccination [14]. However, DC vaccination showed less efficiency than those of peptide vaccination. Mature DCs express CD25 on their surface, and CD25 on DCs has a role in induction of polyclonal T-cell activation. Daclizumab suppresses activation of antigen-specific T cells by inhibition of CD25 on DCs [16]. Thus, daclizumab with a peptide vaccine might be a more effective approach than daclizumab with DC vaccination. Further large-scale clinical studies are necessary.

The authors found that CD25 blockage *in vitro* resulted in loss of FOXP3 expression and loss of CD45RA^{neg} Treg suppressive function, which might mean reprogramming of CD45RA^{neg} Tregs. Since both CD45RA^{neg} Tregs and CD45RA^{neg} Tregs express CD25, it is not clear why daclizumab shows an effect on CD45RA^{neg} Tregs but not on CD45RA$^+$ Tregs. The difference in dependency on IL-2 signals may be one reason. CD45RA$^+$ Tregs express high levels of CTLA4, and the anti-CTLA4 antibody (ipilimumab) may target CD45RA$^+$ Tregs specifically. Ipilimumab has been approved by the FDA for treatment of melanoma patients, and it has shown a significant anti-tumor effect; however, it also caused auto-immune reactions in patients [17]. CD45RA$^+$ Tregs have a higher expression level of FOXP3 and greater immune suppressive potency than CD45RA^{neg} Tregs [18], and suppression of CD45RA$^+$ Tregs may be related to autoimmunity. On the other hand, suppression of CD45RA^{neg} Tregs may

not be sufficient to break immunotolerance but sufficient to augment CTL response. Further study is needed to determine the clinical benefits of daclizumab in comparison with those of ipilimumab.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Executive summary

- Tregs have a role in controlling autoimmunity, and they are related to suppression of anti-tumor immunity.
- There are two subsets of Tregs ($CD45RA^{neg}$ Tregs and $CD45RA^{+}$ Tregs), and anti-CD25 antibody (daclizumab) decreased and reprogrammed $CD45RA^{neg}$ Tregs.
- Inhibition of $CD45RA^{neg}$ Tregs by daclizumab resulted in enhancement of antipeptide vaccine immune response.

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Gene Expression Profiles of Prostate Cancer Stem Cells Isolated by Aldehyde Dehydrogenase Activity Assay

Sachiyo Nishida, Yoshihiko Hirohashi,* Toshihiko Torigoe, Hiroshi Kitamura, Akari Takahashi, Naoya Masumori, Taiji Tsukamoto and Noriyuki Sato

From the Departments of Pathology (YH, TT, AT, NS) and Urology (HK, NM, TT), Sapporo Medical University School of Medicine (SN), Sapporo, Japan

Abbreviations and Acronyms

ALDH = aldehyde dehydrogenase
CIC = cancer initiating cell
CSC = cancer stem-like cell
DEAB = diethylaminobenzaldehyde
DMEM = Dulbecco's modified Eagle's medium
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
PCR = polymerase chain reaction
RT-PCR = reverse transcriptase-PCR

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* Correspondence: Department of Pathology, Sapporo Medical University, South-1 West-17, Chuo-Ku, Sapporo 060-8556, Japan (telephone: +81-11-611-2111, extension 2692; FAX: +81-11-643-2310; e-mail: hirohash@sapmed.ac.jp).

Purpose: Prostate cancer cells include a small population of cancer stem-like/cancer initiating cells, which have roles in cancer initiation and progression. Recently aldehyde dehydrogenase activity was used to isolate stem cells of various cancer and normal cells. We evaluated the aldehyde dehydrogenase activity of the human prostate cancer cell line 22Rv1 (ATCC®) with the ALDEFLUOR® assay and determined its potency as prostate cancer stem-like/cancer initiating cells.

Materials and Methods: The human prostate cancer cell line 22Rv1 was labeled with ALDEFLUOR reagent and analyzed by flow cytometry. $ALDH1^{high}$ and $ALDH1^{low}$ cells were isolated and tumorigenicity was evaluated by xenograft transplantation into NOD/SCID mice. Tumor sphere forming ability was evaluated by culturing in a floating condition. Invasion capability was evaluated by the Matrigel™ invasion assay. Gene expression profiling was assessed by microarrays and reverse transcriptase-polymerase chain reaction.

