ethylene glycol-bis[beta-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid [EGTA], 1 mM dithiothreitol) containing 1 mM ATP at 37 C. The reaction was terminated by washing cells with ice-cold transport buffer, followed by lysis in a lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride). Then, cell lysates were purified by centrifugation at 10 000 × g for 5 min. Glycosylated peptides were collected by incubating 100 μL Con-A Sepharose beads (Pharmacia Biotech) with the lysates at 4 C for 60 min. After washing the beads with lysis buffer five times, radioactivities of the beads were analyzed by a gamma counter.

Minigene construction, transfection and cytotoxicity assays

In order to express peptide antigen F4.2 in endogenous form, we constructed expression minigene vectors, *pF4.2*, *pF4.2ss* and *pF4.2reverse*. *pcDSR -E3* contains an adenovirus E3/19 kDa protein signal sequence (38) under the control of the SR promoter. *pF4.2ss* was constructed by insertion of oligonucleotides corresponding to F4.2 peptide into the *Pst*I and *Xba*I sites of the *pcDSR -E3* expression vector. *pF4.2* and *pF4.2reverse* were constructed by insertion of the F4.2-coding oligonucleotides into the *Eco*RI site of the *pcDSR* expression vector in a sense orientation or an antisense orientation, respectively. Expression vectors were transfected to HOBC8-A31-12 cells or C1R-A31 cells by using Lipofectin reagent (Life Technologies, Gaithersburg, MD, USA) and cultured in the AIM-V serum-free media. In some cases, 200 μg/mL MeDSG was included in the media. Forty-eight hours after the transfection, cells were mixed with TcHST-2 cells for 10 hr at an E/T ratio of 3:1 or 10:1, followed by quantification of tumor necrosis factor (TNF) production. In the case of the ^{51}Cr release assay, target cells were labeled with ^{51}Cr for 1 hr, washed with PBS and co-cultured with TcHST-2 for 10 hr at an E/T ratio of 10:1 in the presence or absence of 200 μg/mL MeDSG, followed by counting radioactivities of ^{51}Cr in the culture supernatants. The expression of the oligonucleotide sequence coding F4.2 in the transfected cells was confirmed by using polymerase chain reaction (PCR) analysis.

RESULTS

HSC73 is co-immunoprecipitated with TAP1

It is known that MHC class I is associated with TAP1 in the ER until antigenic peptides are loaded on the MHC class I molecule (8). Analogously, we speculated that HSP70 might be associated with TAP1 in the cytoplasm. TAP1 (70 kDa) was immunoprecipitated from the lysate of human lymphoblastoid cell line T1 cells but not from the lysate of TAP-deficient line T2 cells, as detected by immunoblotting using anti-TAP1 antibody (Fig. 1a). The same immunoprecipitates were examined to detect HSC73. Although similar levels of HSC73 expression were demonstrated by immunoblotting of T1 lysates and T2 lysates with anti-HSP70 antibody (Fig. 1b, lanes 1,2), HSC73 was detected only in the TAP1-immunoprecipitates from T1 cell lysates (Fig. 1b,

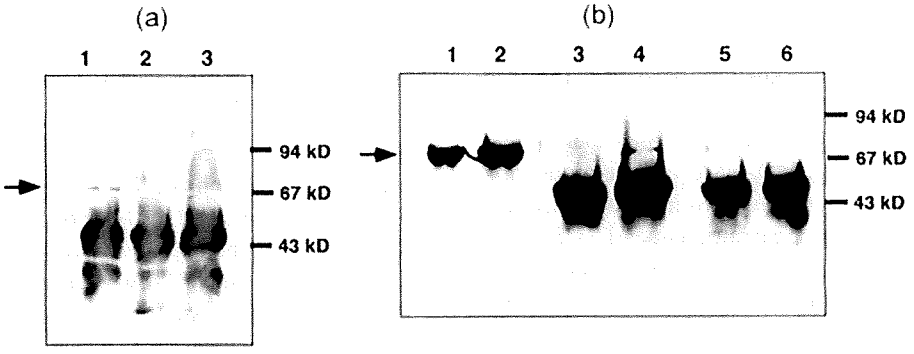

Fig. 1. Co-immunoprecipitation of HSC73 with TAP1 from T1 cell lysates. (a) T1 cells (lanes 1, 2) or T2 cells (lane 3) were lysed by 0.5% CHAPS lysis buffer. The lysates were immunoprecipitated with anti-TAP1 antibody (lanes 1, 3) or control antibody (lane 2). The immunoprecipitates were resolved by 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidine difluoride (PVDF) membranes and analyzed by immunoblotting with anti-TAP1 antibody. Arrowhead indicates the 70 kDa TAP1. (b) T2 lysates (lane 1) or T1 lysates (lane 2) were resolved by 8% SDS-PAGE and analyzed by immunoblotting with anti-HSP70 antibody. In lanes 3–6, immunoprecipitates with anti-TAP1 antibody (lanes 3, 4) or control antibody (lanes 5, 6) were made from T2 lysates (lanes 3, 5) or T1 lysates (lanes 4, 6), resolved by 8% SDS-PAGE and analyzed by immunoblotting with anti-HSP70 antibody. The faster-migrating 50 kDa band in each lane is an immunoglobulin heavy chain. The band corresponding to 73 kDa HSC73 is indicated by an arrowhead.

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lanes 3,4). As no band of HSC73 was detected in the immunoprecipitates with normal rabbit IgG (Fig. 1b, lanes 5,6), it is indicated that HSC73 is associated with TAP1 in T1 cells.

ATP-dependent dissociation of HSC73 from TAP1

HSC73 has an ATP-binding domain and an intrinsic ATPase activity. It can form at least two conformations, the ATP-binding form and the ADP-binding form. It is known that HSC73 can bind to and dissociate from other molecules depending upon the ATP/ADP-dependent conformational change (39). To test if the association of HSC73 with TAP1 is regulated by ATP, TAP1 was immunoprecipitated from T1 cell lysates in the presence of various concentrations of ATP or apyrase, which hydrolyzed ATP. The addition of ATP can facilitate the formation of the ATP-binding conformation of HSC73, whereas hydrolysis of ATP can facilitate the ADP-binding conformation. T1 cells were cultured in the medium supplemented with 150 U/mL IFN- for 48 hr before lysis in order to increase the level of TAP1 expression.

As shown in Figure 2a, similar levels of TAP1 were immunoprecipitated from T1 cell lysates in the presence of ATP or apyrase. Remarkably, the co-precipitation of HSC73 with TAP1 was decreased when 1 mM or 10 mM ATP was added to the cell lysates (Fig. 2b, lanes 2,3). In contrast, depletion of ATP from cell lysates by apyrase did not affect the co-immunoprecipitation levels of HSC73 (Fig. 2b, lanes 4,5). These data imply that the ADP-binding form of HSC73 may have higher affinity to TAP1 than its ATP-binding form.

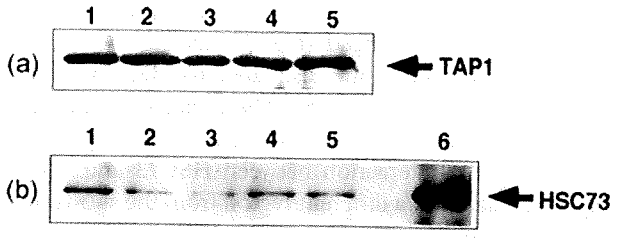

Fig. 2. ATP-dependent dissociation of HSC73 from TAP1. T1 cells were cultured in complete media containing 150 U/mL -interferon for 24 hr and lysed by 0.5% CHAPS lysis buffer. TAP1 was immunoprecipitated from the lysates using anti-TAP1 antibody under the condition of the absence (lane 1) or presence of 1 mM ATP (lane 2), 10 mM ATP (lane 3), 1 U/mL apyrase (lane 4) or 10 U/mL apyrase (lane 5). (a) TAP1-immunoprecipitates were resolved by 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with anti-TAP1 antibody. (b) TAP1-immunoprecipitates or T1 lysates (lane 6) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-HSP70 antibody.

MeDSG downregulates the cell surface MHC class I levels in T1 cells

In order to know the functional significance of the HSC73/TAP1 interaction, we used a stable derivative of a polyamine compound 15-DSG, which contains a spermidine-like structure. 15-DSG acts as an immunosuppressant and has already been used clinically to suppress rejection after organ transplantation (30). There have been a number of reports demonstrating that 15-DSG could inhibit the MHC class I and/or class II expression in some tissues (40–42). Although the molecular mechanism of the immunosuppressive action of 15-DSG remains obscure, it has been revealed that 15-DSG could specifically bind to both HSC73 and HSP90 (29, 43). Thus, we speculated that 15-DSG might affect the function of HSC73 in the presentation of antigenic peptides. In our experiments MeDSG, a methoxy-derivative of 15-DSG, was used, as it is more stable *in vitro* than 15-DSG (44).

T1 cells were cultured for 36 hr in a serum-free medium containing various concentrations of MeDSG, followed by fluorescent activated cell sorting (FACS) analysis of the cell surface levels of MHC class I expression. As shown in Figure 3a, the cell surface levels of MHC class I were downregulated by the treatment with MeDSG. The downregulation was shown to be dependent on the concentration of MeDSG. In another experiment, the levels of MHC class I were compared between T1 cells and TAP-deficient counterpart T2 cells (Fig. 3b). It is noteworthy that the decreased level of MHC class I expression after the treatment

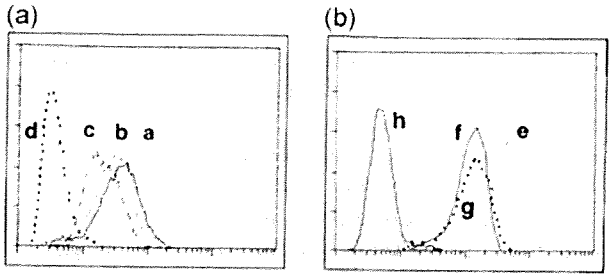

Fig. 3. MeDSG was capable of downregulating the expression of MHC class I molecules on the cell surface of T1 cells. Cells were cultured for 36 hr in AIM-V serum-free media containing various concentrations of MeDSG. After washing once with phosphate-buffered saline (PBS), cells were incubated with anti-human MHC class I antibody W6/32 for 45 min at 4 C, followed by an incubation with fluorescein-isothiocyanate (FITC)-labeled anti-mouse IgG antibody and an analysis on a FACScan flow cytometer. (a) T1 cells cultivated in the media containing various concentrations of MeDSG (a. 0 μg/mL, b. 100 μg/mL, c. 200 μg/mL) were analyzed for the cell surface expression of MHC class I molecules (d. FITC-labeled anti-mouse IgG antibody only). (b) T1 cells cultivated in the absence (e.) or presence of 200 μg/mL MeDSG (g.) or T2 cells (f.) were analyzed for the cell surface expression of MHC class I molecules (h. FITC-labeled anti-mouse IgG antibody only).

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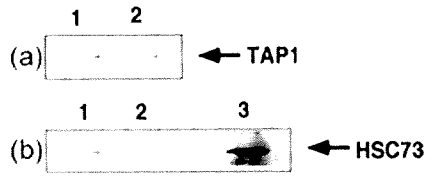

Fig. 4. HSC73 is dissociated from TAP1 in the presence of MeDSG. T1 cells were cultured in media supplemented with 200 μg/mL spermidine or 200 μg/mL MeDSG for 36 hr and lysed by 0.5% CHAPS lysis buffer. TAP1 was immunoprecipitated from the lysates using anti-TAP1 antibody in the presence of 200 μg/mL spermidine (lane 1) or 200 μg/mL MeDSG (lane 2). (a) TAP1-immunoprecipitates from T1 lysates containing spermidine (lane 1) or MeDSG (lane 2) were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed for the relative levels of TAP1 by immunoblotting using anti-TAP1 antibody. The band corresponding to TAP1 is indicated. (b) The immunoblot used in (a) was regenerated by soaking in a stripping buffer and reblocking. T1 lysates (lane 3) and TAP1-immunoprecipitates from T1 lysates containing spermidine (lane 1) or MeDSG (lane 2) were analyzed by immunoblotting with anti-HSP70 antibody. The band corresponding to HSC73 is indicated.

