early endosomes, recycling endosomes and proteasomes, but not in lysosomes or ER (Fig. 6B). To confirm whether exogenous Hsp90 also co-localized with cytosolic proteasome, DCs were incubated with Alexa 594-labeled Hsp90 complexed with OVA. We observed that the exogenous Hsp90 was detected in early endosomes and proteasome subunit LMP-2, but not in lysosomes and recycling endosomes (Fig. 7). These data demonstrated that the part of exogenous Hsp90–OVA complex translocated from early endosomes into the cytosol while it has been unclear whether the Hsp90 and OVA remain in the complex or not, followed by association with proteasomes. Moreover, unlike in the data from localization of Hsp90–Alexa 594-labeled OVA, Alexa 594-labeled Hsp90 did not co-localize with recycling

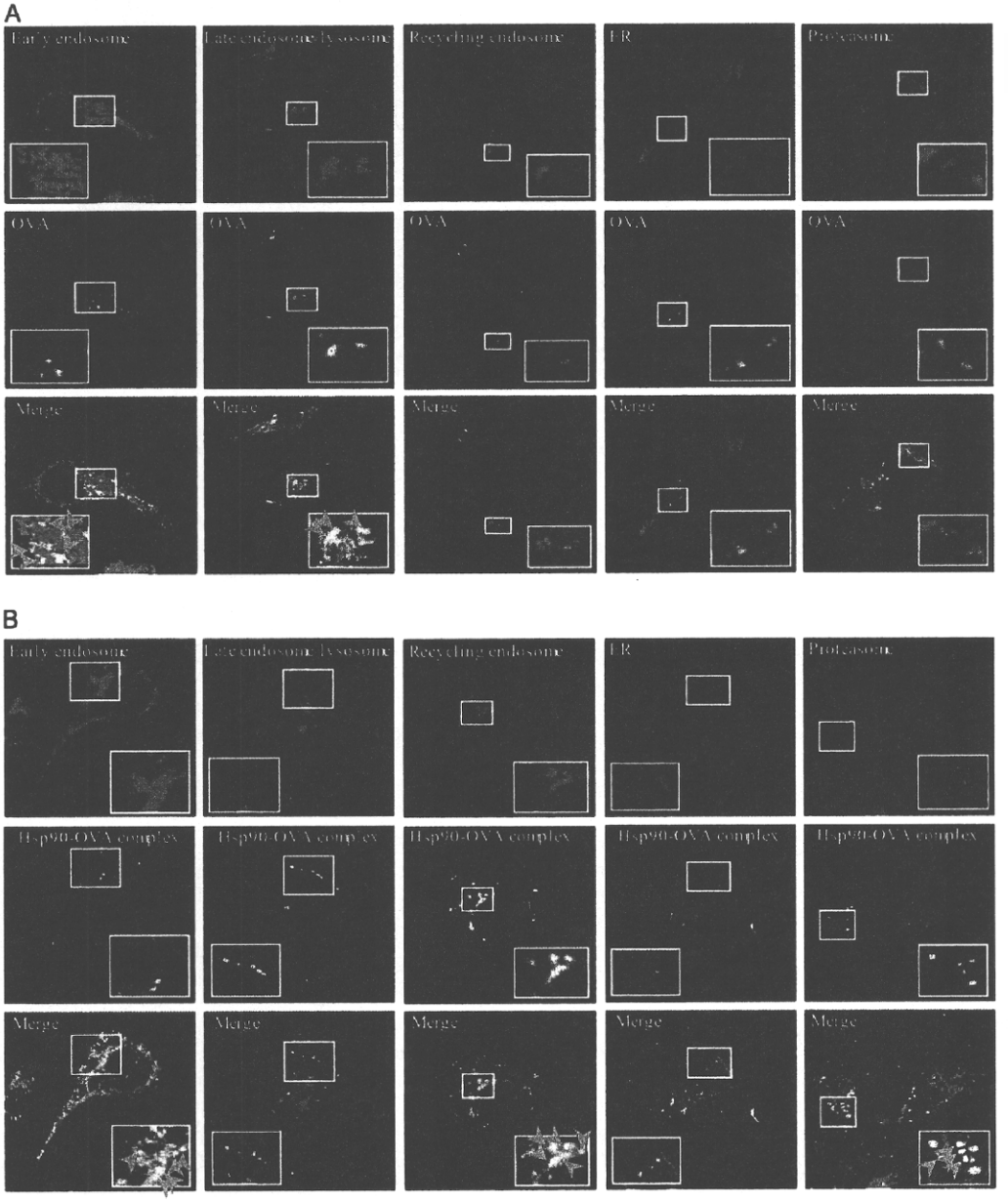

Fig. 6. Exogenous OVA and Hsp90–OVA complex show different subcellular localization after uptake by DCs. Immunocytological localization of OVA and Hsp90–OVA complex. (A, B) DCs were incubated at 37°C with Alexa Fluor 594-labeled OVA (A), Hsp90–Alexa 594-labeled OVA (B) for 1 h, and then washed and fixed. Organelles were stained with an anti-Rab5 pAb for early endosomes, anti-LAMP-1 pAb for late endosomes/lysosomes, anti-Rab11 pAb for recycling endosomes, anti-KDEL mAb for ER and anti-LMP2 pAb for proteasomes, conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy (original magnification ×630). Co-localization of internalized OVA or Hsp90–OVA and each organelle is indicated with arrows. Data are representative of three independent experiments.

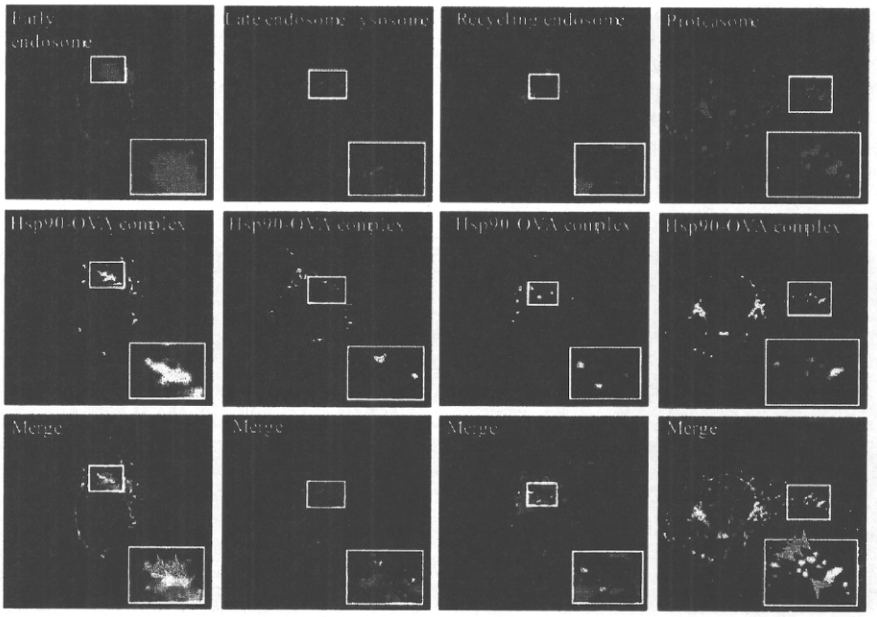

Fig. 7. Exogenous Hsp90 co-localized with cytosolic proteasome. DCs were incubated with Alexa Fluor 594-labeled Hsp90 complexed with OVA for 1 h. Organelles were stained with an anti-Rab5 pAb, anti-LAMP-1 pAb, anti-Rab11 pAb and anti-LMP2 pAb, conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy (original magnification ×630). Co-localization of internalized OVA or Hsp90–OVA and each organelle is indicated with arrows. Data are representative of three independent experiments.

endosomes, suggesting that a part of OVA chaperoned by Hsp90 was released from Hsp90 within early endosomes, followed by translocation to recycling endosomes where recycling MHC class I molecules existed.