Results: $ALDH1^{high}$ cells were detected in 6.8% of 22Rv1 cells, which showed significantly higher tumorigenicity than $ALDH1^{low}$ cells in NOD/SCID mice ($p < 0.05$). Gene expression profiling revealed higher expression of the stem cell related genes *PROM1* and *NKX3-1* in $ALDH1^{high}$ cells than in $ALDH1^{low}$ cells. $ALDH1^{high}$ cells also showed higher invasive capability and sphere forming capability than $ALDH1^{low}$ cells.

Conclusions: Results indicate that cancer stem-like/cancer initiating cells are enriched in the $ALDH1^{high}$ population of the prostate cancer cell line 22Rv1. This approach may provide a breakthrough to further clarify prostate cancer stem-like/cancer initiating cells. To our knowledge this is the first report of cancer stem-like/cancer initiating cells of 22Rv1 using the aldehyde dehydrogenase activity assay.

Key Words: prostate, carcinoma, neoplastic stem cells, aldehyde dehydrogenase, gene expression

PROSTATE cancer is one of the most common lethal malignancies in males. There are some curative treatments for early stage prostate cancer but effective treatment for advanced metastatic prostate cancer has not yet been established. The origin of prostate cancer remains unclear but the recent identification of prostate CSCs has led to speculation that this minor fraction of tumor cells initiates the disease.

Tumors comprise heterogeneous populations of cells. According to cancer stem cell theory a small, distinct cell

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population has higher tumor initiation, self-renewal and differentiation ability.[1,2] Prostate cancer cells are also thought to include a small population of CSCs/CICs, which contribute to cancer initiation and progression. CSCs/CICs are known to be resistant to therapy and are thought to be a major cause of tumor recurrence, metastasis and resistance to castration. Thus, elucidating the genetic properties of CSCs/CICs is essential to establish effective cancer treatment.[3]

Although some cell surface marker combinations, such as $CD44^+/\alpha2\beta1^{high}/CD133^+$, have been used to detect prostate CSCs/CICs,[4] there are some controversial reports.[5,6] It was also suggested that selection by single cell surface markers such as CD133 would be unlikely to identify all putative stem or progenitor cell types.[7,8]

ALDH enzymes have roles in the epithelial development and homeostasis. Deregulation of this class of enzymes is implicated in multiple cancers. Also, ALDH enzymes are related to drug resistance, and cell proliferation, differentiation and response to oxidative stress. ALDH activity has been used to isolate stem-like cell subsets in hematopoietic cells,[9–11] endothelial progenitor cells, and mesenchymal and epithelial stem cells.[12,13] It is becoming increasingly clear that ALDH activity can be used alone or in combination with cell surface markers to identify CSCs/CICs in hematopoietic malignancies and a steadily increasing number of solid carcinomas, including those of the pancreas, lung, colon and breast.[14–17]

We found that a subpopulation of human prostate cancer cell lines with high ALDH activity correlates with enhanced tumorigenicity in vivo. ALDH activity might be a putative marker for prostate CSCs/CICs.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human prostate cancer cell lines 22Rv1, PC3, LNCaP and DU145 (ATCC) were cultured in DMEM (Life Technologies™) supplemented with 10% fetal calf serum. Cells were incubated in a 37C incubator with humidified air and 5% CO_2. Culture medium was changed twice per week.

ALDEFLUOR Assay and Fluorescence Activated Cell Sorting Isolation

Cell ALDH activity was measured using the ALDEFLUOR assay kit according to the manufacturer protocol. Cells were suspended in ALDEFLUOR assay buffer containing the ALDH1 substrate boron-dipyrromethene-aminoacetaldehyde (1 μmol/l/1 $\times$ 10^6 cells) and incubated for 40 minutes at 37C. As a negative control, aliquots of each sample were treated with 50 mmol/l of the specific ALDH1 inhibitor DEAB. Stained cells were analyzed using a FACSAria™ II cell sorter. Sorting gates were established using propidium iodide stained cells for viability.

