proinflammatory cytokines. The capacity of HMGB1 to bind other molecules may be the underlying basis for these observations [57]. Thus, some of these DAMPs have the ability to interact with other molecules, including DNA, RNA, IL-1β, and LPS, and also nucleosomes that augment or modify the function of DAMPs themselves. As mentioned previously, HSPs can also bind a number of endogenous as well as pathogen-associated exogenous molecules and enhance the cytokine effects of these molecules. Recently, we have shown that extracellular Hsp90 can enhance the self-DNA-mediated type I IFN production by DCs. We will discuss how Hsp90 is able to change the fate of associated molecules *via* spatiotemporal regulation in the next section.

SPATIOTEMPORAL REGULATION OF RECEPTOR-LIGAND INTERACTION DETERMINES THE IMMUNE RESPONSES

The innate immune system of vertebrates has evolved to recognize unmethylated CpG dinucleotides within certain sequence contexts (CpG motifs) present at high frequency in bacterial, but not vertebrate, DNA. These sequences are recognized by TLR9, which is expressed primarily by plasmacytoid dendritic cells (pDCs) and B cells, resulting in a large amount of IFN-α production [58, 59]. In mice and humans, CpG motifs in bacterial DNA or synthetic oligodeoxynucleotides (ODNs) trigger an immune cascade, resulting in improved antigen uptake and presentation by APCs. Unlike other TLR receptors, which are present on the cell membrane, TLR9 is present within the endosome. In humans, pDCs uniquely express TLR9. Together with the constitutive expression of IRF7, TLR9 permits pDCs to mount rapid and robust type-I IFN responses to viral/microbial infections [60]. Two classes of synthetic ODNs containing an unmethylated CpG motif have been identified, CpG-A ODN, which stimulates IFN-α production by pDCs, and CpG-B ODN, which does not [59]. Recently, it has been demonstrated that the manner of CpG internalization and the retention time of CpG in endosomes differ between CpG-A and CpG-B, and the retention of the CpG/TLR9 complex in endosomes is the primary determinant of TLR signaling [61, 62]. CpG-A ODNs are characterized by a poly G tail that forms large multimeric aggregates with a diameter about 50 μm. In contrast, CpG-B ODNs are monomeric and do not form such higher order structures. In addition, multimeric CpG-A ODNs are retained for longer periods of time in the early endosomes, whereas CpG-B ODNs rapidly traffic through early endosomes into late endosomes or lysosomes of pDCs. The prolonged retention of multimeric CpG-A ODNs provides extended activation of the TLR9-MyD88-IRF7 signal-transducing complex, which leads to robust IFN-α production. Instead, CpG-B ODN stimulates pDCs to produce IL-6 and TNF-α and induce DC maturation *via* the upregulation of CD80, CD86 and the expression of MHC class II molecules. Thus, spatiotemporal regulation of receptor-ligand interaction is a critical determinant for the type of immune response.

HSP90-MEDIATED SPATIOTEMPORAL REGULATION OF CPG-ODN AND ACTIVATION OF INNATE IMMUNITY

In contrast to the idea that HSP itself acts as an endogenous danger signal, we have a working hypothesis that HSP may empower the chaperoned innate ligands to activate the innate immune response [63]. We have shown that human pDCs pulsed with an Hsp90-CpG-A ODN complex produce a higher amount of IFN-α than CpG-A alone (Fig. **1A**). Furthermore, in contrast to human DCs system, as murine conventional DCs (cDCs) express both TLR7 and TLR9 even though the expression level is low compared to pDCs, we examined the ability of Hsp90 to target and retain chaperoned CpG-A in static early endosomes of murine cDCs, resulting in IFN-α production (Fig. **1B**). We found that Hsp90-chaperoned CpG-A was localized and retained within static early endosomes for longer periods in cDCs, thereby eliciting TLR9 signaling for IFN-α production, but not inflammatory cytokines such as IL-6 and TNF-α. In contrast, CpG-A alone moved into late endosomes and lysosomes within cDCs. Interestingly, not only CpG-A but also CpG-B could stimulate the TLR9 signaling within static early endosomes, resulting in the production of IFN-α. The observed IFN-α production was shown to be TLR9 dependent. Thus, extracellular Hsp90 has the ability to direct associated CpGs into static early endosomes, which leads to IRF7 activation and IFN-α production.