Inhibition of endogenous Hsp90 does not affect the cross-presentation of exogenous Hsp90–OVA complex

Recently, it has been demonstrated that endogenous cytosolic Hsp90 plays an important role in antigen presentation of exogenous as well as endogenous antigens. To investigate whether endogenous Hsp90 was responsible for the cross-presentation of the exogenous Hsp90–OVA complex, we treated DCs with the Hsp90-specific inhibitor radicicol in a cross-presentation assay. The results showed that treatment of DCs with radicicol did not affect the cross-presentation of exogenous Hsp90–OVA, suggesting that endogenous Hsp90 might not responsible for the exogenous Hsp90-mediated cross-presentation (Fig. 8A). Moreover, radicicol treatment did not affect the co-localization of the internalized Hsp90-chaperoned OVA and LMP2 (Fig. 8B). In contrast, high concentration of soluble OVA (200 mg ml^{-1}) was cross-presented by DCs, although it was considerably less efficiently than Hsp90–OVA complex. Interestingly, this cross-presentation was radicicol sensitive, indicating that endogenous Hsp90 played a very important role in the cross-presentation of exogenous OVA (Fig. 8C). However, as it is not clear how endogenous Hsp90 participates in the cross-presentation, we are currently studying the possibility that endocytosed soluble OVA might be translocated by endogenous Hsp90 into the cytosol for proteasomal degradation. It has been demonstrated that treatment with radicicol induces stress responses such as induction of Hsp72 protein. Therefore, we examined whether the concentration of radicicol used in our experiments was effective to induce Hsp72 expression. When DCs were exposed to the radicicol at concentrations of 1.0 μM and 5.0 μM for 2 h, the expression of Hsp72 was clearly induced compared with control cells (Fig. 8D), indicating that concentration of radicicol used in the cross-presentation assay was sufficient enough for the inhibition of Hsp90 function of DCs. Thus, collectively, exogenous Hsp90, but not endogenous Hsp90, might be required for cross-presentation of exogenous antigens chaperoned by Hsp90.

Exogenous Hsp90 contributes to the translocation of chaperoned OVA to proteasomes

We hypothesized that exogenous Hsp90 itself was responsible for the efficient translocation of chaperoned OVA to the site for proteasomal degradation. To test this idea, we introduced an anti-Hsp90 antibody into the cytosol of DCs to inhibit the activities of both endogenous Hsp90 and exogenous Hsp90. We utilized the BioPORTER protein delivery reagent to introduce the anti-Hsp90 mAb into DCs. The BioPORTER/antibody complexes are endocytosed by the cells and then fuse with the endosome releasing the BioPORTER-captured antibody into the cytoplasm. To examine the localization of the transduced antibody, FITC-antibody control protein was mixed with the BioPORTER reagent and added to DCs. After incubation for 4 h, FITC-antibody control protein was mixed with the BioPORTER reagent and added to DCs. After incubation for 4 h, the cells were washed and fixed. Organelles were stained with an anti-Rab5 pAb for early endosomes conjugated with Alexa 594 and visualized with confocal laser microscopy (Fig. 9A). The results showed that the transduced antibody localized in cytosol but not

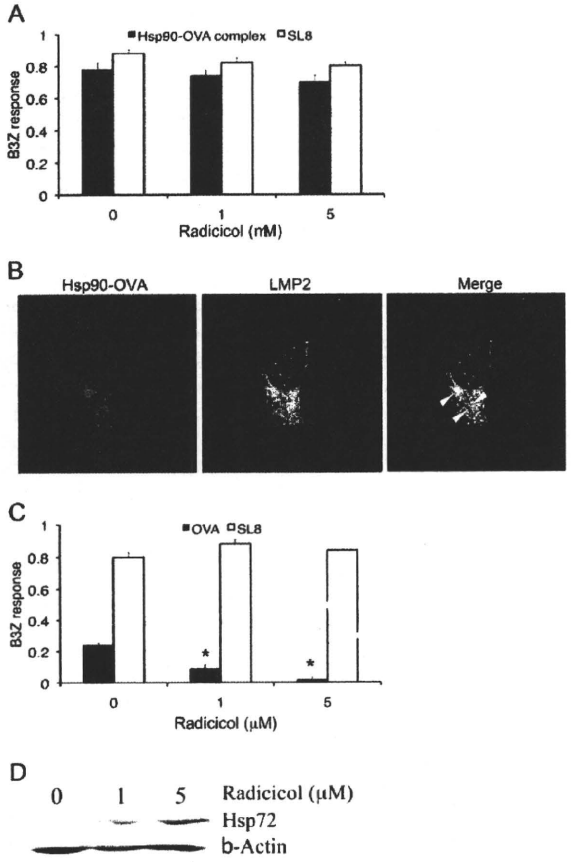

Fig. 8. Inhibition of endogenous Hsp90 does not affect the cross-presentation of exogenous Hsp90–OVA complex. (A) DCs were pre-incubated with radicicol at the indicated doses. Two hours after pre-incubation, the DCs were pulsed with Hsp90 (200 μg ml^{-1})–OVA (200 μg ml^{-1}) or SL8 (1 μM) for 1 h, and fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. (B) DCs were pre-incubated with radicicol at 5 μM. Two hours after pre-incubation, DCs were incubated at 37°C with Hsp90–Alexa Fluor 594-labeled–OVA complex for 1 h, and then washed and fixed. Organelles were stained with an anti-LMP2 pAb conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy. Co-localization of internalized Hsp90–OVA and LMP2 is indicated with arrows. (C) DCs were pre-incubated with radicicol at the indicated doses. Two hours after pre-incubation, the DCs were pulsed with OVA (200 mg ml^{-1}) or SL8 (1 μM) for 1 h, fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. (D) Following treatment of DCs with the indicated concentrations of radicicol for 2 h and culture for 24 h, the cell lysate was harvested. Western blotting of the expression of Hsp72 was done using a specific antibody. The ß-actin level was used as a loading control. Data are shown as means ± SD of three independent experiments. *, $p < 0.01$.

endosomes, suggesting that the transduced anti-Hsp90 antibody could be expected to bind to both exogenous and endogenous Hsp90 in the cytosol. In fact, the transduced Alexa 594-labeled anti-Hsp90 mAb was co-localized with Alexa 488-labeled exogenous Hsp90 within DCs (data not shown). We next examined whether transduction of the anti-Hsp90 mAb affected the intracellular localization of the exogenous Hsp90–OVA complex in DCs by confocal laser microscopy. We observed that when the anti-Hsp90 mAb was transduced into the cytosol of DCs, co-localization of Hsp90–Alexa 594–OVA complex and Alexa 488-labeled proteasome subunit LMP2 was poorly detected. Although it should be elucidated how the transduced antibody affected the Hsp90 activity, these data suggested that transduction of the anti-Hsp90 antibody resulted in marked inhibition of transportation of Hsp90-chaperoned OVA to the proteasome (Fig. 9B). In agreement with these data, transduction of the anti-Hsp90 antibody into DCs, but not the control antibody, resulted in partial inhibition of Hsp90-mediated cross-presentation (Fig. 9C). In contrast, B3Z responses to DCs pulsed with the SL8 peptide with or without antibody transduction were not affected. Since Hsp90-mediated cross-presentation was involved in both the cytosolic and endosomal pathways, this partial inhibition seemed to reflect the inhibition of a cytosolic–proteasome–TAP-dependent pathway, whereas the endosomal pathway might not have been affected. Therefore, we tested whether the transduction of the anti-Hsp90 mAb plus leupeptin treatment of DCs abolished the cross-presentation of the Hsp90–OVA complex. The results showed that these treatments of DCs almost completely inhibited the cross-presentation of Hsp90–OVA (Fig. 9C). Taking into account the co-localization of exogenous Hsp90, OVA and proteasome subunit LMP-2 shown by the confocal laser study and the results from radicicol treatment, it was suggested that exogenous Hsp90 itself might act as a translocator of chaperoned OVA from early endoosme to cytosol for proteasomal degradation.