RNA Preparation and RT-PCR

Total RNA isolation and RT-PCR were performed as described previously.[18] The primer pairs used for RT-PCR analysis were 5′-TTGTGGCAAATCACCAGGTA-3′ and 5′-TCAGATCTGTGAACGCCTTG-3′ for *PROM1(CD133)* with an expected PCR product size of 162 bp, and 5′-GTCCTTCCTCATCCAGGACA-3′, 5′-AAGACCCCAAGTGCCTTTCT-3′ for *NKX3.1* with an expected PCR product size of 224 bp and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for *GAPDH* with an expected product size of 452 bp.

Xenograft Transplantation in NOD/SCID Mice

Isolated $ALDH1^{high}$ and $ALDH1^{low}$ cells were resuspended at concentrations of 1 $\times$ 10^2 to 1 $\times$ 10^4 cells in 100 μl phosphate buffered saline and Matrigel mixture (1:1). $ALDH1^{high}$ and $ALDH1^{low}$ cells were injected subcutaneously into the right and left mid back areas of anesthetized 6-week-old male NOD/SCID mice. The progression of cancer cell growth was monitored weekly. Mice underwent autopsy 50 days after cell injection. Tissues were fixed in formaldehyde and examined histologically.

Immunohistochemical staining was done as described previously.[18] To detect ALDH1 protein we used anti-ALDH1 monoclonal antibody (clone 44/ALDH, BD Transduction Laboratories™) at 200 times dilution.

Assays

Matrigel invasion. Cellular potential for invasiveness was determined using Matrigel invasion chambers (BD™ Biosciences Discovery Labware) according to manufacturer instructions. Briefly, 5,000 cells each were seeded in the upper chambers in serum-free DMEM. The outer chambers were filled with the same medium but with fetal bovine serum as a chemoattractant. Cells were incubated for 48 hours. Invasive cells were stained with hematoxylin, mounted on slides and counted by light microscopy. Each experiment was repeated 3 times.

Sphere formation. A total of 3,000 sorted $ALDH1^{high}$ and $ALDH1^{low}$ cells each were incubated in DMEM/F12 medium (Invitrogen™) supplemented with 20 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (Sigma-Aldrich®) in each well of an Ultra-Low Attachment Surface culture 6-well plate (Corning®). Cell morphology was assayed and pictures were obtained daily under light microscopy.

Gene Expression Profiling

RNA from $ALDH1^{high}$ cells was labeled with Cy5 dye and RNA from $ALDH1^{low}$ cells were labeled with Cy3 dye. The probe mixture was hybridized for 40 hours at 65C to a G4412A Human Genome CGH Microarray Kit 105A (Agilent Technologies, Santa Clara, California). The array was scanned after washing with a G2565BA Microarray Scanner and fluorescent signals were acquired using Feature Extraction software (Agilent Technologies). The average expression ratio of Cy5 to Cy3 was determined per gene.

A dye swap experiment was also done to label $ALDH1^{high}$ and $ALDH1^{low}$ cells with Cy3 and Cy5, respectively. An average ratio of more than 2.0, reproducible in 2 experiments, was determined to indicate differential up-regulation in $ALDH1^{high}$ cells.

Statistical Analysis

Data are shown as the mean ± SD of at least 3 independent experiments. Statistical analysis of data was done using the Student t test with $p \leq 0.05$ considered statistically significant.

RESULTS

ALDH1^{high} Cells

Identification in prostate cancer cell lines. The enzyme ALDH is a useful marker for isolating cancer stem cell populations.[15] We investigated whether ALDH1 activity would be applicable to isolate prostate carcinoma stem cells. ALDH1^{high} cells were detected in all prostate carcinoma cell lines, including 22Rv1, PC3, LNCaP and DU145, at a proportion of 6.8%, 1.2%, 1.3% and 1.5%, respectively (fig. 1, *A*). The proportion of ALDH1^{high} cells was greatest in 22Rv1 cells. To our knowledge the characteristics of ALDH1^{high} cells in 22Rv1 cells have not been reported previously. Thus, we further analyzed ALDH1^{high} cells derived from 22Rv1 cells.