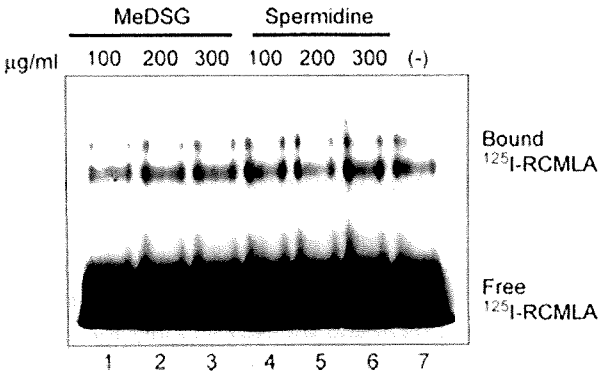

Fig. 5. MeDSG does not affect the interaction between HSC73 and a substrate protein. RCMLA was labeled with 125-iodine by Iodo-beads in 250 μL phosphate-buffered saline (PBS) for 15 min at 20 C. Radiolabeled RCMLA (40 ng) was incubated with 2 μg HSC73 (HSC73/RCMLA mole ratio of 10:1) in 10 μL PBS at 37 C for 1 hr in the absence (lane 7) or presence of the indicated concentrations of MeDSG (lanes 1–3) or spermidine (lanes 4–6) and resolved by 7% native polyacrylamide gel electrophoresis (PAGE). Radiolabeled RCMLA was visualized by autoradiography of 125-iodine. The slower-migrating bands correspond to HSC73-bound RCMLA.

with 200 μg/mL MeDSG was comparable to the level in T2 cells (approximately 35% lower level). These data indicate that MeDSG might change T1 cells to T2-like phenotypes possibly by impairing TAP-dependent transportation of MHC class I antigenic peptides.

MeDSG disrupts the association of HSC73 with TAP1

As one of the major intracellular target molecules of MeDSG is HSC73 (29), we examined the effect of MeDSG on the interaction between HSC73 and TAP1. Spermidine, an analogous polyamine compound, was used as a negative control reagent, as it has a low affinity to HSC73 and has less immunosuppressive activity (43).

TAP1 was immunoprecipitated from T1 cell lysates in the presence of 200 μg/mL spermidine or 200 μg/mL MeDSG. These concentrations had been shown to down-regulate the MHC class I expression of T1 cells (Fig. 3a). The levels of TAP1 immunoprecipitated were identical between the spermidine treatment and the MeDSG treatment as detected by western blotting (Fig. 4a). The same blot was then regenerated and was examined to detect HSC73 by western blotting. Strikingly, HSC73 was not detected in the TAP1-immunoprecipitates from T1 cell lysates treated with MeDSG, whereas it was co-precipitated with TAP1 from the lysates treated with spermidine (Fig. 4b). As protein levels of HSC73 contained in the cell lysates were equal between these cases (data not shown), the data imply that MeDSG can disrupt the association of HSC73 with TAP1, possibly by direct binding to HSC73.

MeDSG does not affect the interaction between HSC73 and a substrate protein

It is well known that HSC73 binds to an unfolded form of lactalbumin, RCMLA, through the C-terminal substrate binding domain. In order to know whether MeDSG can affect the interaction between HSC73 and a substrate protein, we analyzed a direct association between HSC73 and radiolabeled RCMLA in the absence or presence of MeDSG. In native PAGE analysis, HSC73-bound RCMLA was detected as the slower migration band (Fig. 5, lane 7). In the presence of MeDSG at concentrations from 100 μg/mL to 300 μg/mL, the levels of HSC73-bound RCMLA were not changed (Fig. 5, lanes 1–3). A control polyamine, spermidine, also failed to affect the binding affinity (Fig. 5, lanes 4–6). These data indicate that MeDSG does not bind to a substrate-binding region on HSC73 and the interaction between HSC73 and TAP1 may be mediated by a different region from the substrate-binding region.

HSC73-binding assay of synthetic peptides

We have reported that peptides Bw4 and Bw6 have distinct binding affinity to HSC73, although they have very homologous amino acid sequences (35, 45). We designed the model peptides NGT-Bw4 and NGT-Bw6 by the addition of an *N*-linked glycosylation site, NGT, to Bw4 and Bw6, respectively so that the ER-translocated peptides could be collected by Con A Sepharose beads. HSC73-binding affinity of the model peptide was assessed by

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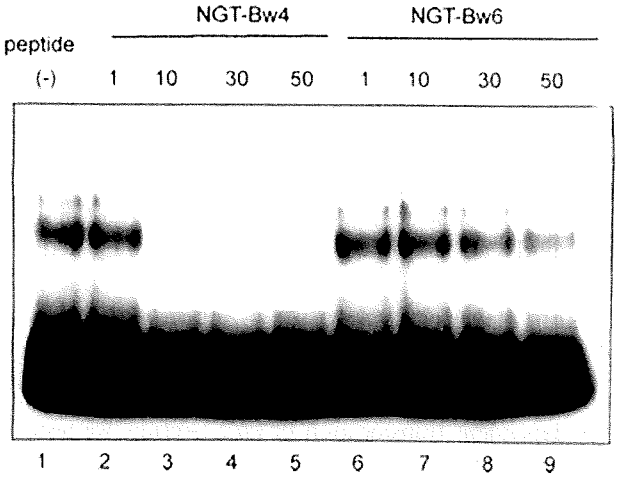

Fig. 6. HSC73-binding assay of synthetic peptides. RCMLA was labeled with 125-iodine by Iodo-beads as described earlier. Radiolabeled RCMLA (40 ng) was incubated with 2 μg HSC73 (HSC73/RCMLA mole ratio of 10:1) in 10 μL PBS at 37 C for 1 hr in the absence (lane 1) or presence of the indicated peptide/HSC73 mole ratio of NGT-Bw4 peptide (lanes 2–5) or NGT-Bw6 peptide (lanes 6–9). The mixtures were resolved by 7% native polyacrylamide gel electrophoresis (PAGE). Radiolabeled RCMLA was visualized by autoradiography of 125-Iodine. The slower-migrating bands correspond to HSC73-bound RCMLA.

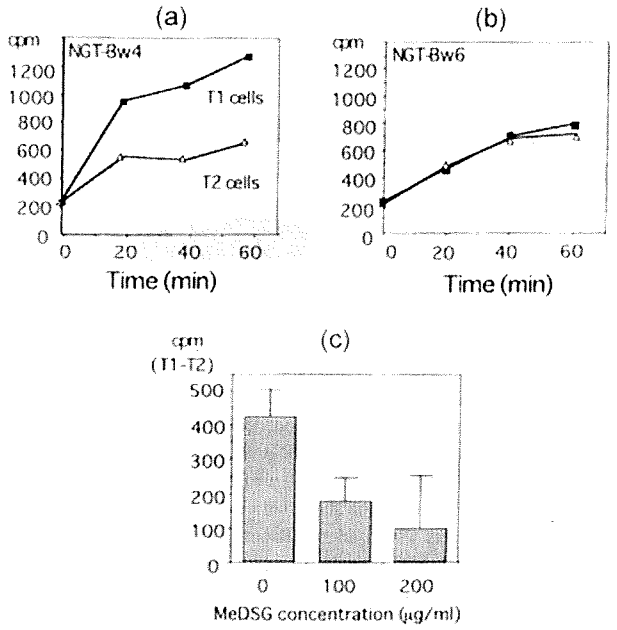

Fig. 7. ER translocation assay of synthetic peptides. 1×10^7 T1 cells or T2 cells were permeabilized by incubating with streptolysin-O as described earlier. Cells were then incubated with 19.0 μM 125-iodine-labeled peptides in the presence of 1 mM ATP. In the case of 0 min, non-permeabilized cells were incubated with the same amount of peptides. After the indicated time, glycosylated peptides were collected by Con A Sepharose beads, followed by counting radioactivity of the beads. (a) Time course of ER-translocation of NGT-Bw4 peptide in T1 cells ( ) or T2 cells ( ). (b) Time-course of ER-translocation of NGT-Bw6 peptide in T1 cells ( ) or T2 cells ( ). (c) Permeabilized T1 cells or T2 cells were incubated with radiolabeled NGT-Bw4 peptide in the transport buffer containing the indicated concentrations of MeDSG. Radioactivities of Con A Sepharose beads recovered from T2 cells were subtracted from those recovered from T1 cells, corresponding to TAP-dependent translocation levels. Bars represent SD from triplicated samples.

competitive binding assay using RCMLA. RCMLA was labeled with 125-iodine, incubated with purified HSC73 in the absence or presence of various amounts of synthetic peptides and separated by a native PAGE. HSC73-bound RCMLA was detected as a slower migrating band (Fig. 6, lane 1). By increasing the amount of NGT-Bw4 peptide, levels of HSC73-bound RCMLA were decreased (Fig. 6, lanes 2–5). In contrast, less dissociation was observed in the addition of NGT-Bw6 peptide (Fig. 6, lanes 6–9). Thus, it was demonstrated that NGT-Bw4 and NGT-Bw6 have a high binding affinity and a low binding affinity to HSC73, respectively.

Analysis of TAP-dependent translocation of synthetic peptides

T1 cells and T2 cells were permeabilized by streptolysin-O and incubated with 125-iodine-labeled synthetic peptide, NGT-Bw4 or NGT-Bw6, at 37 C in the presence of 1 mM ATP. Peptides translocated into the ER are glycosylated and, thus, can be collected by Con A Sepharose beads. The difference between radioactivities of the glycosylated peptide recovered from T1 cells and that from TAP-deficient T2 cells represents the TAP-dependent translocation level of the peptide. NGT-Bw4 was effectively translocated into the ER by TAP during a 20–60 min incubation (Fig. 7a). In contrast, NGT-Bw6 failed to be translocated by TAP (Fig. 7b).

In order to determine if HSC73 is involved in the TAP-dependent translocation of NGT-Bw4 peptide, an ER translocation assay was performed in the absence or presence of 100 μg/mL or 200 μg/mL MeDSG, which induced the dissociation of HSC73 from TAP1. The TAP-dependent translocation of NGT-Bw4 was clearly inhibited by MeDSG in the presence of MeDSG (Fig. 7c). These results indicate that HSC73-binding affinity may affect the efficiency of TAP-dependent translocation into the ER, at least for some peptides, and that the physical association between HSC73 and TAP1 is important for the TAP-dependent translocation of HSC73-bound peptides.

HSC73-binding affinity of a natural MHC class I peptide F4.2

As NGT-Bw4 and NGT-Bw6 are not natural antigenic peptides, we tested if an MHC class I-presentable

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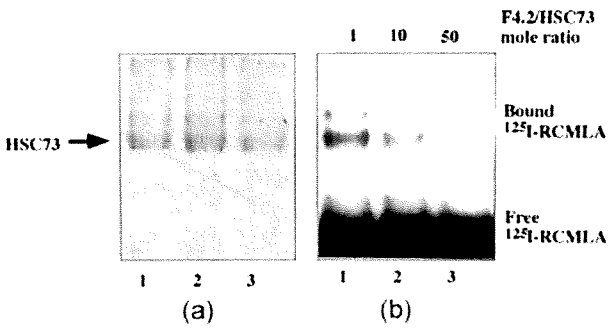

Fig. 8. HSC73-binding assay of antigenic peptide F4.2. RCMLA was labeled with 125-iodine by Iodo-beads as described earlier. Radiolabeled RCMLA (40 ng) was incubated with 2 μg HSC73 in a binding buffer at 37 C for 1 hr in the presence of the indicated peptide/HSC73 mole ratio (lane 1, 1:1; lane 2, 10:1; lane 3, 50:1) of F4.2 peptide. The mixtures were resolved by 7% native polyacrylamide gel electrophoresis (PAGE). (a) HSC73 protein was visualized by staining the gel with Coomassie Brilliant Blue. The protein band corresponding to HSC73 is indicated. (b) Radiolabeled RCMLA was visualized by autoradiography of 125-iodine. The slower-migrating band observed at the position of HSC73 corresponds to HSC73-bound RCMLA.

natural peptide could bind to HSC73. By using acid elution and biochemical analyses, the structure of natural antigenic peptide of gastric signet ring cell carcinoma cells, HST-2, was determined (32). The peptide, named F4.2, is recognized by autologous cytotoxic T-cell clone TcHST-2 in the context of HLA-A31 restriction. F4.2 peptide was synthesized and incubated with 125-I-labeled RCMLA-HSC73 complex. After native PAGE separation, radioactivity was detected at the position of HSC73 (Fig. 8a), representing HSC73-bound RCMLA. Levels of the HSC73-bound RCMLA were decreased by increasing the amount of F4.2 peptide (Fig. 8b). Therefore, it was shown that the natural antigenic peptide F4.2 could bind to HSC73.