Hsp90–OVA complex is cross-presented by DCs in vivo

We examined whether our system worked *in vivo*. We immunized B6C3F1 mice with the Hsp90–OVA complex or OVA alone and evaluated the appearance of $CD11c^+$ DCs that could stimulate B3Z and KZO in the draining lymph nodes. Figure 10A and B show that $CD11c^+$ DCs from mice immunized with Hsp90–OVA elicited strong B3Z responses, whereas they did not cause KZO responses. In contrast, $CD11c^+$ DCs from mice immunized with OVA alone activated KZO, whereas stimulation of B3Z by them led to very weak responses compared with mice immunized with the Hsp90–OVA complex. These results were in agreement with previous findings shown in Fig. 4 that Hsp90–OVA was presented selectively and efficiently through the MHC class I pathway *in vitro*.

Hsp90–OVA complex elicits OVA-specific CTLs in vivo and protects mice from challenge with tumor cells expressing the OVA protein

We tested whether mice immunized with Hsp90–OVA developed OVA-specific CTL responses. C57BL/6 mice were immunized s.c. on days 0 and 7 with the Hsp90 (20 μg)–OVA (20 μg) complex in the absence of an adjuvant. As shown in Fig. 10C, immunization with Hsp90–OVA induced a strong and OVA-specific CTL response. Then, to study the efficacy of Hsp90–OVA to protect mice against grafted E.G7 tumor cells, mice ($n = 10$) were injected s.c. on days 0 and 7 with Hsp90 (20 μg)–OVA (20 μg), Hsp90 (20 μg), OVA (20 μg) or PBS, and 7 days after the second immunization, they were grafted i.d. with 10^5 E.G7 cells. All mice immunized with

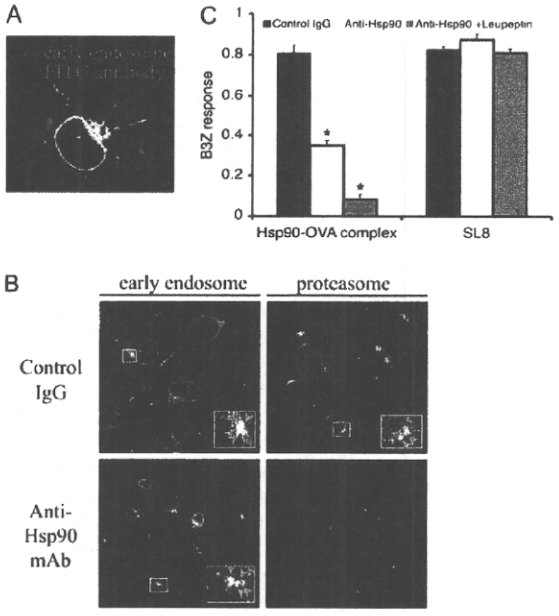

Fig. 9. Exogenous Hsp90 contributes to the translocation of chaperoned antigen from endosomes into cytosol. (A–C) One microgram of a FITC-antibody control protein, anti-Hsp90 mAb or rat IgG_{2a} control protein was mixed with the BioPORTER protein delivery reagent and added to each dish of DCs. After incubation for 4 h, the medium was removed and the cells were washed with RPMI twice to remove any antibodies that did not enter the cells. (A) Localization of transduced FITC-antibody control protein was examined. After incubation for 4 h, organelles were stained with an anti-Rab5 pAb for early endosomes conjugated with Alexa Fluor 594 and visualized with confocal laser microscopy (original magnification ×630). The BioPORTER/antibody complex localized in the cytosol instead of endosomes. (B) After the transduction of an anti-Hsp90 mAb or rat IgG_{2a} control protein, the cells were incubated with Hsp90–Alexa Fluor 594-labeled–OVA for 1 h, and then washed and fixed. Organelles were stained with an anti-Rab5 pAb for early endosomes and anti-LMP2 pAb for proteasomes, conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy (original magnification ×630). Co-localization of internalized Hsp90–OVA and each organelle is indicated with arrows. Data are representative of three independent experiments. (C) After the transduction of an anti-Hsp90 mAb or rat IgG_{2a} control protein, the cells were incubated with leupeptin (200 μM) for 2 h, and then pulsed with Hsp90–OVA or SL8 for another 2 h, fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. Data are shown as means ± SD of three independent experiments. *, $P < 0.01$.

Hsp90, OVA or PBS developed tumors, whereas 60% of mice immunized with Hsp90–OVA remained tumor free (Fig. 10D). These results showed that the Hsp90-antigen peptide complex induced a strong CTL response to the chaperoned peptide and that this response was sufficiently strong to generate anti-tumor effects.

Discussion

Our results have revealed a novel mode of involvement of Hsp90 in antigen presentation by DCs: exogenous Hsp90 preferentially introduces the chaperoned antigen into the MHC class I pathway, resulting in efficient cross-presentation. Because there is a classical paradigm that extracellular antigens are presented by MHC class II molecules, it seemed significant to find that Hsp90 changed the fate of the associated antigen on antigen presentation. Recent reports demonstrated that Hsp70– and gp96–antigen complex facilitated antigen presentation in association with both MHC class I and class II molecules (13–15). Here, we examined whether an Hsp90–protein complex elicited $CD4^+$ T-cell responses through MHC class II presentation as well as $CD8^+$ T-cell responses and we found that the Hsp90–OVA complex induced preferential $CD8^+$ T-cell responses. Therefore, we further examined how Hsp90 directs the associated OVA to the cross-presentation pathway compared with the intracellular routing of OVA alone using laser confocal microscopy. We found that the Hsp90–OVA complex entered the MHC class I pathway, which is comprised of an endosomal pathway and proteasome–TAP-dependent pathway. In contrast, the soluble OVA was sorted into an endosome–lysosome pathway, which is regarded as a classical MHC class II pathway. Thus, the Hsp90–OVA complex and OVA followed different routes and it is very important to elucidate the role of Hsp90 in cross-presentation for the development of HSP-based cancer vaccines. We assumed that exogenous Hsp90 was taken up by receptor-dependent endocytosis. In fact, the Calderwood group has recently reported that the scavenger receptor expressed by endothelial cells-1 (SREC-1) acts as the Hsp90 receptor for cross-presentation expressed on DCs (16). Lakadamyali *et al.*(17) have demonstrated that early endosomes are comprised of two distinct populations: a dynamic population that matures rapidly toward the late endosome and lysosome, and a static early endosome that matures much more slowly. Interestingly, Burgdorf *et al.* (18) have demonstrated that a mannose receptor introduces exogenous OVA specifically into an $EEA\text{-}1^+$, $Rab5^+$-static early endosomal compartment for subsequent cross-presentation. In contrast, OVA endocytosed by a scavenger receptor did not co-localize with EEA1; instead, it co-localized with LAMP-1 in the lysosome as shown here, leading to presentation in the context of MHC class II molecules. Thus, we expect that Hsp90-specific receptors such as SREC-1 might introduce the Hsp90–OVA complex into the static early endosome for cross-presentation. Therefore, it should be examined whether the Hsp90–OVA complex is localized to the static early endosome after uptake through Hsp90 receptors, including SREC-1. More importantly, it should be clarified whether such Hsp90 receptors for cross-presentation bear the sorting motifs, which are responsible for introducing the Hsp90–OVA complex into the static early endosome. Identification and characterization of Hsp90 receptors have important implications for further understanding the biology of cross-presentation.