Tumor initiating ability in vivo. To determine whether ALDH1^{high} cells derived from 22Rv1 cells were enriched with CSCs/CICs we performed xenograft transplantation of ALDH1^{high} and ALDH1^{low} cells in NOD/SCID mice. A total of 1,000 ALDH1^{high} and 1,000 ALDH1^{low} cells had initiated tumors by day 49. Mean tumor size was 3,955 ± 3,374 and 335 ± 663 mm^3, respectively (fig. 1, *B* and *C*). Tumors derived from ALDH1^{high} cells were significantly larger than those derived from ALDH1^{low} cells ($p < 0.05$). There was no significant difference in the number of xenografts derived from ALDH1^{high} and ALDH1^{low} cells. For 1×10^2, 1×10^3 and 1×10^4 ALDH1^{high} cells 0, 3 and 3 tumors were initiated per 4 injections while for 1×10^2, 1×10^3 and 1×10^4 ALDH1^{low} cells 0, 2 and 4, respectively, were initiated. A significant difference was found in tumors derived from 10,000 cells. No tumor initiation was observed in mice injected with 100 ALDH1^{high} and 100 ALDH1^{low} cells.

ALDH1 Immunohistochemical Staining

Tumors derived from ALDH1^{high} and ALDH1^{low} cells showed poorly differentiated adenocarcinoma. There was no notable histological difference between the tumors (fig. 1, *D*). We performed immunohistochemical staining using anti-ALDH1 anti-

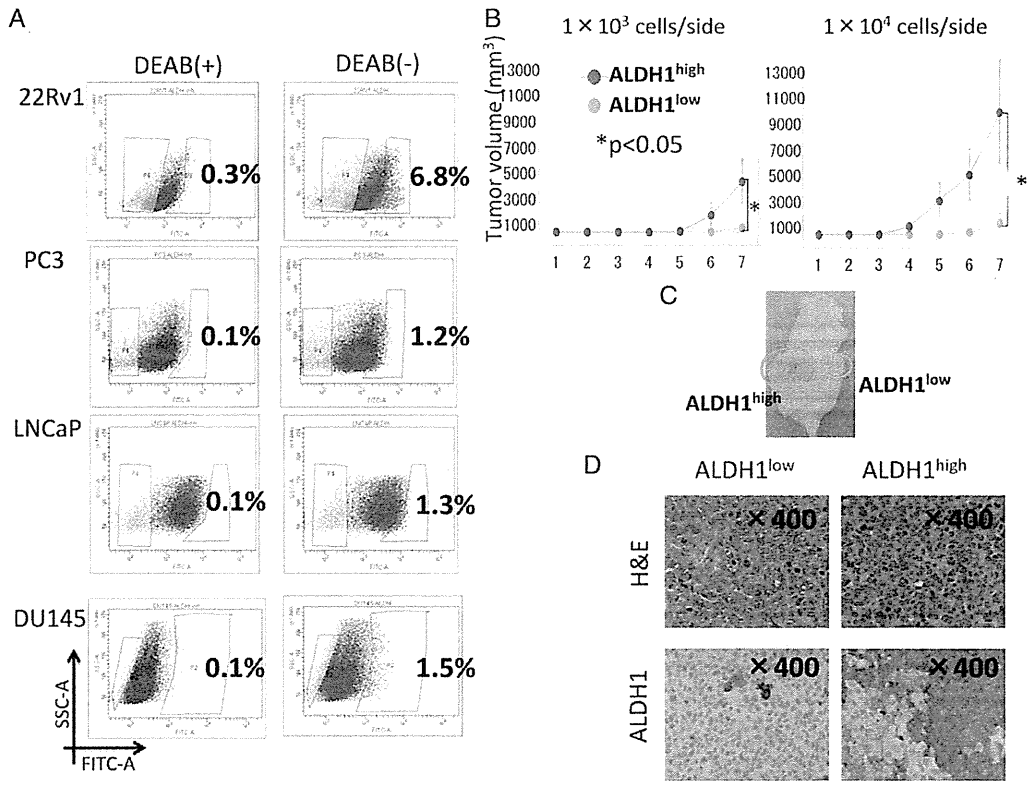

Figure 1. *A*, ALDEFLUOR assay of prostate carcinoma cell lines. ALDH1^{high} cells were detected in 22Rv1, PC3, LNCaP and DU145 prostate carcinoma cell lines. *SSC-A*, single strand conformation analysis. *FITC-A*, fluorescein isothiocyanate analysis. Percents indicate ALDH positivity. *B* and *C*, tumor initiating ability of ALDH1^{high} and ALDH1^{low} cells derived from 22Rv1 cells. *B*, tumor growth curves of cells injected in NOD/SCID mice. *C*, representative view of mouse tumors. *D*, histology of tumors derived from ALDH1^{high} and ALDH1^{low} cells.

body to determine ALDH1 protein expression in tumors derived from ALDH1^{high} and ALDH1^{low} cells. Positivity to anti-ALDH1 antibody was extremely high in tumors derived from ALDH1^{high} cells compared to that in tumors derived from ALDH1^{low} cells (fig. 1, *D*).