Cytosolic F4.2 peptide can be presented by HLA-A31

TcHST-2 cells are capable of responding to pulsed F4.2 peptide in a highly sensitive TNF production assay (32). In addition, we have shown that TcHST-2 also responds to the endogenous form of F4.2 peptide expressed by transfection of minigene vector *pF4.2ss* encoding the signal sequence plus F4.2 peptide (Fig. 9a). F4.2 peptide expressed by the minigene *pF4.2ss* can be translocated into the ER through the translocon protein by the signal peptide. Using a TNF production assay, we tested if TcHST-2 could respond to cytosolic F4.2 peptide expressed by transfection of minigene *pF4.2* encoding just F4.2 peptide without the signal peptide (Fig. 9a). *pF4.2reverse* was constructed by insertion of the F4.2-coding oligonucleotides into the expression vector in an antisense orientation. Expression vectors were transfected to HOBC8-A31-12 cells, which express HLA-A31, followed by co-culture with TcHST-2 cells and TNF production assay. As shown in Figure 9b, *pF4.2*-transfected cells, as well as *pF4.2ss*-transfected cells, were recognized by TcHST-2 cells, whereas *pF4.2reverse*-transfected cells were not. These results indicate that cytosolic F4.2 without the signal peptide can be translocated into the ER through TAP.

Presentation of cytosolic F4.2 is inhibited by MeDSG treatment

In order to elucidate the involvement of HSC73 in the TAP-dependent translocation of F4.2 peptide, we tested the effect of MeDSG on the presentation of endogenous F4.2 peptide. C1R-A31 cells were transfected with either *pF4.2* or *pF4.2ss* minigene expression vector and cultured in the absence or presence of 200 μg/mL MeDSG. Forty-eight hours after the transfection, cytotoxicity by TcHST-2 was examined by ^{51}Cr release assay. HST-2 cells and K562 cells were used as positive control target cells and negative control target cells, respectively (Fig. 9c, bars 1,2). Both *pF4.2*-transfected cells and *pF4.2ss*-transfected cells were lysed by TcHST-2 similarly. In the presence of MeDSG, however, only *pF4.2ss*-transfected cells were susceptible to killing by TcHST-2 (Fig. 9c, bars 5,7). Taken together with the result showing that HSC73 binds to F4.2, it is indicated that MeDSG-induced disruption of the HSC73-TAP complex results in the incomplete ER-translocation of the HSC73-bound antigenic peptide. Furthermore, our data imply that MeDSG may not affect the signal peptide-mediated translocation of antigenic peptides.

DISCUSSION

It has been speculated so far that HSP70 might be involved in antigen presentation. This idea came from evidence produced by us or other groups showing that: (i) MHC class I-bound antigenic peptide with an N-terminal short flanking sequence, which might be a cytoplasmic precursor of the antigenic peptide, was isolated from murine tumor cell lysates as a HSP70-binding peptide (18); (ii) HSP70 could bind to some antigenic peptides and present them to T cells (18, 20, 46); and (iii) the vaccination of HSP70 plus tumor-derived peptides could enhance the anti-tumor immunity in animals (47–50). Moreover, there are some reports demonstrating the involvement of HSP70 in the TAP-independent presentation of cytosolic antigens and in the MHC class II antigen presentation (51, 52). In our experiments, it was revealed that HSC73, the constitutively expressed HSP70 family protein, was associated with TAP1 in an ATP-dependent manner, which was consistent with the report of Chen and Androlewicz (53).

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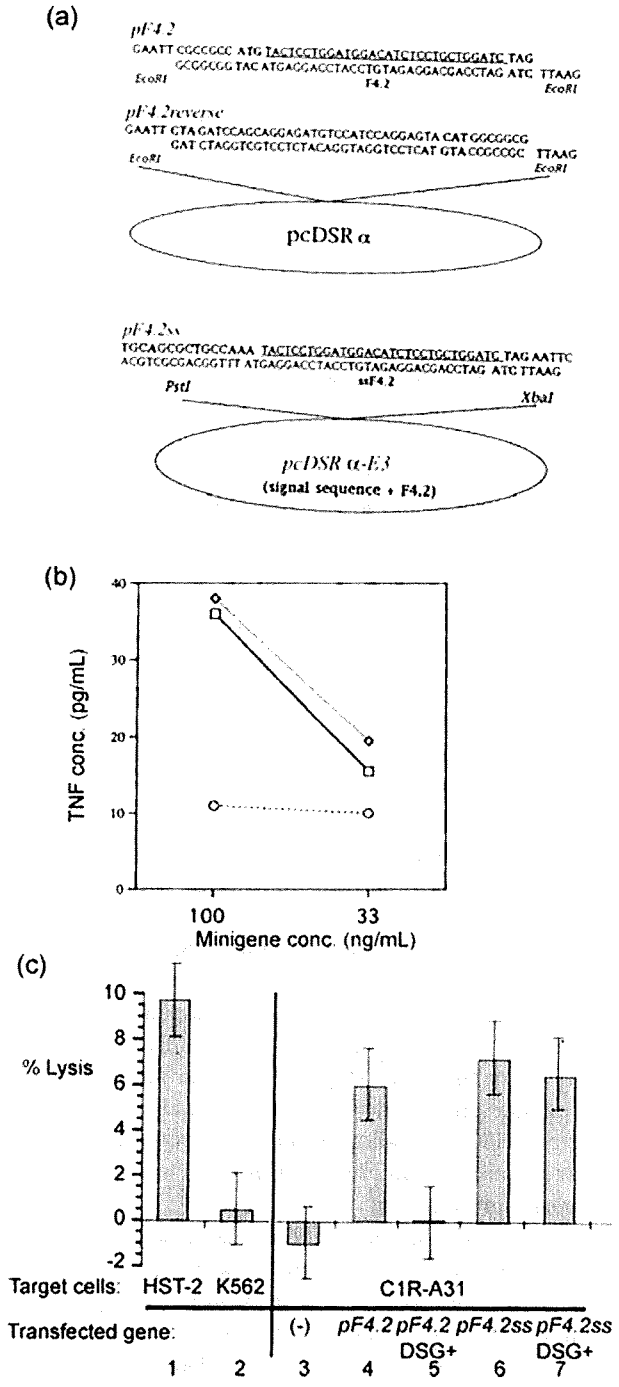

Fig. 9. T-cell recognition of gene-transferred F4.2 peptide and the effect of MeDSG. (a) Construction and nucleotide sequence of minigene vectors. Construction and nucleotide sequence (underline) of *pF4.2*, *pF4.2reverse* and *pF4.2ss* minigene vectors are illustrated. *pF4.2* and *pF4.2reverse* were constructed by insertion of the F4.2-coding oligonucleotides into the *EcoRI* site of the *pcDSR* expression vector in a sense orientation or an antisense orientation, respectively. *pF4.2ss* was constructed by insertion of F4.2-coding oligonucleotides into the *PstI* and *XbaI* sites of the *pcDSR -E3* expression vector containing an adenovirus E3/19 kDa protein signal sequence. (b) Cytosolic F4.2 peptide can be presented by HLA-A31. HOBC8-A31-12 cells that express HLA-A31 were transfected with 100 ng/mL or 33 ng/mL *pF4.2*, *pF4.2reverse* or *pF4.2ss*. Forty-eight hours after the transfection, cells were incubated with TcHST-2 for 10 hr at an E/T ratio of 3:1 and then tumor necrosis factor (TNF) production was determined. , *pF4.2ss*; , *pF4.2*; , *pF4.2reverse*. (c) Effect of MeDSG on the cytotoxic susceptibility ofminigene-transfected cells by TcHST-2. C1R-A31 cells that express HLA-A31 were transfected with 100 ng/mL *pF4.2* or *pF4.2ss* minigene expression vector by Lipofectin reagent and cultured in the absence (bars 4, 6) or presence of 200 μg/mL MeDSG (bars 5, 7) for 48 hr. Then, HST-2 cells (bar 1), K562 cells (bar 2) and C1R-A31 cells without (bar 3) or with minigene transfection (bars 4–7) were labeled with ^{51}Cr and were mixed with TcHST-2 for 10 hr at an E/T ratio of 10:1, followed by counting radioactivities of ^{51}Cr in the culture supernatants. Error bars represent SD calculated from triplicated samples

HSC73 is a cytosolic protein functioning as a molecular chaperone (54). It has a peptide binding domain in the C-terminal region, which has a high affinity to peptides containing several hydrophobic amino acids (55). Isolation and sequencing of MHC class I-bound antigenic peptides revealed that most of the peptides contain hydrophobic amino acids, some of which are important as anchor residues to the groove formed on the MHC class I molecule. The hydrophobic peptides become solubilized by binding to chaperone proteins such as heat shock proteins. In addition, chaperone-bound peptides may be protected from degradation by cytosolic peptidases. In the ER, antigenic peptides are transferred from TAP to MHC class I molecule through tapasin-mediated interaction between these two molecules (9, 11). It has been reported so far that molecular chaperones such as gp96 and calreticulin bind to antigenic peptides in the ER and may mediate their transfer from TAP to MHC class I heavy chain (17, 56). In contrast to the event in the ER, less is known about the feeding mechanism of cytoplasmic peptides to TAP. We tried to detect a physical interaction between TAP and proteasome; however we failed (O. Fujiwara and T. Torigoe, unpublished data, 2002). Thus, it is possible that antigenic peptides produced by proteasomes in the cytosol could be carried to TAP by some cytosolic chaperones such as HSC73 and HSP90. Actually, evidence has been reported that N-terminally extended peptides are associated with the cytosolic chaperone, TriC, and are protected from degradation by cytosolic peptidases (57). In addition, Yamano *et al.* provided evidence that HSP90 and PA28 accelerated the processing and presentation of MHC class I-bound peptides (58).

In order to determine the functional significance of the interaction between HSC73 and TAP1, we used an immunosuppressant, MeDSG, a stable analog of

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15-DSG, which could specifically bind to HSC73 (29, 59). Treatment of T1 cells with MeDSG resulted in the downregulation of the cell surface levels of MHC class I expression. The downregulatory effect of MeDSG was specific in the expression of MHC class I, as other surface molecules such as CD3 and LFA-1 were not changed by treatment of cells with the same concentration of MeDSG (data not shown). These data are consistent with a previous report showing a decreased expression of MHC class I by 15-DSG in a rat allograft model and cultured mouse cells (23, 42). The decreased MHC class I levels of T1 cells after MeDSG treatment were very similar to the levels of T2 cells, which lacked TAP molecules. MHC class I becomes unstable and the cell surface levels are decreased if TAP-dependent translocation of antigenic peptides is impaired (4). Therefore, it was suggested that MeDSG might inhibit antigen presentation at the TAP level.

15-DSG binds to both HSC73 and HSP90. Based on previous reports, there may be two 15-DSG-binding regions in HSC73 (60). One resides in the ATP-binding region and the other is the C-terminus EEVD domain, which is conserved among HSP70-family proteins. HSP90 also contains the same sequence in its C-terminus. Previously, Nadler *et al.* demonstrated that 15-DSG could inhibit the nuclear translocation of transcriptional factor nuclear factor-kappa B (NF- B) by competitively blocking the association of HSC73 with NF- B (59). This was the first report elucidating the molecular mechanism of immunosuppression by 15-DSG. In the present study, we showed evidence that MeDSG could not affect the interaction between HSC73 and a substrate protein. Although MeDSG contains a spermidine-like structure, it is known that spermidine had low affinity to HSC73 and less immunosuppressive activity (43). We confirmed that spermidine could neither downregulate the MHC class I expression in T1 cells (data not shown) nor inhibit the interaction between HSC73 and TAP1. However, it cannot be ruled out that the inhibitory effect of MeDSG to antigen presentation might be mediated by binding of MeDSG to HSP90, as it is definitive that HSP90 also serves as a chaperone for antigenic peptides in the cytosol (58, 61). Therefore, we then executed an ER-translocation study using two model peptides that have distinct binding affinity to HSC73. HSC73-bound peptide NGT-Bw4 could be translocated by TAP, whereas low-affinity peptide NGT-Bw6 failed. As amino acid sequences of these peptides are highly homologous and are identical, especially at their N-terminal and C-terminal residues which are known to affect the binding affinity to TAP (62), it is likely that the difference in the TAP-mediated translocation results from the distinct binding affinity to HSC73. Taken together, it is indicated that MeDSG could dissociate HSC73 from TAP, leading to a decrease of TAP-dependent translocation of HSC73-chaperoned peptides.