In our previous study, we observed that an exogenous Hsp90–'precursor peptide' complex localized to the early endosome but not the ER (12). Because our published data indicated that the Hsp90–peptide complex was cross-presented by a TAP-independent endosome-recycling pathway and this Hsp90-mediated cross-presentation was lactacystin-insensitive, we did not examine whether the Hsp90–precursor peptide (13mer) complex reached the proteasome. In cross-presentation of the Hsp90–OVA protein complex, we showed that part of the OVA chaperoned by

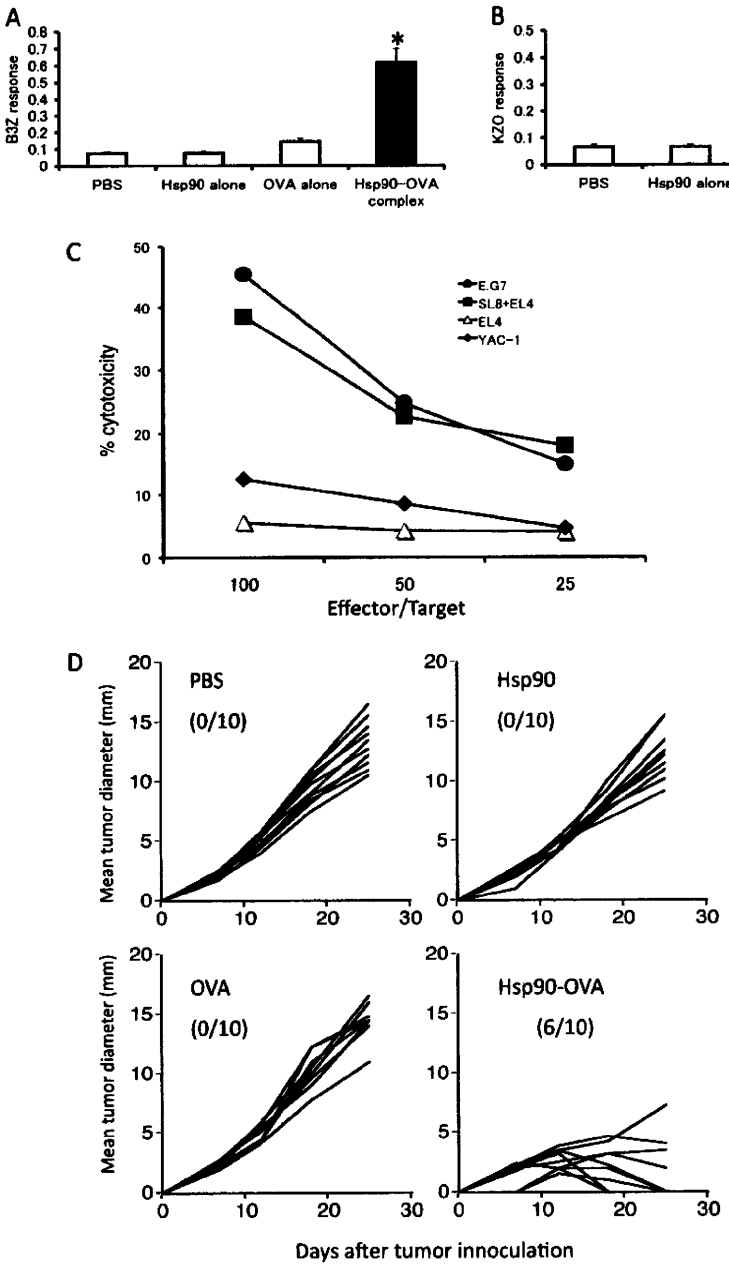

Fig. 10. Immunization with Hsp90–OVA complex elicits OVA-specific CTL responses *in vivo*. (A and B) B6C3F1 mice were immunized in their footpads with Hsp90 alone, OVA alone or a complex of them. Draining popliteal lymph nodes were removed after 4 h, and co-cultured overnight with B3Z (A) or KZO (B). The B3Z or KZO response was measured by the absorbance at 595 nm. Data are shown as means ± SD of three independent experiments. *, $P < 0.01$. (C) C57BL/6 mice were immunized s.c. with Hsp90–OVA, Hsp90 or OVA on days 0 and 7. Seven days after immunization, induction of SL8-specific CTL against E.G7 and EL-4 pulsed with SL8 peptide was measured by a standard 4-h chromium release assay. Cytotoxicity against E.G7 and EL4 loaded with SL8 (SL8 + EL4) showed statistically significant differences compared with EL4 or YAC-1 cells at all effector to target ratios ($P < 0.01$). Data are presented as means of triplicate samples and are representative of three independent experiments. (D) C57BL/6 mice were injected s.c. on days 0 and 7 with Hsp90–OVA, Hsp90, OVA or PBS. Seven days after the second immunization, mice were challenged i.d. with 1×10^5 E.G7 cells. Tumor size was measured in two dimensions twice a week. Then number of mice in which the tumor was rejected is indicated in parentheses. Data are representative of three independent experiments. Mean tumor diameter and survival on day 25 in the group of mice immunized with Hsp90–OVA showed the significant differences as compared with the other groups ($P < 0.001$ versus all other groups).

Hsp90 was transported out to cytosol for proteasomal degradation. These differences may reflect the need for proteasomal processing. More specifically, endosomal protease was sufficient to generate the SL8 epitope (8mer) from the precursor peptide, whereas OVA protein underwent both endosomal and proteasomal processing to generate the epitope. Further examination is required to elucidate the precise mechanism for the decision making on the destination of Hsp90-chaperoned antigens.