ALDH1^{high} Cell Gene Expression Profile

RT-PCR was done to evaluate the mRNA expression of *PROM1* and *NKX3-1*, which are considered prostate cancer stem cell markers.[19,20] *Nkx3.1* encodes a homeodomain transcription factor that represents the earliest specific marker for the prostate epithelium in organogenesis and is also frequently downregulated during prostate cancer initiation.[21] Only luminal CARNs, which are castration resistant Nkx3.1 expressing cells that represent a rare luminal epithelial population with stem cell properties during prostate regeneration, express Nkx3.1 in regressed epithelium.[20] Gene expression profiling revealed higher *PROM1* and *NKX3-1* expression levels in ALDH1^{high} cells (fig. 2, *A*). RT-PCR was done for the reprogramming factors *POU5F1, SOX2, KLF4* and *NANOG* but no significant difference was found in mRNA expression.

Assays

Matrigel invasion. Prostate cancer has a propensity for lymph node and bone metastasis. We performed an invasion assay to determine the metastatic potential of ALDH1^{high} and ALDH1^{low} cells. There was a significant difference in the mean number of invading cells per membrane between ALDH1^{high} and ALDH1^{low} cells (fig. 2, *B*). The invasive capability of ALDH1^{high} cells was more than threefold higher than that of ALDH1^{low} cells ($p < 0.05$).

Prostasphere formation. Nonadherent sphere forming assays are increasingly used to evaluate the stem cell phenotype in normal tissue and in putative CSCs/CICs. Photosphere forming ability was evaluated using 3,000 ALDH1^{high} and ALDH1^{low} cells each incubated in Ultra-Low Attachment Surface culture dishes containing growth factors (epidermal growth factor and basic fibroblast growth factor). On day 3 ALDH1^{high} cells showed spherical colony formation but no sphere formation was observed in ALDH1^{low} cell cultures (fig. 2, *C*).

Identifying ALDH1^{high} Cell Related Genes

To address the gene expression profiles of ALDH1^{high} cells we performed microarray screening using