Why, however, are DNA-Hsp90 complexes selectively retained in early endosomes but not in late endosomes or lysosomes in DCs? We found that endocytosed CpG-A-Hsp90 complexes were selectively transferred into $Rab5^+$, $EEA\text{-}1^+$-static early endosomes. Very recently, Lakadamyali *et al.* have shown that early endosomes are comprised of two distinct populations, a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome and a static population that matures much more slowly [64]. Cargos destined for degradation, including LDL, EGF, and influenza virus, are internalized and targeted to the $Rab5^+$, $EEA1^-$ dynamic population of early endosomes, thereafter trafficking to $Rab7^+$ late endosomes. In contrast, the recycling transferrin is delivered to $Rab5^+$, $EEA1^+$ static early endosomes, followed by translocation to $Rab11^+$ recycling endosomes. They also found that cargos trafficked into these static early endosomes were retained for longer periods and not translocated into late endosomes and lysosomes. Thus, our observation that the CpG-A-Hsp90 complex was retained in the static early endosomes, leading to sustained activation of DCs and IFN-α production, is consistent with their findings (Fig. **2**).

HSP90 AS A POSSIBLE ACCELERATOR FOR AUTOIMMUNE DISEASE

pDCs normally do not respond to self-DNA, which may reflect the fact that viral/bacterial DNA sequences contain multiple CpG nucleotides that bind and activate