In addition, HSP–'peptide' complex-mediated cross-presentation has recently been well demonstrated by many groups, including ours. In contrast, there are only a few reports with regard to HSP–'protein' complex-mediated cross-presentation. Subjeck *et al.* have reported that an Hsp70 family member grp170–tyrosinase protein complex promotes cross-presentation and induces an anti-tumor effect. However, the intracellular route after uptake of the grp170–tyrosinase complex by DCs has not been explored. Thus, the precise mechanism, such as a translocator or a channel responsible for the translocation of the HSP–'antigen protein' complex from endosomes into cytosol for proteasomal degradation remains to be clarified. It has been suggested that, for TAP-dependent cross-presentation, exogenous antigen that translocates across the endosomal membrane to the cytosol is in a relatively unfolded state because membrane pores (~5–8 Å in a diameter) are not large enough for the passage of Hsp90 and OVA (>90 kDa with hydrodynamic radii >30 Å) (19, 20). Therefore, mildly acidic pH in the early endosome (pH 6.3) may be required to partially unfold the Hsp90–OVA complex to translocate it across the endosomal membrane and it may be renatured in the cytosol. Moreover, as the Hsp90–OVA complex is expected to be taken up via receptors such as SREC-1, the acidic condition might be necessary to dissociate the Hsp90–OVA complex from such receptors. In addition, Amigorena *et al.* have demonstrated that high pH (~7) in the phagosomes is required to prevent the excessive degradation of endocytosed antigen by lysosomal proteases for efficient cross-presentation. In line with these observations, we showed that the Hsp90–OVA complex was targeted to the early endosome, whose pH is believed to be relatively high (pH 6.5), leading to translocation of the Hsp90–OVA complex into cytosol without excessive degradation. However, our data using chloroquine suggested that mildly acidic pH (6.5) in the early endosome was required, probably because of the partial unfolding of the Hsp90–OVA complex as well as dissociation from Hsp90 receptors as described above. In our manuscript, we aimed at determining the underlying mechanism for this antigen translocation necessary for cross-presentation. We have observed that (i) the exogenous Hsp90–OVA complex co-localized with proteasome subunit LMP2 and (ii) blocking of interaction of the exogenous Hsp90–OVA complex and proteasomes by the introduction of an anti-Hsp90 antibody using BioPORTER resulted in the inhibition of cross-presentation. Although it was considered that the transduced anti-Hsp90 mAb also bound to endogenous Hsp90 as well as exogenous Hsp90, our data indicated that inhibition of the endogenous Hsp90 by radicicol did not affect the cross-presentation of the exogenous Hsp90–OVA complex. Furthermore, radicicol treatment did not affected the co-localization of the Hsp90–OVA complex and LMP2. Thus, exogenous Hsp90 seems to play an important role in cytoplasmic translocation of the endocytosed Hsp90–antigen complex. It has been shown that cytoplasmic Hsp90 is essential for the translocation of the diphteria toxin catalytic domain (21), *Clostridium botulinum* C2 toxin (22), fibroblast growth factor (FGF)-1 and FGF-2 (23). Thus certain toxins and growth factors depend on Hsp90 for efficient translocation from endosomes to cytosol. Very recently, Ichiyanagi *et al.* (20) have demonstrated that endogenous Hsp90 plays an essential role in cross-presentation of both exogenous OVA and cell-associated OVA by DCs. In agreement with their data, we showed that cross-presentation of soluble OVA was sensitive to the Hsp90 inhibitor radicicol, indicating that the endogenous Hsp90 might act as a translocator to the proteasome–TAP-dependent cross-presentation pathway for the exogenous antigen. These findings also suggest that endogenous Hsp90, as well as exogenous Hsp90, might help the exogenous Hsp90–OVA complex translocate into cytosol at the cytosolic face for cross-presentation. More importantly, it should be revealed how Hsp90–OVA complex escapes across the endosomal membrane. Experimental evidence supports existence of chaperones and co-chaperones such as Hsp90 and Hsc 73 on the endolysosomal membrane participating in lysosomal proteolytic pathways such as chaperone-mediated autophagy (24, 25). Moreover, Hsp90 forms multichaperone complex, which is consisted of Hsc73, p23 and Hop (26). Therefore, it is possible that these cytoplasmic chaperone complex might play an important role in this Hsp90-mediated cross-presentation and that endogenous Hsp90 might help to translocate the exogenous antigen from endosomal compartment to cytosolic proteasome for degradation. More importantly, it has been suggested that Sec61 complex played an important role in the translocation of exogenous antigens to cytosol for proteasomal degradation (27, 28). Therefore, it should be clarified whether the Hsp90–OVA complex is pumped out to cytosol through Sec61 complex. Furthermore, association of Hsp90 with the 20S proteasome has been shown to influence proteasomal enzymatic activity (29). Yamano *et al.* (30) have demonstrated that antigen processing by proteasomes is regulated by Hsp90 and PA28. They showed that up-regulation of Hsp90 and PA28 enhanced the antigen processing of OVA. Given our observation of the association of exogenous Hsp90 and the proteasome, exogenous Hsp90 might regulate proteasomal activity to create antigenic determinants. Together, our data show that TAP-dependent cross-presentation may require exogenous Hsp90 for the delivery of exogenous antigens into the cytosol for proteasomal proteolysis. The Hsp90 appears to represent an attractive candidate for vaccine development due to its ability to target DCs and to induce specific CTL without the need for an adjuvant.

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ORIGINAL PAPER

Autoantibody against hypoxia-inducible factor prolyl hydroxylase-3 is a potential serological marker for renal cell carcinoma

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Abstract
Purpose To verify the efficacy of a serum autoantibody against hypoxia-inducible factor prolyl hydroxylase-3 (PHD3) as a serological marker for RCC.
Methods Serum samples and surgically resected tumor tissue specimens were obtained from 22 patients with primary RCC, 15 of whom underwent radical nephrectomy and 7 partial nephrectomy. Preoperative serum samples were obtained just before tumor resection. Postoperative serum samples were obtained from 17 patients at least 1 month after tumor removal. Serum samples were also obtained from 26 healthy volunteers. Titers of the anti-PHD3 antibody (Ab) were determined by enzyme-linked immunosorbent assay.
Results Serum anti-PHD3 Ab titers were significantly higher in patients with RCC than in healthy volunteers (0.610 ± 0.023 vs. 0.591 ± 0.031, $P = 0.0001$). Using a cutoff point of 0.599, sensitivity, specificity, and positivity for prediction of RCC were 86.4, 57.7, and 63.3%, respectively. In all 17 patients, titers of serum anti-PHD3 were decreased after the surgical resection compared with those before operation (0.622 ± 0.023 vs. 0.580 ± 0.024, $P = 0.0003$).
Conclusions The present study suggests that the anti-PHD3 Ab may be a novel serological marker for RCC and the titer may reflect the tumor burden in each individual.

Keywords Renal cell carcinoma · Tumor marker · Autoantibody · PHD3 protein

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Introduction

In the United States, it was estimated that in 2009 there were 57,760 new cases of kidney cancer and 12,980 cancer-related deaths (Jemal et al. 2009). Renal cell carcinoma (RCC) accounts for around 95% of kidney cancers and is the most lethal of the genitourinary tract malignancies. The development and diffusion of diagnostic imaging technology have contributed to discovery of an increased number of small tumors and decreased death from RCC. However, the cost-effectiveness of screening using ultrasonography or computed tomography is still controversial due to the relatively low incidence of RCC (Campbell et al. 2007). Therefore, discovery of diagnostic biomarkers of RCC in serum or urine samples is much awaited. Biomarkers may also be helpful for differential diagnosis of radiographically equivalent renal tumors such as complicated cysts and angiomyolipoma lacking a fat component. In addition, they may be useful to distinguish metastatic or recurrent disease from nonspecific nodular lesions in follow-up examinations after surgical treatment.

For various other cancers, several autoantibodies against cancer-specific antigens have been investigated as serological tumor markers (Barua et al. 2007; Larsson et al. 2006; Nilsson et al. 2001; Shimada et al. 2000, 2002; Suzuki et al. 2004; Takeda et al. 2001a, b; Tan and Zhang 2008; Zhong et al. 2008). We previously reported that an autoantibody

against a cancer-specific antigen could be detected in sera of patients with RCC (Kitamura et al. 2007). In addition, we found that hypoxia-inducible factor prolyl hydroxylase-3 (PHD3) was frequently overexpressed in cancer tissue of renal cell carcinomas (RCCs) with high specificity to cancer tissue and demonstrated its usefulness as a novel tumor antigen in immunotherapy for RCC (Sato et al. 2008).

PHD3 is one member of the PHD family, which is involved in the degradation of hypoxia-inducible factor (HIF) proteins in cooperation with von-Hippel Lindau protein under normoxic conditions. HIF, a transcriptional factor, induces the expression of more than 60 target genes such as vascular endothelial growth factor (VEGF) and erythropoietin, which play roles in tumor progression and contribute to tumor aggressiveness (Maynard and Ohh 2007) under hypoxic conditions.