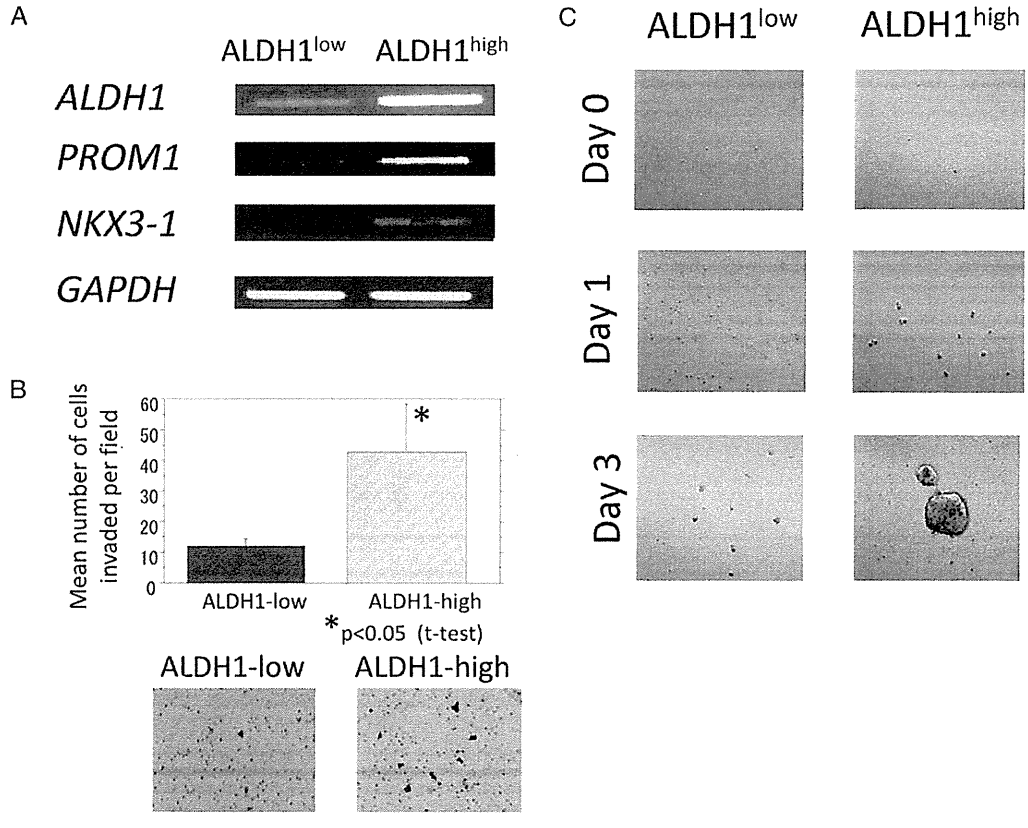

Figure 2. ALDH1^{high} and ALDH1^{low} cells. *A*, RT-PCR of stem cell markers. *B*, invasion assay. *C*, prostasphere formation.

ALDH1^{high} and ALDH1^{low} cells. More than 200 genes were up-regulated in ALDH1^{high} cells compared with the expression levels of those genes in ALDH1^{low} cells. Gene up-regulation was confirmed by RT-PCR (fig. 3).

DISCUSSION

In this study 1) it was possible to obtain an ALDH1^{high} cell population from the prostate cancer cell line 22Rv1, 2) the ALDH1^{high} cell population showed higher tumorigenic capability than the ALDH1^{low} cell population, 3) ALDH1^{high} cells could form spherical colonies, 4) ALDH1^{high} cells were more invasive than ALDH1^{low} cells and 5) factors related to prostate cancer stem cells were up-regulated in the ALDH1^{high} cell population.

Pioneering studies of prostate cancer led to the identification of CD44+/α2β1high/CD133+ prostate cancer stem cells.[4] Prostate CSCs/CICs from human prostate cancer cell lines were isolated using similar combinations of cell surface markers. For example, CD44+/α2β1high/CD133+ cells were isolated from the DU145 cell line[22] and CD44+CD24− cells were isolated from the LNCaP cell line.[23]