To confirm further that MeDSG inhibits the MHC class I antigen presentation, we performed experiments using a natural antigenic peptide, F4.2, which was identified from human gastric cancer cell line HST-2 (32). F4.2 is presented by HLA-A31 and recognized by cytotoxic T-cell clone, TcHST-2. We found that F4.2 peptide had high affinity to HSC73. By using minigenes coding F4.2, it was shown that the presentation of the cytosolic peptide chaperoned by HSC73 was inhibited by MeDSG. In contrast, TAP-independent presentation of F4.2 peptide, possibly through translocon, was not affected by MeDSG. However, it cannot be ruled out that HSC73 might function as a molecular chaperone for TAP itself and, therefore, MeDSG might disturb the structure and function of TAP.

Meanwhile, it is considered that the repertoire of MHC class I peptides could be affected by: (i) the MHC class I binding motif; (ii) the binding affinity to TAP; and (iii) the proteasomal cleavage site of antigenic proteins. Our data proposed another possibility that the HSC73-binding affinity might also affect the repertoire of MHC class I peptides. Our findings are likely to be important in the field of tumor immunotherapy, especially in the design of antigenic peptides for developing peptide vaccines. There is much evidence that CTL fail to kill tumor cells even though they are successfully induced by pulsing synthetic peptides to antigen-presenting cells. It is possible that some peptides might not be translocated into the ER if the peptides have low HSC73-binding affinity, as in the case of NGT-Bw6. As discussed previously, there are multiple cytosolic chaperones for antigenic peptides, such as TriC, HSP90, PA28 and HSC73, each of which is involved in the processing and transportation of antigenic peptides independently or cooperatively (57, 58, 61). Therefore, HSC73-binding affinity of the peptides and the proper function of HSC73 is not the only factor limiting the peptide presentation.

Our data showing the ATP-dependent dissociation of HSC73 from TAP1 present some important implications for the molecular machinery of the peptide-feeding mechanism to TAP. HSC73 has an ATP-binding domain in the N-terminal region and an intrinsic ATPase activity. It is known that the ADP-binding form of HSC73 has a higher affinity to hydrophobic peptides than the ATP-binding form (63). The contact of empty (peptide-free) HSC73 to peptides can stimulate the intrinsic ATPase activity of HSC73, resulting in the formation of a stable ADP-HSC73-peptide complex (64). In the presence of ATP, HSC73-bound ADP is exchanged to ATP, and the peptide is released from the ATP-binding HSC73 (65). Thus, HSC73 has an association/dissociation cycle with a substrate peptide depending upon the presence of ATP and

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the intrinsic ATPase activity. As the ADP-binding form of HSC73, which carries a peptide, has higher affinity to TAP than the ATP-binding form, the HSC73-bound antigenic peptide can be brought to close proximity to TAP. It is reported that DSG binds to HSC73 in its C-terminal EEVD domain, which could affect the conformation of the ATP-binding domain (59, 66). Therefore, HSC73 might interact with TAP by the same domain, leading to ADP-ATP exchange. In the presence of ATP, peptides can be released from HSC73, resulting in the transfer of peptides to TAP. Following the ATP-dependent feeding of the peptides, ATP-binding HSC73 (empty form) may be dissociated from TAP to bind to other peptides. It is possible that HSC73 may feed the cytosolic antigenic peptides by such an ATP-dependent cycle.

Where and how can HSC73 catch antigenic peptides produced by proteasome? In this context, it is of interest to test whether ATP-binding HSC73 is associated with proteasome. Further experiments focusing on the interaction between proteasome and chaperone will clarify the molecular machinery of antigenic peptide transportation in the cytoplasm.

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REFERENCES

1. Michalek M.T., Grant E.P., Gramm C., Goldberg A.L., Rock K.L. (1993) A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* 363: 552–4.
2. Kelly A., Powis S.H., Kerr L.A., Mockridge I., Elliott T., Bastin J., Uchanska Ziegler B., Ziegler A., Trowsdale J., Townsend A. (1992) Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex. *Nature* 355: 641–4.
3. Powis S.J., Townsend A.R., Deverson E.V., Bastin J., Butcher G.W., Howard J.C. (1991) Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature* 354: 528–31.
4. Spies T., Cerundolo V., Colonna M., Cresswell P., Townsend A., DeMars R. (1992) Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature* 355: 644–6.
5. Neefjes J.J., Momburg F., Hammerling G.J. (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter [published erratum appears in Science 1994 Apr 1;264(5155):16]. *Science* 261: 769–71.
6. Shepherd J.C., Schumacher T.N., Ashton Rickardt P.G., Imaeda S., Ploegh H.L., Janeway C.A. Jr., Tonegawa S. (1993) TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective [published erratum appears in Cell 1993 Nov 19;75(4):613]. *Cell* 74: 577–84.
7. Androlewicz M.J., Ortmann B., van Endert P.M., Spies T., Cresswell P. (1994) Characteristics of peptide and major histocompatibility complex class I/beta 2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc Natl Acad Sci USA* 91: 12716–20.
8. Grandea A.G.r., Androlewicz M.J., Athwal R.S., Geraghty D.E., Spies T. (1995) Dependence of peptide binding by MHC class I molecules on their interaction with TAP. *Science* 270: 105–8.
9. Ortmann B., Androlewicz M.J., Cresswell P. (1994) MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature* 368: 864–7.
10. Sadasivan B., Lehner P.J., Ortmann B., Spies T., Cresswell P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* 5: 103–14.
11. Suh W.K., Cohen Doyle M.F., Fruh K., Wang K., Peterson P.A., Williams D.B. (1994) Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science* 264: 1322–6.
12. Ortmann B., Copeman J., Lehner P.J., Sadasivan B., Herberg J.A., Grandea A.G., Riddell S.R., Tampe R., Spies T., Trowsdale J., Cresswell P. (1997) A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science* 277: 1306–9.
13. Nair S., Wearsch P.A., Mitchell D.A., Wassenberg J.J., Gilboa E., Nicchitta C.V. (1999) Calreticulin displays in vivo peptide-binding activity and can elicit CTL responses against bound peptides. *J Immunol* 162: 6426–32.
14. Lindquist J.A., Jensen O.N., Mann M., Hammerling G.J. (1998) ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. *Embo J* 17: 2186–95.
15. Morrice N.A., Powis S.J. (1998) A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. *Curr Biol* 8: 713–6.
16. Srivastava P.K., Udono H. (1994) Heat shock protein-peptide complexes in cancer immunotherapy. *Curr Opin Immunol* 6: 728–32.
17. Srivastava P.K., Udono H., Blachere N.E., Li Z. (1994) Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics* 39: 93–8.
18. Ishii T., Udono H., Yamano T., Ohta H., Uenaka A., Ono T., Hizuta A., Tanaka N., Srivastava P.K., Nakayama E. (1999) Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *J Immunol* 162: 1303–9.

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19. Kishi A., Ichinohe T., Hirai I., Kamiguchi K., Tamura Y., Kinebuchi M., Torigoe T., Ichimiya S., Kondo N., Ishitani K., Yoshikawa T., Kondo M., Matsuura A., Sato N. (2001) The cell surface-expressed HSC70-like molecule preferentially reacts with the rat T-cell receptor Vdelta6 family. *Immunogenetics* 53: 401–9.
20. Takashima S., Sato N., Kishi A., Tamura Y., Hirai I., Torigoe T., Yagihashi A., Takahashi S., Sagae S., Kudo R., Kikuchi K. (1996) Involvement of peptide antigens in the cytotoxicity between 70-kDa heat shock cognate protein-like molecule and CD3+, CD4−, CD8−, TCR-alpha beta- killer T cells. *J Immunol* 157: 3391–5.
21. Tamura Y., Tsuboi N., Sato N., Kikuchi K. (1993) 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J Immunol* 151: 5516–24.
22. Udono H., Srivastava P.K. (1994) Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol* 152: 5398–403.
23. Binder R.J., Blachere N.E., Srivastava P.K. (2001) Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. *J Biol Chem* 276: 17 163–71.
24. Nihei T., Sato N., Takahashi S., Ishikawa M., Sagae S., Kudo R., Kikuchi K., Inoue A. (1993) Demonstration of selective protein complexes of p53 with 73 kDa heat shock cognate protein, but not with 72 kDa heat shock protein in human tumor cells. *Cancer Lett* 73: 181–9.
25. Nihei T., Takahashi S., Sagae S., Sato N., Kikuchi K. (1993) Protein interaction of retinoblastoma gene product pRb110 with M(r) 73,000 heat shock cognate protein. *Cancer Res* 53: 1702–5.
26. Inoue A., Torigoe T., Sogahata K., Kamiguchi K., Takahashi S., Sawada Y., Saijo M., Taya Y., Ishii S., Sato N. (1995) 70-kDa heat shock cognate protein interacts directly with the N-terminal region of the retinoblastoma gene product pRb. Identification of a novel region of pRb-mediating protein interaction. *J Biol Chem* 270: 22 571–6.
27. Hartl F.U. (1991) Heat shock proteins in protein folding and membrane translocation. *Semin Immunol* 3: 5–16.
28. Kawasaki C., Okamura S., Shimoda K., Nemoto K., Niho Y. (1995) In vitro study of deoxymethylspergualin on functions of lymphocytes and bone marrow cells from healthy volunteers. *J Antibiot Tokyo* 48: 243–7.
29. Nadler S.G., Tepper M.A., Schacter B., Mazzucco C.E. (1992) Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp70 family of heat shock proteins. *Science* 258: 484–6.
30. Amemiya H. (1995) Immunosuppressive mechanisms and action of deoxyspergualin in experimental and clinical studies. Japanese Collaborative Transplant Study Group of NKT-01. *Transplant. Proceedings* 27: 31–2.
31. Fujii H., Takada T., Nemoto K., Abe F., Fujii A., Talmadge J.E., Takeuchi T. (1992) Deoxyspergualin, a novel immunosuppressant, markedly inhibits human mixed lymphocyte reaction and cytotoxic T-lymphocyte activity in vitro. *Int J Immunopharmacol* 14: 731–7.
32. Suzuki K., Sahara H., Okada Y., Yasoshima T., Hirohashi Y., Nabeta Y., Hirai I., Torigoe T., Takahashi S., Matsuura A., Takahashi N., Sasaki A., Suzuki M., Hamuro J., Ikeda H., Wada Y., Hirata K., Kikuchi K., Sato N. (1999) Identification of natural antigenic peptides of a human gastric signet ring cell carcinoma recognized by HLA-A31-restricted cytotoxic T lymphocytes. *J Immunol* 163: 2783–91.
33. Hosken N.A., Bevan M.J. (1990) Defective presentation of endogenous antigen by a cell line expressing class I molecules. *Science* 248: 367–70.
34. Yasoshima T., Sato N., Hirata K., Kikuchi K. (1995) The mechanism of human autologous gastric signet ring cell tumor rejection by cytotoxic T lymphocytes in the possible context of HLA-A31 molecule. *Cancer* 75: 1484–9.
35. Nossner E., Goldberg J.E., Naftzger C., Lyu S.C., Clayberger C., Krensky A.M. (1996) HLA-derived peptides which inhibit T cell function bind to members of the heat-shock protein 70 family. *J Exp Med* 183: 339–48.
36. Fourie A.M., Sambrook J.F., Gething M.J. (1994) Common and divergent peptide binding specificities of hsp70 molecular chaperones. *J Biol Chem* 269: 30 470–8.
37. Androlewicz M.J., Anderson K.S., Cresswell P. (1993) Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc Natl Acad Sci USA* 90: 9130–4.
38. Anderson K., Cresswell P., Gammon M., Hermes J., Williamson A., Zweerink H. (1991) Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I- restricted cell-mediated lysis. *J Exp Med* 174: 489–92.
39. Brot N., Redfield B., Qiu N.H., Chen G.J., Vidal V., Carlino A., Weissbach H. (1994) Similarity of nucleotide interactions of BiP and GTP-binding proteins. *Proc Natl Acad Sci USA* 91: 12 120–4.
40. Hoeger P.H., Tepper M.A., Faith A., Higgins J.A., Lamb J.R., Geha R.S. (1994) Immunosuppressant deoxyspergualin inhibits antigen processing in monocytes. *J Immunol* 153: 3908–16.
41. Takasu S., Sakagami K., Morisaki F., Kawamura T., Haisa M., Oiwa T., Inagaki M., Hasuoka H., Kurozumi Y., Orita K. (1991) Immunosuppressive mechanism of 15-deoxyspergualin on sinusoidal lining cells in swine liver transplantation: suppression of MHC class II antigens and interleukin-1 production. *J Surgical Res* 51: 165–9.
42. Waaga A.M., Ulrichs K., Krzymanski M., Treumer J., Hansmann M.L., Rommel T., Muller-Ruchholtz W. (1990) The immunosuppressive agent 15-deoxyspergualin induces tolerance and modulates MHC-antigen expression and interleukin-1 production in the early phase of rat allograft responses. *Transplant Proceedings* 22: 1613–4.
43. Nadeau K., Nadler S.G., Saulnier M., Tepper M.A., Walsh C.T. (1994) Quantitation of the interaction of the immunosuppressant deoxyspergualin and analogs with Hsc70 and Hsp90. *Biochemistry* 33: 2561–7.
44. Fujii H., Takada T., Nemoto K., Abe F., Takeuchi T. (1989) Stability and immunosuppressive activity of deoxyspergualin in comparison with deoxymethylspergualin. *Transplant Proc* 21: 3471–3.
45. Maeda H., Sahara H., Mori Y., Torigoe T., Kamiguchi K., Tamura Y., Hirata K., Sato N. (2007) Biological heterogeneity of the peptide-binding motif of the 70-kDa heat shock protein by surface plasmon resonance analysis. *J Biol Chem* 282: 26 956–62.
46. Blachere N.E., Li Z., Chandawarkar R.Y., Suto R., Jaikaria N.S., Basu S., Udono H., Srivastava P.K. (1997) Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med* 186: 1315–22.
47. Bendz H., Ruhland S.C., Pandya M.P., Hainzl O., Riegelsberger S., Brauchle C., Mayer M.P., Buchner J., Issels R.D., Noessner E. (2007) Human heat shock protein 70 enhances tumor antigen presentation through complex formation and intracellular antigen delivery without innate immune signaling. *J Biol Chem* 282: 31688–702.
48. Kammerer R., Stober D., Riedl P., Oehninger C., Schirmbeck R., Reimann J. (2002) Noncovalent association with stress protein