In the current study, we verified the availability of an anti-PHD3 antibody (Ab) as a serological marker for RCC.

Materials and methods

After informed consent was obtained from the 22 patients with RCC, serum samples and surgically resected tumor tissue specimens were collected from the 15 patients who underwent radical nephrectomy and 7 who underwent partial nephrectomy. The study was performed after approval of the institutional review board. The median age at operation for the 11 men and 11 women was 63.5 years (range 35–82). Histological examination was done with hematoxylin and eosin-stained slides, and clinical stage was assigned according to the 2002 TNM classification of malignant tumors (Sobin and Wittekind 2002). Preoperative blood samples were collected just before tumor resection, and postoperative ones were obtained from 17 patients 1 month after surgery. We also collected blood samples from 26 healthy volunteers (age range 22–66 years) after receipt of informed consent. After centrifugation, sera were divided into aliquots and stored at −80°C. Tumor tissue specimens were immersed in RNAlater solution (Ambion, Austin, TX) immediately after removal and incubated at 4°C overnight, then stored at −80°C.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed by the following procedure. Total ribonucleic acid (RNA) was isolated from tumor tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA). A complementary deoxyribonucleic acid (cDNA) mixture was synthesized from 2 μg of total RNA by reverse transcription using SuperScript III and oligo (dT) primer (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR amplification was done in 50 μL of PCR mixture containing 1 μL of cDNA mixture, 1 μL of KOD Plus DNA polymerase (Toyobo, Osaka, Japan), and 15 pmol primers. The PCR mixture was initially incubated at 94°C for 2 min followed by 35 cycles of denaturation as 94°C for 15 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. The primer pair 5′-CATCCCTGTCTTGT GTGTGG-3′ (forward) and 5′-CCAACAGCCCTGGATT AAGA-3′ (reverse) was employed for specific detection of PHD3. The expected size of the PCR product was 420 bp. As an internal control, glyceraldehyde-3-phosphate dehydrogenase expression was detected using forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′ with an expected PCR product of 452 bp. The PCR products were visualized with ethidium bromide staining under ultraviolet light following electrophoresis on 1.0% agarose gel.

Recombinant PHD3 protein was produced and purified as described previously (Sato et al. 2008; Yagihashi et al. 2003). For production of the protein, the RT-PCR product amplified from the initial 300 bp of the PHD3 gene was used. The calculated molecular weight was 11.0 kDa. Enzyme-linked immunosorbent assay (ELISA) was performed for detection of the anti-PHD3 Ab in each serum as previously described (Yagihashi et al. 2003). The absorbance was measured at 450 nm. Data were obtained in triplicate for each sample. Western blot analysis of serum anti-PHD3 Ab was also performed to confirm the specificity of immunoreactivity to PHD3 protein. Recombinant PHD3 protein and bovine serum albumin were boiled in Laemmli buffer and resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA), which were then incubated with serum samples diluted 1:10 followed by a 1:2,000 dilution of goat anti-human immunoglobulin G F(ab')2 conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark). The immunocomplex was visualized by enhanced chemiluminescence according to the manufacturer's specifications (Amersham Biosciences, Piscataway, NJ).

We compared the values for the anti-PHD3 Ab between two groups by using the Mann-Whiney U test and the Wilcoxon signed-rank test. $P < 0.05$ was considered to indicate statistical significance. Receiver operating characteristics (ROC) analysis was used to determine the area under the curve (AUC), sensitivity, and specificity for prediction of RCC.

Results

RT-PCR analysis revealed that PHD3 was overexpressed in 21 (95.4%) of the 22 RCC specimens. The results of RT-PCR analysis and ELISA detecting the serum anti-PHD3 Ab, and the relationship to clinicopathologic characteristics are summarized in Table 1. All patients with stage

Table 1 Clinicopathological characteristics, PHD3 mRNA expression and serum anti-PHD3 antibody titers in patients with renal cell carcinoma

No.	Histological type	Clinical stage	PHD3 mRNA expression	Anti-PHD3 antibody	
				Before	After
1	Clear	T1bN0M0	Negative	0.625	N.A.
2	Clear	T1bN0M0	Positive	0.617	0.555
3	Clear	T1aN0M0	Positive	0.598	0.566
4	Clear	T1aN0M0	Positive	0.607	0.589
5	Clear	T2N0M0	Positive	0.611	0.571
6	Clear	T1aN0M0	Positive	0.607	0.602
7	Clear	T1aN0M0	Positive	0.620	0.584
8	Clear	T1bN0M0	Positive	0.638	0.598
9	Spindle	T2N1M1	Positive	0.633	0.574
10	Clear	T1aN0M0	Positive	0.648	0.600
11	Clear	T1aN0M0	Positive	0.660	0.598
12	Clear	T2N0M0	Positive	0.678	0.651
13	Clear	T1aN0M0	Positive	0.609	N.A.
14	Clear	T1aN0M0	Positive	0.600	0.576
15	ACDK	T1aN0M0	Positive	0.595	N.A.
16	Clear	T1aN0M0	Positive	0.599	0.551
17	Chromophobe	T1aN0M0	Positive	0.607	0.565
18	Clear	T1bN0M0	Positive	0.603	0.564
19	Clear	T1aN0M0	Positive	0.579	N.A.
20	Clear	T2N0M0	Positive	0.623	0.560
21	TFE3 fusion	T1aN0M0	Positive	0.630	0.564
22	Clear	T1aN0M0	Positive	0.621	N.A.

Clear clear cell carcinoma, *Spindle* spindle cell carcinoma, *ACDK*, RCC associated with acquired cystic disease of the kidney in a chronic hemodialysis patient, *Chromophobe* chromophobe cell carcinoma, TFE3 fusion, *RCC* associated with Xp11.2/TFE3 gene fusion, *Before* before surgery, *After* after surgery, *N.A.* not available

I–II disease underwent complete surgical resection. On the other hand, one stage IV patient (Case 9) had multiple metastases to the lung and abdominal lymph nodes at the time of radical nephrectomy; therefore, she did not become free from disease after the operation. There was no significant correlation between disease stage and the anti-PHD3 Ab titer.

The median absorbance values of ELISA detecting the anti-PHD3 Ab in sera from patients with RCC and healthy volunteers were 0.614 (0.579–0.678) and 0.597 (0.473–0.621), respectively (Fig. 1a). There was a significant difference between them ($P = 0.0007$). The cutoff value for positivity in the anti-PHD3 ELISA was determined to be 0.599, according to ROC-AUC analysis (Fig. 1b). On the basis of this criterion, the sensitivity, specificity, and positive predictive value for RCC were 86.4, 57.7, and 63.3%, respectively.

Western blot analysis for the serum anti-PHD3 Ab in a patient with RCC demonstrated immunoreactivity to recombinant PHD3 protein. On the other hand, there was no immunoreactivity in serum from a healthy volunteer with a low titer in the anti-PHD3 ELISA (Fig. 1c).

The titers of the anti-PHD3 Ab before and after surgical resection were compared individually in 17 patients with RCC (Fig. 2). The anti-PHD3 Ab titer was decreased after the operation in all of them, with statistical significance ($P = 0.0003$), and 14 (83.4%) showed conversion to a seronegative condition with a cutoff value of 0.599.