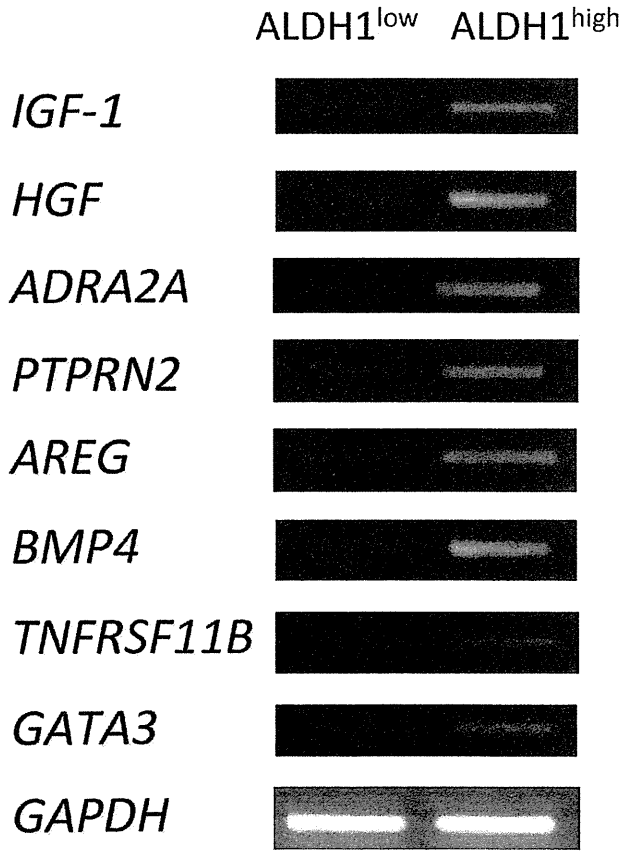

Figure 3. RT-PCR of microarray positive genes

Despite significant progress in prostate CSC/CIC research there is a limitation about the specificity of these markers. A major concern is that CD44 and CD133 are CSC/CIC markers in various tissues.[19,24,25] Thus, these markers are not specific for prostate CSCs/CICs. Another recent study showed that mouse CD133 is widely expressed in luminal cells while human CD133 is less broadly expressed in benign tissue but up-regulated in inflammation regions in tumors, suggesting no association with CSCs/CICs.[7] Wang et al reported that CARNs represent a rare luminal epithelial cell population with stem cell properties and, thus, *NKX3-1* may be considered a key marker of prostate regeneration.[20] However, *NKX3-1* is not a cell surface marker and not necessarily a specific marker of prostate CSCs/CICs. Therefore, to our knowledge reliable surface markers of prostate CSCs/CICs have not been established. It is important to find a new marker or technique to identify prostate CSCs/CICs.

Deregulation of ALDH enzyme activity is implicated in the pathophysiology of various hematological and epithelial cancers.[12,13] Thus, as detected by the ALDEFLUOR assay, high ALDH activity can be used as a functional marker to isolate CSCs/CICs in several types of epithelial cancers, including those of the breast, lung and colon.[14,15,17] ALDH1^{high} cells derived from LNCaP and PC3 prostate cancer cell lines seem to have CSC/CIC characteristics.[26]

To our knowledge we report for the first time that ALDH1^{high} cells could also be isolated from 22Rv1 prostate cancer cells by the ALDEFLUOR assay and this cell population has stem cell characteristics. However, we did not further analyze the other cell lines since it was difficult to obtain sufficient ALDH1^{high} cells from the PC3, LNCaP and DU145 cell lines due to the small proportion of ALDH1^{high} cells.

It was recently reported that ALDH activity and CD44 indicated increased tumorigenic and metastatic potentials in the PC3 and LNCaP prostate cancer cell lines.[27] In our experiment CD44 was not used to detect CSCs/CICs. Further investigations using this cell surface marker might provide more information.

Our study revealed the properties of prostate CSCs/CICs and clarified prostate CSC/CIC gene expression. Gene expression profiling using cDNA microarrays revealed many interesting genes. For example, some genes were metabolic or detoxifying enzymes, suggesting that ALDH1^{high} cells might have properties to counteract anticancer drugs, as do CSCs/CICs in other organs. We also detected the 2 growth factors *HGF* and *IGF1*. Hepatocyte growth factor is secreted by mesenchymal cells and acts on epithelium as a multifunctional cyto-

kine. Due to its ability to stimulate mitogenesis, cell motility and matrix invasion hepatocyte growth factor has a central role in angiogenesis, tumorigenesis and tissue regeneration. Insulin-like growth factor I is a member of a family of proteins involved in mediating growth and development. Thus, these factors may have important roles in prostate CSCs/CICs. Further investigation is needed.

CONCLUSIONS

In in vitro and in vivo experimental systems $ALDH1^{high}$ cells derived from prostate carcinoma cells by the ALDEFLUOR assay were suitable as a prostate CSC/CIC model. Further molecular and biological analysis of ALDH1 and a longitudinal clinical study in a large population are needed to validate its prognostic value and develop a novel strategy to improve the effectiveness of prostate cancer treatment.

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Tumor and Stem Cell Biology

Cancer Research

HSP DNAJB8 Controls Tumor-Initiating Ability in Renal Cancer Stem–like Cells

Satoshi Nishizawa[1,3], Yoshihiko Hirohashi[1], Toshihiko Torigoe[1], Akari Takahashi[1], Yasuaki Tamura[1], Takashi Mori[1,3], Takayuki Kanaseki[1], Kenjiro Kamiguchi[1], Hiroko Asanuma[1,2], Rena Morita[1], Alice Sokolovskaya[1], Junichi Matsuzaki[1], Ren Yamada[1], Reona Fujii[3], Harm H. Kampinga[5], Toru Kondo[4], Tadashi Hasegawa[2], Isao Hara[3], and Noriyuki Sato[1]