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facilitates cross-priming of CD8 + T cells to tumor cell antigens by dendritic cells. *J Immunol* 168: 108–17.
49. Tamura Y., Peng P., Liu K., Daou M., Srivastava P.K. (1997) Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 278: 117–20.
50. Ueda G., Tamura Y., Hirai I., Kamiguchi K., Ichimiya S., Torigoe T., Hiratsuka H., Sunakawa H., Sato N. (2004) Tumor-derived heat shock protein 70-pulsed dendritic cells elicit tumor-specific cytotoxic T lymphocytes (CTLs) and tumor immunity. *Cancer Sci* 95: 248–53.
51. Panjwani N., Akbari O., Garcia S., Brazil M., Stockinger B. (1999) The HSC73 molecular chaperone: involvement in MHC class II antigen presentation. *J Immunol* 163: 1936–42.
52. Schirmbeck R., Bohm W., Reimann J. (1997) Stress protein (hsp73)-mediated, TAP-independent processing of endogenous, truncated SV40 large T antigen for Db-restricted peptide presentation. *Eur J Immunol* 27: 2016–23.
53. Chen D., Androlewicz M.J. (2001) Heat shock protein 70 moderately enhances peptide binding and transport by the transporter associated with antigen processing. *Immunol Lett* 75: 143–8.
54. Rassow J., Voos W., Pfanner N. (1995) Partner proteins determine multiple functions of Hsp70. *Trends in Cell Biol* 5: 207–12.
55. Gierasch L.M. (1994) Molecular chaperones. Panning for chaperone-binding peptides. *Curr Biol* 4: 173–4.
56. Basu S., Srivastava P.K. (1999) Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J Exp Med* 189: 797–802.
57. Kunisawa J., Shastri N. (2003) The group II chaperonin TRiC protects proteolytic intermediates from degradation in the MHC class I antigen processing pathway. *Mol Cell* 12: 565–76.
58. Yamano T., Murata S., Shimbara N., Tanaka N., Chiba T., Tanaka K., Yui K., Udono H. (2002) Two distinct pathways mediated by PA28 and hsp90 in major histocompatibility complex class I antigen processing. *J Exp Med* 196: 185–96.
59. Nadler S.G., Eversole A.C., Tepper M.A., Cleaveland J.S. (1995) Elucidating the mechanism of action of the immunosuppressant 15-deoxyspergualin. *Ther Drug Monit* 17: 700–3.
60. Nadler S.G., Dischino D.D., Malacko A.R., Cleaveland J.S., Fujihara S.M., Marquardt H. (1998) Identification of a binding site on Hsc70 for the immunosuppressant 15- deoxyspergualin. *Biochem Biophys Res Commun* 253: 176–80.
61. Kunisawa J., Shastri N. (2006) Hsp90alpha chaperones large C-terminally extended proteolytic intermediates in the MHC class I antigen processing pathway. *Immunity* 24: 523–34.
62. Uebel S., Kraas W., Kienle S., Wiesmuller K.-H., Jung G., Tampe R. (1997) Recognition principle of the TAP-transporter disclosed by combinatorial peptide libraries. *Proc Natl Acad Sci USA* 94: 8976–81.
63. Beckmann R.P., Mizzen L.E., Welch W.J. (1990) Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* 248: 850–54.
64. Jordan R., McMacken R. (1995) Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins. *J Biol Chem* 270: 4563–9.
65. Szabo A., Langer T., Schroder H., Flanagan J., Bukau B., Hartl F.U. (1994) The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system-DnaK, DnaJ, and GrpE. *Proc Natl Acad Sci USA* 91: 10 345–9.
66. Freeman B.C., Myers M.P., Schumacher R., Morimoto R.I. (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *Embo J* 14: 2281–92.

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Research

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Clinical and immunological evaluation of anti-apoptosis protein, survivin-derived peptide vaccine in phase I clinical study for patients with advanced or recurrent breast cancer

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Abstract

Background: We previously reported that survivin-2B, a splicing variant of survivin, was expressed in various types of tumors and that survivin-2B peptide might serve as a potent immunogenic cancer vaccine. The objective of this study was to examine the toxicity of and to clinically and immunologically evaluate survivin-2B peptide in a phase I clinical study for patients with advanced or recurrent breast cancer.

Methods: We set up two protocols. In the first protocol, 10 patients were vaccinated with escalating doses (0.1–1.0 mg) of survivin-2B peptide alone 4 times every 2 weeks. In the second protocol, 4 patients were vaccinated with the peptide at a dose of 1.0 mg mixed with IFA 4 times every 2 weeks.

Results: In the first protocol, no adverse events were observed during or after vaccination. In the second protocol, two patients had induration at the injection site. One patient had general malaise (grade 1), and another had general malaise (grade 1) and fever (grade 1). Peptide vaccination was well tolerated in all patients. In the first protocol, tumor marker levels increased in 8 patients, slightly decreased in 1 patient and were within the normal range during this clinical trial in 1 patient.

With regard to tumor size, two patients were considered to have stable disease (SD). Immunologically, in 3 of the 10 patients (30%), an increase of the peptide-specific CTL frequency was detected. In the second protocol, an increase of the peptide-specific CTL frequency was detected in all 4 patients (100%), although there were no significant beneficial clinical responses. ELISPOT assay showed peptide-specific IFN-! responses in 2 patients in whom the peptide-specific CTL frequency in tetramer staining also was increased in both protocols.

Conclusion: This phase I clinical study revealed that survivin-2B peptide vaccination was well tolerated. The vaccination with survivin-2B peptide mixed with IFA increased the frequency of peptide-specific CTL more effectively than vaccination with the peptide alone, although neither vaccination could induce efficient clinical responses. Considering the above, the addition of another effectual adjuvant such as a cytokine, heat shock protein, etc. to the vaccination with survivin-2B peptide mixed with IFA might induce improved immunological and clinical responses.

Background

The incidence of breast cancer has continuously increased in Japan, similar to European countries and the USA, whereas mortality from breast cancer has declined, indicating improving survival because of the development of early diagnosis [1-3]. However, metastatic recurrence still occurs, and once the cancer has spread beyond the breast and locoregional nodal areas it is felt to be incurable [4]. In the case of metastatic recurrence, the prevailing treatment is systemic chemotherapy, which is fraught with various adverse effects. Thus, we considered the availability of immunotherapy, which is generally reported to be safe, for advanced or recurrent breast cancer.

Tumor cells express antigens that can be recognized by the host's immune system. In the past decade, many antigenic peptides, which can be recognized by CTLs, have been identified [5-9]. As a result, clinical trials of peptide-based immunotherapy for cancer have taken place. Melanoma antigen peptides were the first to be tested in phase I and phase II studies for active immunization of metastatic melanoma patients [10,11]. Recently, there are reports of clinical trials for various cancers, including colorectal cancer [12], esophageal cancer [13], pancreatic cancer [14], among others. However, most clinical trials did not demonstrate sufficient anti-tumor clinical responses. Thus, it is necessary to establish peptide-based immunotherapy that can induce sufficient clinical responses.

Survivin was initially isolated as one of the inhibitors of the apoptosis protein family with only one baculovirus inhibitor of apoptosis protein (IAP) repeat domain [15]. Survivin is aberrantly expressed in various cancer cells but is undetectable in normal differentiated adult tissues, with the exception of the testis, thymus and placenta. We have previously reported that survivin-2B, a splicing variant of survivin, is expressed in various tumor cell lines [16], and the survivin-2B80-88 (AYACNTSTL) peptide derived from the exon 2B-encoded region is recognized by CD8+ CTLs in the context of HLA-A24 molecules [16]. In addition, we recently reported further evidence that survivin-2B80-88 peptide might serve as a potent immunogenic cancer vaccine for various cancer patients [17]. In that report, we demonstrated that overexpression of survivin was detected in surgically resected primary tumor specimens of most breast cancers in an immunohistochemical study. In addition, HLA-A24/survivin-2B80-88 tetramer analysis revealed that there were an increased number of CTL precursors in peripheral blood mononuclear cells (PBMCs), and in vitro stimulation of PBMCs from 6 breast cancer patients with survivin-2B80-88 peptide led to increases of the CTL precursor frequency. Furthermore, CTLs specific for this peptide were successfully induced in PBMCs from all 7 HLA-A24+ patients (100%) with breast cancers and exhibited cytotoxicity against HLA-A24+/survivin+ adenocarcinoma cells [17]. On the basis of these studies, we started a phase I clinical study of vaccination with survivin-2B peptide for patients with advanced or recurrent breast cancer.

Methods

Patient selection

The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan. All patients gave informed consent before being enrolled. Patients enrolled in this study were required to conform to the following criteria: (1) to have histologically confirmed breast cancer, (2) to be HLA-A*2402 positive, (3) to be survivin-positive in the carcinomatous lesions by immunohistochemistry, (4) to be between 20 and 85 years old, (5) to be unresectable advanced cancer or recurrent cancer and (6) to have Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 3. Exclusion criteria included (1) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks, (2) the presence of other cancers that might influence the prognosis, (3) immunodeficiency or a history of splenectomy, (4) severe cardiac insufficiency, acute infection, or hemat-

opoietic failure, (5) ongoing breast-feeding and (6) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, Sapporo Medical University Primary Hospital from July 2003 through November 2005.

Peptide preparation

The peptide, survivin-2B80-88 with the sequence **AYACNTSTL**, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA). The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by high pressure liquid chromatography analysis.

The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 ml of physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and stored at -80°C until just before use.

Incomplete Freund's Adjuvant (IFA) preparation

Montanide ISA 51 (SEPPIC Inc., NJ, USA) was used as incomplete Freund's adjuvant (IFA).

Patient treatment

In this phase I clinical study, two protocols were used. One was a basic protocol, namely, with survivin-2B peptide alone, and the other used survivin-2B peptide mixed with IFA. In this trial, the primary endpoint was safety. The second endpoint was investigations about anti-tumor effects and clinical and immunological monitoring.