Discussion

PHDs are involved in the degradation of hypoxia-inducible factor (HIF) proteins in cooperation with von-Hippel Lindau (VHL) protein. A member of the family, PHD3 is frequently overexpressed in RCCs, but barely in normal tissues (Sato et al. 2008). The putative expression mechanism is stable accumulation of HIF protein due to VHL gene inactivation. In this study, however, PHD3 overexpression at the messenger RNA (mRNA) level was equally observed even in RCC tissues unrelated to mutation or epigenetic modification of the VHL gene such as in chromophobe cell carcinoma and spindle cell carcinoma. Tissue hypoxia may also lead to PHD3 expression, because the catalytic activity of PHD3 is oxygen-dependent, and hypoxia may induce accumulation of unhydroxylated and undegradated HIF proteins (Appelhoff et al. 2004; Cioffi et al. 2003; Hirsilä et al. 2003). One patient (Case 1) had a high titer of the anti-PHD3 Ab despite the lack of expression of PHD3 mRNA in the RCC tissue. RT-PCR for

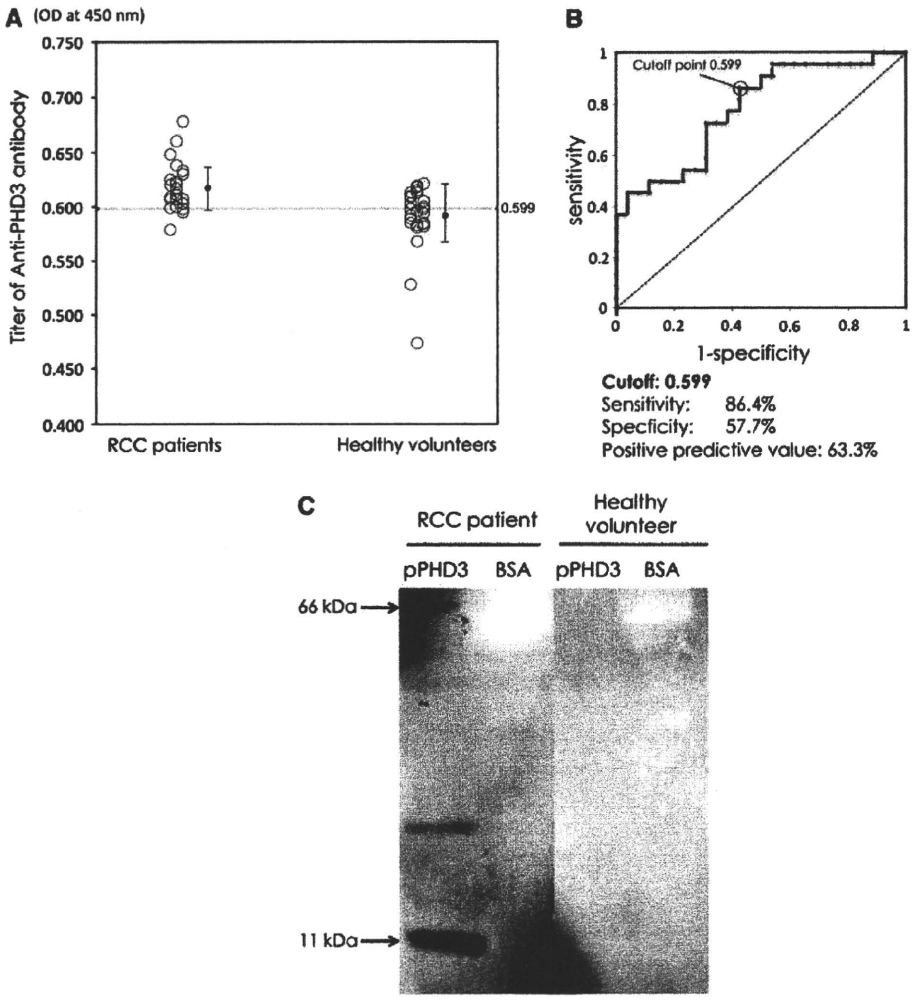

Fig. 1 **a** Serum anti-PHD3 Abs of patients with RCC and healthy volunteers. *Error bars* indicate mean ± standard deviation. **b** Receiver operating characteristic plot for cutoff point of serum anti-PHD3 Ab titer to predict RCC. **c** Western blot analysis for immunoreactivity of serum to PHD3 recombinant protein. *Lanes 1* and *2*, serum from a patient with RCC. *Lanes 3* and *4*, serum from a healthy volunteer. The *bars* of 66 kDa and 11 kDa indicate molecular sizes of bovine serum albumin and recombinant protein of PHD3, respectively. pPHD3, PHD3 recombinant protein; BSA, bovine serum albumin

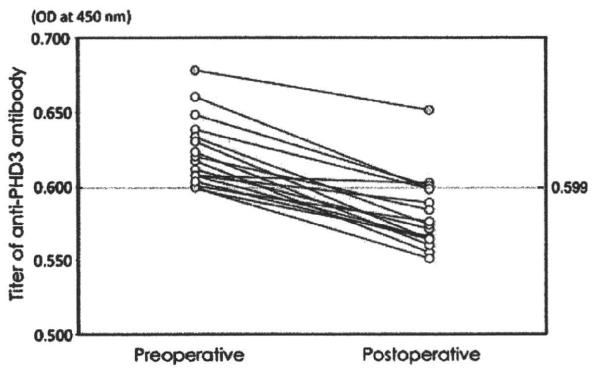

Fig. 2 Changes of serum anti-PHD3 Ab titers after surgical resection

detection of PHD3 was performed with only a small part of the RCC tissue, so it is speculated that we may have failed to obtain a PHD3-positive area.

Various autoantibodies against cancer-specific antigens have been investigated to verify their potential as serological tumor markers. Autoantibodies against p53, IMP, p62, Koc, c-myc, cyclin-B1, and survivin were reported to be useful for detection of malignant tumors of various origins such as those of the esophagus, colorectum, bladder, prostate, stomach, liver, pancreas, lung, breast, and ovary (Barua et al. 2007; Larsson et al. 2006; Nilsson et al. 2001; Shimada et al. 2000, 2002; Suzuki et al. 2004; Takeda et al. 2001a, b; Tan and Zhang 2008: Zhong et al. 2008). In RCC, we previously reported that livin, a member of the inhibitor of apoptosis protein family, was recognized as a tumor-specific antigen in patients and anti-livin autoantibodies were detected in their sera (Kitamura et al. 2007). In addition, we found that PHD3 was recognized as a cancer-specific antigen and might be a target in immunotherapy for RCC (Sato et al. 2008). To the best of our knowledge, this is the first report demonstrating the efficacy of an autoantibody against a cancer-specific antigen as a serological marker for RCC.

The current study has limitations. Many individuals were included in the marginal zone, and 42.3% of the

healthy volunteers were positive for serum anti-PHD3 Abs, with a cutoff point of 0.599. The assay possibly detected nonspecific immune reactions. That is probably also the reason why there was a discrepancy between the result of the ELISA and the seronegativity shown by Western blotting in a healthy volunteer. Furthermore, PHD3 may be expressed in normal tissues other than those screened in our previous study (Sato et al. 2008). Although the specificity was low, our results showed the feasibility of an autoantibody against an anti-tumor antigen as a serological marker for RCC. The anti-PHD3 Ab can potentially facilitate the efficient selection of candidates for further radiographical examinations and may contribute to improvement of the cost-effectiveness of screening examinations for RCC. Further studies, in which patients are followed for long periods and the serum anti-PHD3 Ab level is regularly tested, are needed to determine its clinical usefulness.