Abstract

Cancer stem–like cells (CSC) are a small population of cancer cells with superior tumor initiating, self-renewal, and differentiation properties. In this study, we show that the cancer-testis antigen and HSP40 family member DNAJB8 contributes to the CSC phenotype in renal cell carcinoma (RCC). DNAJB8 overexpression increased the percentage of side population (SP) cells representing CSCs in RCC cells, enhancing their tumor-initiating ability. Conversely, attenuation of DNAJB8 decreased SP cells and reduced tumor-initiating ability. The utility of DNAJB8 as an immunologic target was established in DNA vaccination experiments. Compared with immunization with the tumor-associated antigen survivin, which was expressed in both CSCs and non-CSCs in RCC, immunization with Dnajb8 expression plasmids yielded stronger antitumor effects. Together, our findings suggest that DNAJB8 plays a role in CSC maintenance and that it offers a candidate for CSC-targeting immunotherapy in RCC. *Cancer Res; 72(11); 2844–54. ©2012 AACR.*

Introduction

Renal cell carcinoma (RCC) is one of the most resistant forms of cancers to both radiotherapy and chemotherapy. In recent years, molecular targeted therapies have been developed and have shown significant objective responses (1–3), and they have been incorporated into current standard therapies of metastatic RCC; however, these molecular targeted therapies have not provided durable responses. RCC is regarded as an immunogenic malignancy and has well-documented responses to some cytokines such as interleukin-2 (IL-2) and IFN-α, and some patients have shown significant responses to treatments with these cytokines (4–6). However, the results have been limited by such nonspecific immunotherapy; therefore, cancer-specific immunotherapy may become a new modality for patients with metastatic RCC. Tumor-associated antigens (TAA) that can be recognized by CTLs have been investigated (7, 8), and some of these antigens induced objective tumor regression in patients with malignant melanoma (9, 10). In advanced cases, however, complete elimination of tumor cells by limited numbers of effector CTLs is difficult because a solid tumor contains an enormous number of tumor cells (about 5×10^8 cells per gram; ref. 11).

Cancer cells in solid carcinomas display heterogeneity in many aspects of their phenotypes, and only a small population of cells, called cancer stem–like cells/cancer-initiating cells (CSC/CIC), express stem cell phenotype and have high tumor-initiating ability (cancer stem cell hypothesis; refs. 12–14). CSCs/CICs are resistant to chemotherapy and radiotherapy by various mechanisms, and these characteristics of CSCs/CICs are thought to be related to posttherapeutic recurrence (15). Thus, therapy targeting the small population of CSCs/CICs might be a reasonable approach for treatment of resistant and advanced cancers. Some immunologic effector cells, including natural killer (NK) cells and γδT cells, have been reported to be able to efficiently recognize the CSC/CIC population (14). Furthermore, we have reported that CTLs can efficiently recognize human colon CSCs/CICs (16). We have categorized TAAs that can be recognized by CTLs into 3 groups: (i) CSC antigens, which are expressed in CSCs/CICs but not in non-CSCs/CICs (e.g., MAGE-A3 and MAGE-A4); (ii) shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs (e.g., CEP55, SURVIVIN); and (iii) non-CSC antigens, which are expressed in non-CSCs/CICs but not in CSCs/CICs (e.g., AMACR, HIFPH3; ref. 17).

In this study, we found that a HSP40 family protein, DnaJ (Hsp40) homolog, subfamily B, member 8 (DNAJB8), is expressed preferentially in the CSC/CIC population cancer cells, including RCC and the testis among normal tissues, indicating

Authors' Affiliations: [1]Department of Pathology, [2]Division of Surgical Pathology, Sapporo Medical University School of Medicine, Sapporo; [3]Department of Urology, Wakayama Medical University, Wakayama; [4]Department of Stem Cell Biology, Ehime University Proteo-Medicine Research Center, Ehime, Japan; and [5]Department of Radiation and Stress Cell Biology, University Medical Center Groningen, Groningen, The Netherlands

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Authors: Yoshihiko Hirohashi, Department of Pathology, Sapporo Medical University, School of Medicine, South-1 West-17, Chuo-Ku, Sapporo 060-8556, Japan. Phone: 81-11-611-2111 (ext. 2692); Fax: 81-11-643-2310; E-mail: hirohash@sapmed.ac.jp; and Toshihiko Torigoe, E-mail: torigoe@sapmed.ac.jp

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