In the first protocol, the vaccination schedule was as follows. Vaccinations with survivin-2B peptide were given subcutaneously (s.c.) four times at 14-day intervals. To set up a dose-escalation trial, the patients were separated into the following two groups: in group 1 patients were vaccinated with 0.1 mg of the peptide and in group 2 patients were vaccinated with 1.0 mg of the peptide. Each group included five patients. Escalation to the next dose was allowed if side effects did not exceed grade 3. If patients whose disease was not far advanced hoped for continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination.

In the second protocol, survivin-2B peptide at a dose of 1 mg/1 ml and IFA at a dose of 1 ml were mixed immediately before vaccination. Then the patients were vaccinated subcutaneously (s.c.) four times at 14-day intervals.

Delayed-type hypersensitivity (DTH) skin test

A DTH skin test was performed at each vaccination. The peptide (10 "g) solution in physiological saline (0.1 ml) or physiological saline alone (0.1 ml) was separately injected intradermally (i.d.) into the forearm opposite the vaccination site. A positive reaction was defined as a more than 4 mm diameter area of erythema and induration 48 hr after the injection.

Toxicity evaluation

Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC).

Clinical response evaluation

Physical examinations and hematological examinations were conducted before and after each vaccination. Tumor markers (CEA, CA15-3, NCC-ST-439, and ICTP) were examined monthly.

Tumor size was evaluated by computed tomography (CT) scans or MRI in comparison with the size before the first vaccination and that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response (PR) was defined as a 30% decrease from the baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for CR, PR, or PD. Patients who received less than four vaccinations were excluded from all evaluations in this study.

In vitro stimulation of PBMC

PBMCs were isolated from blood samples by Ficoll-Conray density gradient centrifugation. Then they were frozen and stored at -80°C. As needed, frozen PBMCs were thawed and incubated in the presence of 30 "l/ml survivin-2B peptide in AIM-V medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/ml 1 hr, 2 days, 4 days and 6 days after the addition of the peptide. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

Tetramer staining

FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) tetramer and PE-labeled HLA-A*2402-Survivin-2B80-88 peptide tetramer were purchased from MBL Inc.(Japan). For flow cytometric analysis, PBMCs, which were stimulated in vitro as above, were stained with the tetramers at 37°C for 20 min, followed by staining with FITC- or PerCP-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (BD Biosciences). The frequency of

CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.

ELISPOT assay

ELISPOT plates were coated sterilely overnight with an IFN-! capture antibody (Beckton Dickinson Biosciences) at 4°C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 hr at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well), which were stimulated in vitro as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) (5×10^4 cells/well), which had been preincubated with or without survivin-2B80-88 peptide (10 "g/ml) and with an HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 hours, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-! antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Germany).

Results

Patient profiles

In the first protocol with survivin-2B peptide alone, 12 patients were initially enrolled in the study (Additional file 1), but two (cases 7 and 10) discontinued halfway through the protocol. One patient (case 7) had local recurrence, brain and lung metastases from bilateral breast cancer and was removed from the study after 3 vaccinations since new brain metastasis appeared and she required radiation therapy. Another patient (case 10) had lymph node metastases from right breast cancer. She was removed from the study after 3 vaccinations because of enlargement of lymph node metastases. Neither of the treatment interruptions was due to adverse effects of the vaccination. Ten patients received the complete regimen including four vaccinations and were evaluated. They were all women, whose average age was 49 years (range, 34–71).

In the second protocol with survivin-2B peptide mixed with IFA, five patients were initially enrolled in the study (Additional File 2), but one (case 2) discontinued halfway through the protocol. This patient had lung and liver metastases from right breast cancer and was removed from the study after 3 vaccinations because of exacerbated liver function resulting from advanced liver metastases. In this protocol, there were no patients who dropped out because of adverse events due to the peptide vaccination. Four patients received the complete regimen including four vaccinations and were evaluated. They were all women, whose average age was 52 years (range, 36–71).

Safety

Peptide vaccination was well tolerated in all patients. In patients vaccinated with the peptide alone, no adverse events were observed during or after vaccination (Additional File 3). Of the patients vaccinated with the peptide mixed with IFA, two (cases 1 and 3) had induration at the injection site (Additional File 4). One (case 4) had general malaise (grade 1) and one (case 5) had general malaise (grade 1) and fever (grade 1). No other severe adverse events were observed during or after vaccination.

Clinical responses

Table 3 summarizes the clinical outcomes for the 10 patients in the first protocol with survivin-2B peptide alone. In all patients except two (cases 1 and 9), the tumor marker levels were increased. In one patient (case 1), the levels of CEA, CA15-3 and NCC-ST-439 were within the normal range during this clinical trial. The level of ICTP was decreased from 7.2 ng/ml just before the first vaccination to 5.5 ng/ml after the fourth vaccination. However, this change was not considered a significant decrease. In case 9, all tumor marker levels were within the normal range during this clinical trial. As for tumor size, two patients (cases 3 and 5) were considered to have SD. In one patient (case 9) who had bone metastases, the area of bone metastases did not increase in bone scintigraphy, and new metastatic foci did not appear during this trial. In this patient, although there was no aggravation of the clinical condition, we could not estimate the clinical response by our criteria because bone metastases were not able to be evaluated in CT images. The other patients were considered to have progressive disease (PD). In this protocol, if patients whose disease was not far advanced hoped for the continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination. There were 2 patients (cases 3 and 9) who were vaccinated for more than one year. In case 3, with bone and lymph node metastases, vaccination continued 42 times for 20 months (Fig. 1). For this period, new metastatic foci did not appear and there was almost no increase in the size of the metastatic lesions in this patient. Tumor marker levels did not increase rapidly (Fig. 1). In addition, she maintained good quality of life because there was no adverse effect.

Table 4 summarizes the clinical outcomes for the 4 patients in the second protocol with survivin-2B peptide mixed with IFA. In all patients, the tumor marker levels were increased. As for tumor size, all patients were considered to have PD.

DTH skin test

A DTH skin test was performed at each vaccination and assessed 48 hr later. A positive reaction was defined as erythema and induration more than 4 mm in diameter. In

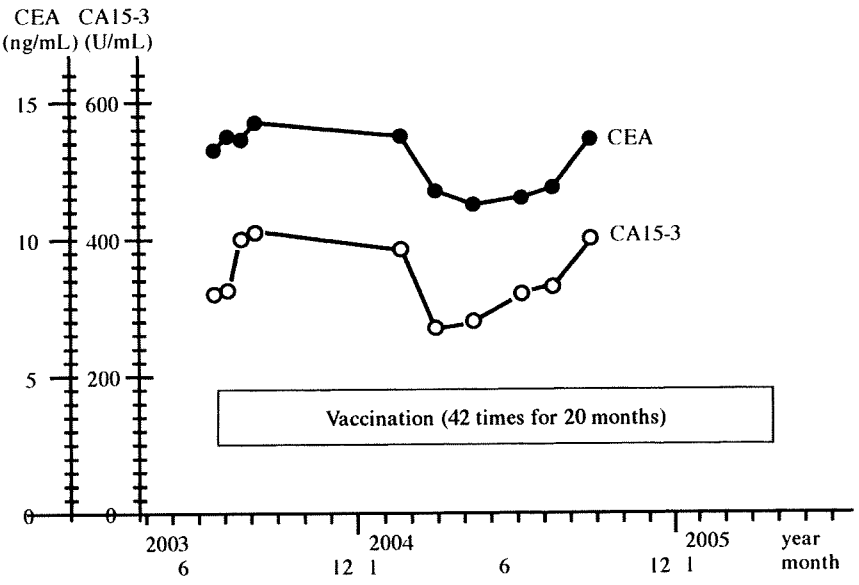

Figure 1
The changes in tumor marker levels in case 3 in the first protocol. For case 3, with bone and lymph node metastases, vaccination continued 42 times for 20 months. In this period new metastatic foci did not appear and there was almost no increase in size of the metastatic lesions. Tumor marker levels did not increase rapidly.

the first protocol with survivin-2B peptide alone, 2 of the 10 patients (20%) exhibited a positive DTH reaction at least once during the study. In the second protocol with peptide mixed with IFA, a positive DTH reaction was observed in 1 of the 4 patients (25%).

Tetramer staining assay and ELISPOT assay

To determine if the survivin-2B peptide vaccination could bring about specific immune responses in the patients, we analyzed the peptide-specific CTL frequency by using the HLA-A24/peptide tetramer. The change of tetramer-positive CTL frequency was evaluated by comparison with that before the first vaccination and that after the fourth vaccination as follows: detected and undetected. Detected was defined as an increase of twofold or more. Undetected was defined as a less than twofold increase. In the first protocol with the peptide alone, a change was considered to be detected in 3 patients (30%) (Table 3). On the other hand, in the second protocol with peptide mixed with IFA, it was considered to be detected in all 4 patients (100%) (Table 4). In Figure 2, the peptide-specific CTL frequency in the second protocol is indicated as the percentage of tetramer-positive CTL cells among CD8-positive T cells before the first vaccination and after the fourth vaccination.

ELISPOT assay of CD8-positive T cells separated from the patients' PBMCs showed peptide-specific IFN-! responses in 2 patients, one of whom (case 8) was in the first protocol while the other (case 5) was in the second protocol. To be more precise, in case 5 in the second protocol, in the wells that were preincubated without the survivin-2B peptide and with the HIV peptide, spots were almost not visualized. On the other hand, in the wells that had been preincubated with survivin-2B peptide, many spots were visualized (Fig. 3). In these two patients, the peptide-specific CTL frequency was also increased by the vaccination with survivin-2B peptide.

Discussion

Recently, a large number of tumor antigens and epitopes recognized by CTLs have been identified, and reports of clinical trials utilizing peptide vaccination are increasing [10,18-20]. We demonstrated that survivin was expressed in a large proportion of various cancer specimens, and the survivin-2B-derived peptide could induce a CTL response in the context of HLA-A24 [16,17]. In addition, we showed an elevation in CTL precursor frequencies in PBMCs of HLA-A24+ cancer patients by using an HLA-A24/survivin-2B peptide tetramer. On the basis of the above studies, we started a phase I clinical study of survivin-2B peptide vaccine therapy for patients with advanced or recurrent colorectal cancer in 2003 [12]. In