All patients with RCC displayed decreases in the titer of the autoantibody after tumor resection, as shown in studies of an anti-p53 Ab in esophageal squamous cell carcinoma (Shimada et al. 2000, 2002) and colorectal adenocarcinoma (Takeda et al. 2001a, b). This finding suggests that the titer of the anti-PHD3 autoantibody may reflect the volume of the disease. In addition, Shimada et al. (2000, 2002) reported that persistent seropositivity for an anti-p53 Ab was associated with future disease recurrence in patients with esophageal cancer, which might indicate small residual cancer after surgery. In this study, some patients with clinically organ-confined disease still had high titers of the anti-PHD3 Ab after surgical resection, though the titers decreased. Although changes in the titer may be associated with the amount of cancer in each individual, we could not conclude that postoperative titers of serum anti PHD3 Ab can predict microscopic residual cancer or future disease recurrence. To confirm that, monitoring of the PHD3 Ab titer in follow-up and verification of the association between the disease recurrence and increase in the titer is necessary.

Conclusions

We demonstrated that the anti-PHD3 Ab titer was significantly higher in the sera of patients with RCC than in healthy volunteers. In addition, the anti-PHD3 Ab titers decreased in all patients with RCC after surgical resection. Although there are some limitations, these findings suggest that the anti-PHD3 Ab is a potential serological marker for RCC.

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Conflict of interest We declare that we have no conflict of interest.

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Autologous CTL response against cancer stem-like cells/ cancer-initiating cells of bone malignant fibrous histiocytoma

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Summary

Malignant fibrous histiocytoma (MFH) of bone is an aggressive tumor with high rates of local recurrence and metastasis. Therefore, development of novel therapeutic approaches is critical to further improve the prognosis. We previously reported that side population (SP) cells of the bone MFH cell line MFH2003 have the characteristics of cancer stem-like cells/cancer-initiating cells (CSCs/CICs). In the present study, for the establishment of immunotherapy targeting CSCs, we analyzed cell surface immune molecules on SP cells of MHF2003 and an autologous CTL responses against SP cells of MFH2003 in the tumor-microenvironment and peripheral circulating lymphocytes using autologous tumor-infiltrating lymphocytes and autologous CTL clone derived from peripheral blood, respectively. We found that SP cells of MFH2003 expressed HLA class I molecules on the cell surface. Autologous tumor-infiltrating lymphocyte line TIL2003 recognized both SP and MP cells of MFH2003. Next, we induced CTL clone Tc4C-6 by mixed lymphocyte tumor cell culture using autologous peripheral blood mononuclear cells and freshly isolated SP cells of MFH2003, followed by a limiting dilution procedure. Tc4C-6 showed specific cytotoxicity against SP cells of MFH2003. Moreover, cytotoxicity against SP cells was blocked by anti-HLA class I W6/32. In conclusion, these findings support the idea that CSCs of bone MFH are recognized by autologous CTLs in the tumor microenvironment and peripheral circulating lymphocytes. CTL-based immunotherapy could target CSCs of bone sarcoma to help prevent tumor recurrence.

Introduction

Malignant fibrous histiocytoma (MFH) of bone is a rare primary neoplasm, accounting for less than 5% of the primary bone malignancies[1, 2]. MFH of bone is histologically composed of fibroblasts and pleomorphic cells with a prominent storiform pattern. It is an aggressive tumor with high rates of local recurrence and metastasis and the prognosis is poor, with a five-year survival rate of less than 60%[3, 4]. Therefore, development of novel therapeutic approaches is critical to further improve the outcomes of patients with this tumor.

All of the neoplastic cells within a tumor have been thought to be capable of tumorigenic growth capacity. However, recent studies have demonstrated that malignant tumors could be generated by a distinct subpopulation of tumor cells, so-called cancer stem cells/cancer-initiating cells (CSCs/CICs), which have self-renewal ability, differentiation potential, and tumorigenic capacity[5, 6]. Thus, CSCs should represent an optimal therapeutic target to achieve complete eradication of tumor cells. However, CSCs have been found to resist standard therapeutic modalities, including radiation and drugs[7, 8].

Recently, many clinical trials of CTL-based immunotherapy using peptide vaccination have demonstrated the potency of this new therapeutic modality for various cancers that resist standard chemotherapy. However, it remains unknown whether CTL-based immunotherapy can kill CSCs. Previously, we demonstrated that side population (SP) cells in a bone MFH cell line (MFH2003) have CSC characteristics[9]. SP cells of MFH2003 showed cancer-initiating ability, with *in vitro* sphere formation and *in*

vivo tumorigenesis in NOD/SCID mice. In the present study, for the characterization of the immunogenicity of CSCs, we analyzed autologous CTL responses against SP cells of MFH2003 in the tumor-microenvironment and peripheral circulating blood using autologous tumor-infiltrating lymphocytes and a CTL clone, respectively.

Materials and Methods

This study was approved under the institutional guidelines for the use of human subjects in research. The patients and their families, as well as healthy donors, gave informed consent for the use of blood samples and tissue specimens in our research.

Cell lines and Culture

The cell lines used were a bone human malignant fibrous histiocytoma (MFH) cell line (MFH2003), osteosarcoma (OS2000, KIKU, NY, Huo9, HOS, U-2OS and Saos2), erythroleukemia (K562) and an Epstein-Barr virus-transformed B cell line (LG2-EBV, B2003-EBV). OS2000, KIKU MFH2003 and B2003-EBV were established in our laboratory[10-12]. The other cell lines were kindly donated or purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and from American Type Culture Collection (Manassa, VA). MFH2003 and OS2000 were cultured with Iscove's modified Dulbecco's Eagle's medium (IMDM; GIBCO BRL, Grand Island, NY) containing 10% FBS and LG-2-EBV, B2003-EBV, and K562 were cultured with RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FBS. The others were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% FBS in a 5% CO_2 incubator at 37°C.

Purification of side population cells

Cancer stem-like cells of MFH2003 were purified by side population analysis as previously described[9]. Briefly, the cell suspensions were labeled with dye Hoechst

33342 dye (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Hoechst33342 was added at a final concentration of 5.0μg/ml in the presence or absence of verapamil (75μM; Sigma-Aldrich) as an inhibitor of the ABC transporter. The cells were incubated at 37°C for 90 min with continuous shaking. At the end of the incubation, the cells were washed with ice-cold PBS with 5% FBS, centrifuged at 4°C, and resuspended in ice-cold PBS containing 5% FBS. Propidium iodide (at the final concentration of 1μg/ml; Life Technologies Corp, Carlsbad, CA) was used to gate viable cells. Flow cytometry and cell sorting were performed using a FACS Aria Ⅱ (BD Biosciences, Bedford, MA). The Hoechst 33342 dye was excited at 357 nm and its fluorescence was analyzed using dual wavelengths (blue, 402-446 nm; red, 650-670 nm).

When the proportion of SP cells was low (≤5%), the SP cells were sorted and brought into in vitro culture in 10ml of IMDM containing 10% FBS for enrichment. After more than 14 days, SP analysis and cell sorting were performed again.

Analysis of expression status of cell surface molecules

Expression of cell surface molecules was assayed as previously described[12] using an anti-HLA-A24 mAb (C7709A2.6), anti-HLA-B&C mAb (B1.23.2), anti-HLA class Ⅰ mAb (W6/32), anti-HLA-class Ⅱ mAb (L243), and an anti-CD80 mAb (MAB104). Bulk, SP and main population (MP) cells of MFH2003, LG2-EBV cells, B2003-EBV cells and K562 cells were incubated with appropriate mAbs, for 40 min on ice. Then these cells were incubated with FITC-labeled second antibody and analyzed using a FACSCaliber (BD Bioscience).