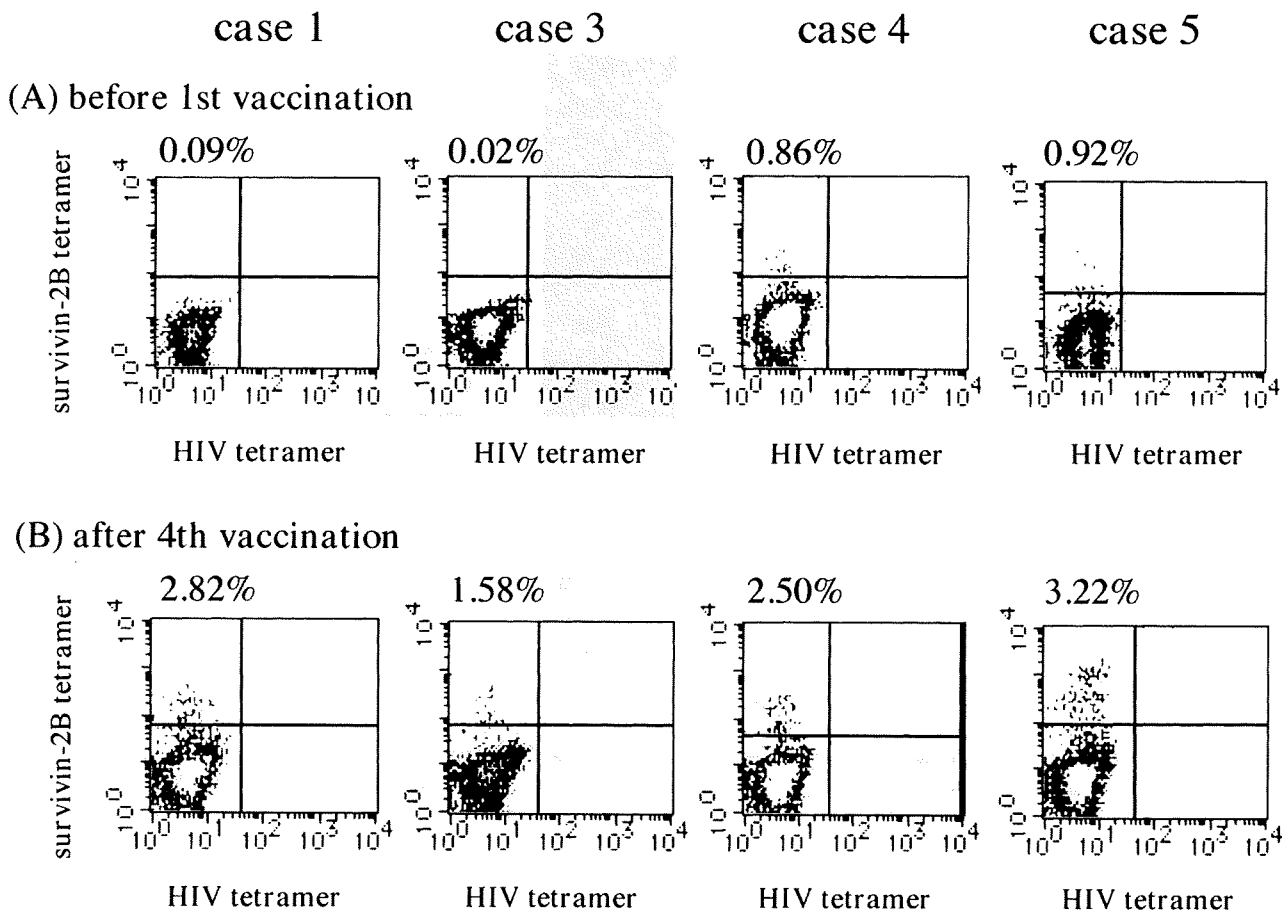

Figure 2

Tetramer staining before the first vaccination and after the fourth vaccination in the second protocol. FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) tetramer and PE-labeled HLA-A*2402-Survivin-2B80-88 peptide tetramer were uesd. For flow cytometric analysis, PBMCs, which were stimulated *in vitro*, were stained with the tetramers at 37°C for 20 min, followed by staining with FITC- or PerCP-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (BD Biosciences). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells. The peptide-specific CTL frequency is indicated as the percentage of tetramer-positive CTL cells among CD8-positive T cells before the first vaccination and after the fourth vaccination. The peptide-specific CTL frequency after the fourth vaccination (B) was compared with that before the first vaccination (A). In the second protocol with the peptide mixed with IFA, the peptide-specific CTL frequency was increased in all 4 patients (100%).

this study, we vaccinated patients with survivin-2B peptide alone, and reported the safety of this peptide vaccination and the potency of anti-tumor effects induced by the peptide vaccination. At that time, we started this phase I clinical study of peptide vaccine therapy for patients with advanced or recurrent breast cancer as well. In this study, we vaccinated patients with not only survivin-2B peptide alone but also survivin-2B mixed with IFA in order to induce greater anti-tumor effects. We recently established immunological monitoring methods using a tetramer staining assay and ELISPOT assay. Thus, in this study, we also conducted immunological monitoring using these techniques.

Survivin is an ideal tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells [21]. The first survivin-derived peptides were characterized in 2000 [22,23]. Subsequently, there

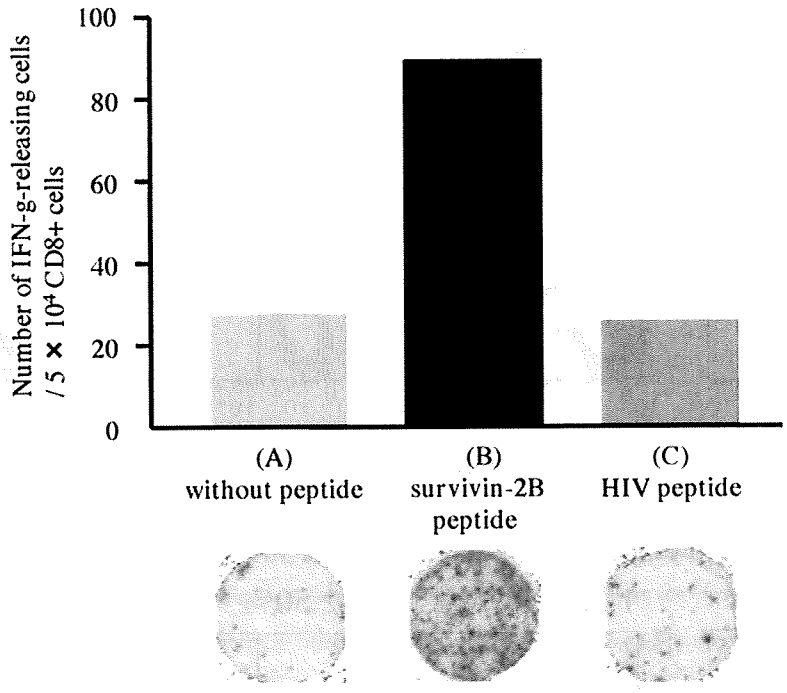

Figure 3
ELISPOT assay after the fourth vaccination of case 5 in the second protocol. In the wells that were preincubated without survivin-2B peptide (A) or with an HIV peptide (C), spots were almost not visualized. On the other hand, in the wells that were preincubated with survivin-2B peptide (B), many spots were visualized. These findings demonstrate that CD8-positive T cells separated from the patients' PBMCs had a peptide-specific IFN-! response.

have been many reports about survivin peptide responses. Grube et al. [24] reported that HLA-A2.1-restricted survivin peptide induced CD8+ T cell reactivity in patients with multiple myeloma. Andersen et al. [25] reported the detection of HLA-A24 restricted and survivin peptide-specific CD8-positive cells by IFN-! ELISPOT assay and perforin ELISPOT assay in patients with breast cancer, melanoma and renal cancer. Abrogating the function of survivin not only limits the proliferative potential and viability of tumor cells directly [26], but also inhibits tumor angiogenesis [27]. Xiang et al. [28] reported that a DNA vaccine targeting survivin lead to eradication of pulmonary metastases by a combinational effect inducing tumor cell apoptosis and suppressing tumor angiogenesis in a murine model. Thus, survivin is a suitable target for immune therapy for cancer [26,29]. Recently, a number of survivin epitopes restricted to several additional HLA-molecules have been identified [22,25,29-34], and several clinical trials of immunotherapy based on survivin-derived peptides have been initiated. Wobser et al. [35] reported complete remission of liver metastasis of pancreatic cancer under vaccination with an HLA-A2 restricted survivin peptide. In addition, a phase I/II trial with HLA-A1, -A2 and -B35 restricted survivin peptides for patients with advanced cancer is ongoing. Fuessel et al. [27] reported a phase I clinical trial for patients with prostate cancer in which they evaluated a vaccination with DCs loaded with five different prostate cancer-associated antigens (survivin, prostate-specific antigen [PSA], prostate-specific membrane antigen [PSMA], and prostein, transient receptor potential p8 [trp-p8]) and concluded that the concept was safe and feasible. Besides the above-mentioned investigations, various clinical trials are ongoing now.

At present, 4 splicing variants of survivin (survivin-#Ex3, survivin-2$, survivin-2B, and survivin-3B) have been identified. Espinosa et al. [36] reported that the expression of survivin-#Ex3 and survivin-2B was higher in cervical cancer samples. There is also a report that survivin-2B was dominantly expressed in gastric cancer [37]. Futakuchi et al. [38] reported that the ratios of survivin-2B/survivin and survivin-#Ex3/survivin in malignant cervical tissue samples were significantly higher than those in normal cervical tissue templates. Moreover, the ratio of survivin-2B/survivin was increased in patients with higher stages and with pelvic lymph node metastasis. These reports might support the idea that survivin-2B is the ideal

target of immunotherapy for cancer patients [17,39], especially for those with advanced or recurrent cancer. On the other hand, Mahotka et al. [40] reported that the ratio of survivin-2B/survivin was decreased in the late stages of renal cell carcinoma. Yamada et al. [41] reported that there was no significant difference in the ratio of survivin-2B/survivin in malignant brain tumors and gliomas compared with nonglioma. There is a hypothesis that the relevant ratios of the survivin and splicing variants may regulate the ultimate apoptotic activities of cancer cells and determine their biological behaviours and responses to apoptosis-inducing treatment [37,40]. Nevertheless, the exact roles and expression of survivin splicing variants and their interplay in various cancers are as yet unclear because of the high complexity of its regulation [36,42]. We previously demonstrated that the expression of survivin-2B was detected in a variety of tumor cell lines but not in normal tissues except in the thymus, although low levels of survivin expression were detected by reverse transcription-PCR analysis [16]. In addition, we reported that survivin-2B-specific CTLs could be induced efficiently from PBMCs of HLA-A24-positive survivin-positive cancer patients [17]. As described above, we are sure that survivin-targeting immunotherapy with survivin-2B peptide should be a reasonable strategy.

A dose-escalation trial was chosen to estimate a safe and optimal dose in the first protocol with survivin-2B peptide alone. We used 0.1 mg and 1.0 mg dosage groups, each consisting of five patients. No adverse events were observed in either group. In addition, for the patients (cases 3 and case 9) who were vaccinated 42 times and 38 times respectively, adverse events were not observed during or after the vaccination. Thus, we concluded that the survivin-2B peptide vaccine was safe and could be repeatedly injected into patients without severe adverse events. In comparison between patients who were vaccinated with 0.1 mg and 1.0 mg of the peptide, there was almost no difference in clinical responses. However, peptide-specific immune responses in tetramer staining and ELISPOT assay were frequently induced in patients vaccinated with 1.0 mg of the peptide in comparison with patients vaccinated with 0.1 mg of the peptide. Therefore, we decided that the optimal dose of the peptide was 1.0 mg. IFA has sustained-release effect, which can enhance the anti-tumor effect of the peptide injected subcutaneously. So, in the second protocol which its purpose was to induce the more effective anti-tumor effect by the survivin-2B peptide, IFA was used mixed with 1.0 mg of the peptide. In this protocol, two patients (cases 1 and 3) had induration. This was due to IFA trapped in the subcutaneous lesion. Patient 4 had general malaise (grade 1), and patient 5 had general malaise (grade 1) and fever (grade 1). No other severe adverse events were observed during or after vaccination. Therefore, we concluded that the vaccine using survivin-2B peptide mixed with IFA was safe, as was the peptide alone.

Positive delayed-type hypersensitivity (DTH) reactions were observed in 2 of the 10 patients (20.0%) in the first protocol and in 1 of the 4 patients (25.0%) in the second protocol at least once during the vaccination. Some reports have suggested a positive correlation between DTH and clinical [43] or immunological responses [44]. In this study, in case 5 in the first protocol a positive DTH reaction was observed and the change of tumor size was considered to indicate SD, while the tumor marker level was considered to have increased, although immunological responses were not induced. However, neither clinical nor immunological responses were totally associated with a positive DTH reaction in this study.

In the first protocol with survivin-2B peptide alone, none of patients in the 0.1 mg peptide group had tetramer response and that 3 of the 5 patients (30%) in the 1.0 mg peptide group had increased the tetramer-specific CTL frequency. On the other hand, in the second protocol with survivin-2B peptide mixed with IFA, all patients had a significant increase of the tetramer-positive CTL frequency. These results might demonstrate that the addition of IFA could enhance the immunological responses to the survivin-2B peptide. In addition, these findings might also indicate that the addition of another effectual adjuvant such as a cytokine, heat shock protein [45], etc. to the vaccination with survivin-2B peptide mixed with IFA could more effectively enhance the immunological and clinical responses to the peptide. At present a phaseII clinical study of survivin-2B peptide vaccine therapy, in which the peptide is combined with IFA and IFN-$, is ongoing in our group.

In the second protocol with the peptide mixed with IFA, although all patients had an increase of the tetramer-positive CTL frequency, only one patient had a peptide specific IFN-! response in the ELISPOT assay. In tetramer staining, the frequency of the peptide-specific CTL was investigated. In the ELISPOT assay, the function of the peptide-specific CTL was investigated. It is possible that the peptide-specific CTL induced by the vaccination might not function well due to immune escape mechanisms in the effector phase. Thus, this might be one of the reasons why the CTL response to the vaccination was not sufficient to induce clinical responses. This also could imply a dysfunction of the host immune system or an immunosuppressive effect of the tumor microenvironment, including the down-regulation of HLA-class I molecules on tumor cells. Therefore, we have recently begun to investigate a novel strategy to overcome the immune escape in peptide vaccine therapy.

Conclusion

In conclusion, this phase I clinical study revealed that the administration of not only survivin-2B peptide alone but also the peptide in combination with IFA for patients with advanced or recurrent breast cancer was well tolerated. Vaccination with survivin-2B peptide mixed with IFA increased the frequency of the peptide-specific CTL more effectively than vaccination with the peptide alone, although neither vaccination could induce an efficient clinical response. Thus, the addition of IFA might enhance the immunological response to the peptide vaccination. Considering the above, the addition of another effectual adjuvant such as a cytokine, heat shock protein [45], etc. to the vaccine using survivin-2B peptide mixed with IFA might induce improved immunological and clinical responses.

List of abbreviations

PBMCs: peripheral blood mononuclear cells, CTL: cytotoxic T lymphocyte, IAP: inhibitor of the apoptosis proteins, ECOG: Eastern Co-operative Oncology Group, IFA: incomplete Freund's adjuvant, DTH: delayed-type hypersensitivity, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT[1] performed peptide vaccine preparation, and contributed largely to designing the phase I clinical study, coordination of this study and analysis of all results. TT[1] and YI contributed to the medical examination and vaccination of patients, and interpretation of clinical data. TO, TK, FH, TF, KY, YK and NT contributed to the medical care. TT2 and YH contributed to designing the peptide vaccine. TT[2] contributed to interpretation of immunological data. TT[2], AY, HA and KS contributed to analysis of immunological responses, such as tetramer staining, ELISPOT assay, etc. MO, YM, NN contributed to registration of patients. NS and KH contributed largely to control over the clinical system and immunological study, respectively, and they also contributed to reviewing the manuscript. All authors have read and approved the final manuscript. (TT[1]: Tetsuhiro Tsuruma, TT[2]: Toshihiko Torigoe)

Additional material

Additional file 1

Table 1: Profiles of patients enrolled in the first protocol with survivin-2B peptide alone. The data showed profiles of patients enrolled in the first protocol with survivin-2B peptide alone.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S1.pdf]

Additional file 2

Table 2: Profiles of patients enrolled in the second protocol with survivin-2B peptide mixed IFA. The data showed profiles of patients enrolled in the second protocol with survivin-2B peptide mixed IFA.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S2.pdf]

Additional file 3

Table 3: Outcome in the first protocol with survivin-2B peptide alone. This data showed the clinical and immunological evaluation in the first protocol.
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[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S3.pdf]

Additional file 4

Table 4: Outcome in the second protocol with survivin-2B peptide mixed IFA. This data showed the clinical and immunological evaluation in the second protocol.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S4.pdf]

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Written consent for publication was obtained from the patient or their relative.

References

1. Talback M, Stenbeck M, Rosen M, Barlow L, Glimelius B: **Cancer survival in Sweden 1960–1998 – developments across four decades.** *Acta Oncol* 2003, **42:**637-659.
2. Edwards BK, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW: **Annual report to the nation on the status of cancer, 1975–2002, featuring population-based trends in cancer treatment.** *J Natl Cancer Inst* 2005, **97:**1407-1427.
3. Sant M, Francisci S, Capocaccia R, Verdecchia A, Allemani C, Berrino F: **Time trends of breast cancer survival in Europe in relation to incidence and mortality.** *Int J Cancer* 2006, **119:**2417-2422.
4. Gralow JR: **Breast cancer 2004: Progress and promise on the clinical front.** *Phys Med* 2006, **21(Suppl 1):**2.
5. Boon T, Coulie PG, Eynde B Van den: **Tumor antigens recognized by T cells.** *Immunol Today* 1997, **18:**267-268.
6. Rosenberg SA: **A new era for cancer immunotherapy based on the genes that encode cancer antigens.** *Immunity* 1999, **10:**281-287.
7. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Lienard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jager E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, Bruggen P van der, Boon T: **Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1.** *Int J Cancer* 1999, **80:**219-230.
8. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE: **Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma.** *Nat Med* 1998, **4:**321-327.
9. Kawaguchi S, Wada T, Ida Y, Sato Y, Nagoya S, Tsukahara T, Kimura S, Sahara H, Ikeda H, Shimozawa K, Asanuma H, Torigoe T, Hiraga H, Ishii T, Tatezaki SI, Sato N, Yamashita T: **Phase I vaccination trial**

of SYT-SSX junction peptide in patients with disseminated synovial sarcoma. *J Transl Med* 2005, **3:**1-9.
10. Bruggen P van der, Traversari C, Chomez P, Lurquin C, De Plasen E, Eynde B Van den, Knuth A, Boon T: **A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.** *Science* 1991, **254:**1643-1647.
11. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA: **Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor.** *Proc Natl Acad Sci USA* 1994, **91:**3515-3519.
12. Tsuruma T, Hata F, Torigoe T, Furuhata T, Idenoue S, Kurotaki T, Yamamoto M, Yagihashi A, Ohmura T, Yamaguchi K, Katsuramaki T, Yasoshima T, Sasaki K, Mizushima Y, Minamida H, Kimura H, Akiyama M, Hirohashi Y, Asanuma H, Tamura Y, Shimozawa K, Sato N, Hirata K: **Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer.** *J Transl Med* 2004, **2:**19-29.
13. Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, Sato A, Noguchi Y, Kawabata R, Yasuda T, Doki Y, Kumon H, Iwatsuki K, Shiku H, Monden M, Jungbluth AA, Ritter G, Murphy R, Hoffman E, Old LJ, Nakayama E: **T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein.** *Cancer Immun* 2007, **7:**9-19.
14. Yanagimoto H, Mine T, Yamamoto K, Satoi S, Terakawa N, Takahashi K, Nakahara K, Honma S, Tanaka M, Mizoguchi J, Yamada A, Oka M, Kamiyama Y, Itoh K, Takai S: **Immunological evaluation of personalized peptide vaccination with gemcitabine for pancreatic cancer.** *Cancer Sci* 2007, **98:**605-611.
15. Ambrosini G, Adida C, Altieri DC: **A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma.** *Nat Med* 1997, **3:**917-921.
16. Hirohashi Y, Torigoe T, Maeda A, Nabeta Y, Kamiguchi K, Sato T, Yoda J, Ikeda H, Hirata K, Yamanaka N, Sato N: **An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin.** *Clin Cancer Res* 2002, **8:**1731-1739.
17. Idenoue S, Hirohashi Y, Torigoe T, Sato Y, Tamura Y, Hariu H, Yamamoto M, Kurotaki T, Tsuruma T, Asanuma H, Kanaseki T, Ikeda H, Kashiwagi K, Okazaki M, Sasaki K, Sato T, Ohmura T, Hata F, Yamaguchi K, Hirata K, Sato N: **A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins.** *Clin Cancer Res* 2005, **11:**1474-1482.
18. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, Mori K, Hayashi A, Yamana H, Shirouzu K: **Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients.** *Cancer Res* 1999, **59:**4056-4063.
19. Nishizaka S, Gomi S, Harada K, Oizumi K, Itoh K, Shichijo S: **A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma.** *Cancer Res* 2000, **60:**4830-4837.
20. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Gladhaug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G: **Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: Clinical and immunological responses in patients with pancreatic adenocarcinoma.** *Int J Cancer* 2001, **92:**441-450.
21. Schmidt SM, Schag K, Muller MR, Weck MM, Appel S, Kanz L, Grunebach F, Brossart P: **Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells.** *Blood* 2003, **15:**571-576.
22. Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, Rammensee HG, Rieber EP: **Generation of survivin-specific CD8+ T effector cells by dendritic cells pulsed with protein or selected peptides.** *Cancer Res* 2000, **60:**4845-4849.
23. Andersen MH, Becker JC, Straten P: **Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients.** *Cancer Res* 2001, **61:**869-872.
24. Grube M, Moritz S, Obermann EC, Rezvani K, Mackensen A, Andressen R, Holler E: **CD8+ Tcell reactive to survivin antigen in patients with multiple myeloma.** *Clin Cacncer Res* 2007, **13:**1053-1060.
25. Andersen MH, Soerensen RB, Becker JC, Straten P: **HLA-A24 and survivin: possibilities in therapeutic vaccination against cancer.** *J Transl Med* 2006, **4:**38.
26. Andersen MH, Becker JC, Straten PT: **Regulators of apoptosis: suitable targets for immune therapy of cancer.** *Nat Rev Drug Discov* 2005, **4:**399-409.
27. Fuessel S, Meye A, Schmitz M, Zastrow S, Linne C, Richter K, Lobel B, Hakenberg OW, Hoelig K, Rieber EP, Wirth MP: **Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: results of a phase I clinical trial.** *Prostate* 2006, **66:**811-821.
28. Xiang R, Mizutani N, Luo Y, Chiodoni C, Zhou H, Mizutani M, Ba Y, Becker JC, Reisfeld RA: **A DNA vaccine targeting survivin combines apoptosis with suppression of angiogenesis in lung tumor eradication.** *Cancer Res* 2005, **65:**553-561.
29. Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, Straten PT: **Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients.** *Cancer Res* 2001, **61:**5964-5968.
30. Reker S, Becker JC, Svane IM, Ralfkiaer E, Straten PT, Andersen MH: **HLA-B35-restricted immune responses against survivin in cancer patients.** *Int J Cancer* 2004, **108:**937-941.
31. Shangary S, Johnson DE: **Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family.** *Leukemia* 2003, **17:**1470-1481.
32. Reker S, Meier A, Holten-Andersen L, Svane IM, Becker JC, Straten P, Andersen MH: **Identification of novel survivin-derived CTL epitopes.** *Cancer Biol Ther* 2004, **3:**173-179.
33. Siegel S, Steinmann J, Schmitz N, Stuhlmann R, Dreger P, Zeis M: **Identification of a survivin-derived peptide that induces HLA-A0201-restricted antileukemia cytotoxic T lymphocytes.** *Leukemia* 2004, **18:**2046-2047.
34. Andersen MH, Becker JC, Straten P: **Regulations of apoptosis: suitable targets for immune therapy of cancer.** *Nat Rev Drug Discov* 2005, **4:**399-409.
35. Wobser M, Keikavoussi P, Kunzmann V, Weininger M, Andersen MH, Becker JC: **Complete remission of liver metastasis of pancreatic cancer under vaccination with a HLA-A2 restricted peptide derived from the universal tumor antigen survivin.** *Cancer Immunol Immunother* 2006, **55:**1294-1298.
36. Espinosa M, Cantu D, Herrera N, Lopez CM, De la Garza JG, Maldonado V, Melendez-Zajgla J: **Inhibitors of apoptosis proteins in human cervical cancer.** *BMC Cancer* 2006, **6:**45-54.
37. Cheng Z, Hu L, Fu W, Zhang Q, Liao X: **Expression of survivin and its splice variants in gastric cancer.** *J Huazhong Univ Sci Technolog Med Sci* 2007, **27:**393-398.
38. Futakuchi H, Ueda M, Kanda K, Fujino K, Yamaguchi H, Noda S: **Transcriptional expression of survivin and its splice variants in cervical carcinoma.** *Int J Gynecol Cancer* 2007, **17:**1092-1098.
39. Ichiki Y, Hanagiri T, Takenoyama M, Baba T, Fukuyama T, Nagata Y, Mizukami M, So T, Sugaya M, Yasuda M, So T, Sugio K, Yasumoto K: **Tumor specific expression survivin-2B in lung cancer as a novel target of immunotherapy.** *Lung Cancer* 2005, **48:**281-289.
40. Mahotka C, Krieg T, Krieg A, Wenzel M, Suschek CV, Heydthausen M, Gabbert HE, Gerharz CD: **Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas.** *Int J Cancer* 2002, **100:**30-36.
41. Yamada Y, Kuroiwa T, Nakagawa T, Kajimoto Y, Dohi T, Azuma H, Tsuji M, Kami K, Miyatake S: **Transcriptional expression of survivin and its splice variants in brain tumors in humans.** *J Neurosurg* 2003, **99:**738-745.
42. Vegran F, Boidot R, Oudin C, Defrain C, Rebucci M, Lizard-Nacol S: **Association of p53 gene alterations with the expression of antiapoptosis survivin splice variants in breast cancer.** *Oncogene* 2007, **11:**290-297.
43. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D: **Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells.** *Nat Med* 1998, **4:**328-332.
44. Hildenbrand B, Sauer B, Kalis O, Stoll C, Freudenberg MA, Niedermann G, Giesler JM, Juttner E, Peters JH, Haring B, Leo R, Unger C, Azemar M: **Immunotherapy of patients with hormone-refractory prostate carcinoma pre-treated with interferon-gamma and vaccinated with autologous PSA-peptide loaded dendritic cells – a pilot study.** *Prostate* 2007, **67:**500-508.