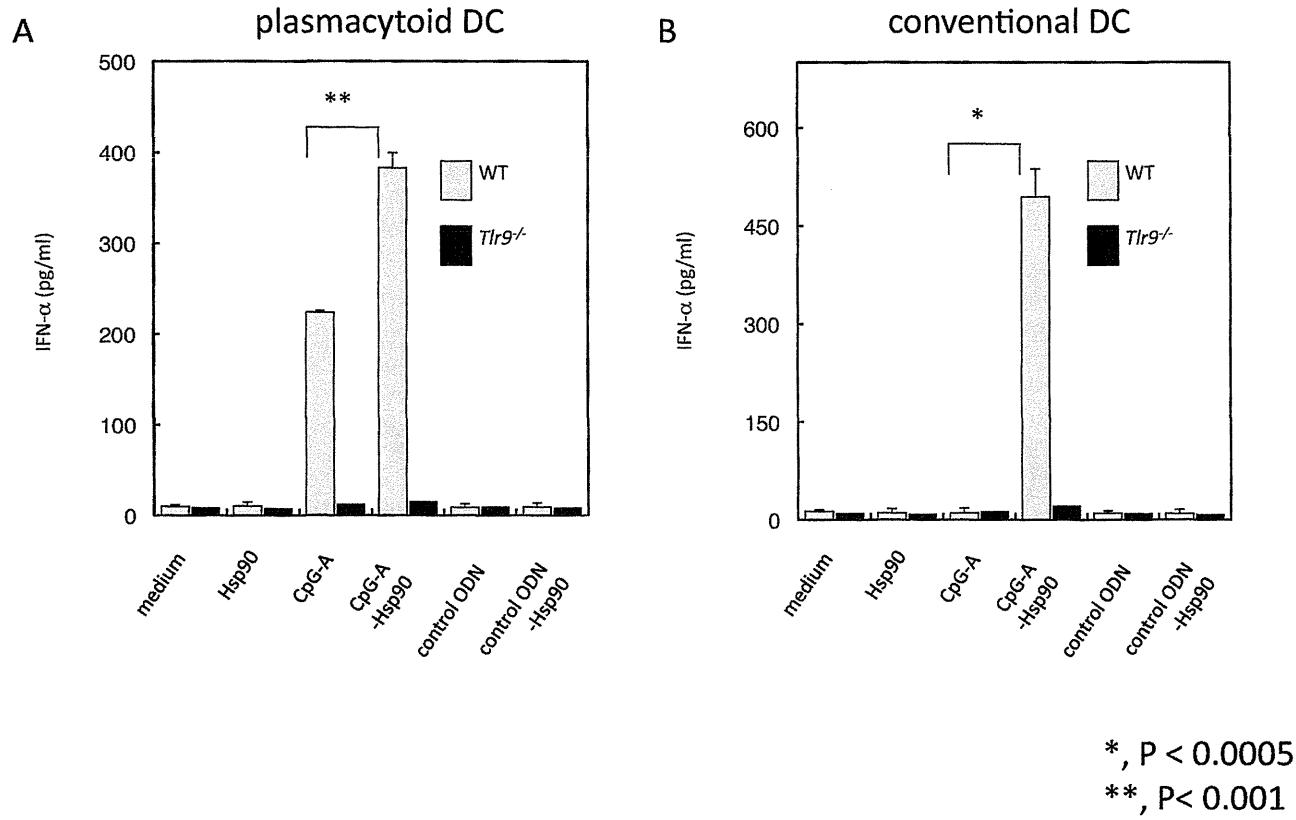

Fig. (1). Hsp90-CpG-A complexes enhance induction of IFN-α by DCs. (**A**) The Hsp90-CpG-A complexes enhance the IFN-α production 2-fold more than CpG-A alone by plasmacytoid DCs. (**B**) Conventional DCs produce the IFN-α when stimulated with Hsp90-CpG-A complex but not CpG-A alone. Hsp90 alone do not have any effects on IFN-α production. The IFN-α production is abrogated when used DCs isolated from *Tlr9*$^{-/-}$ mice, indicating that IFN-α production is TLR9 dependent.

TLR9, whereas mammalian self-DNA contains fewer such motifs, which are most likely masked by methylation. Recent evidence, however, suggests that self-DNA has the potential to trigger TLR9, but may fail to do so because it fails to access the TLR9-containing endolysosomal compartments. One of the mechanisms to which this effect is attributed is the fact that DNase easily and rapidly breaks down extracellular DNA, thereby hampering self-DNA localization into endocytic compartments. The importance of this mechanism in preventing autoimmune responses is shown by the fact that mice deficient in DNase II develop systemic lupus erythematosus (SLE)-like syndrome [65]. Recently, it has been demonstrated that pDCs do sense and respond to self-DNA in human autoimmune diseases. Therefore, we examined whether Hsp90 targets self-DNA into the static early endosome, resulting in IFN-α production by human pDCs [63]. Upon Hsp90-mediated enforced endosomal translocation, both human self-DNA and CpG-ODN could activate DCs *via* TLR9 to produce IFN-α. Previous studies have demonstrated the presence of autoantibodies to Hsp90 [66, 67] and enhanced expression of Hsp90 in PBMC of patients with active SLE [68, 69], suggesting a role of Hsp90 in the pathogenesis. In addition, Hsp90 has been shown to localize both in the cytoplasm and nucleus [70]. Moreover, under stressful conditions, it has been shown that cytosolic Hsp90 translocates to the nucleus [71]. This suggests that Hsp90 may bind self-DNA within the nucleus. When cells undergo necrosis, self-DNA associated with endogenous Hsp90 could be released into extracellular space and might trigger IFN-α production by pDCs. Our findings support the idea that Hsp90, an endogenous danger signal found in the sera from SLE patients, is the key mediator of pDC activation in SLE. Thus, Hsp90 may activate innate immunity to self-DNA by forming a complex with self-DNA that is delivered to and retained within early endocytic compartments of pDCs to trigger TLR9 and induce IFN production. Thus, we determined a fundamental mechanism by which pDCs sense and respond to self-DNA coupled with Hsp90. Our data suggest that, through this pathway, pDCs drive autoimmunity in autoimmune diseases. Several host factors other than Hsp90 that can convert self-DNA into a trigger of DC activation have been reported. Endogenous antimicrobial peptide LL37 (also known as CAMP) [72], autoantibodies [73] and HMGB1 [57] have been demonstrated to do so by forming a complex with host-derived DNA. Together, these findings indicate that the ability of some of DAMPs to convert self-DNA into a trigger of high levels of IFN-α production depends on its capacity to concentrate and retain DNA

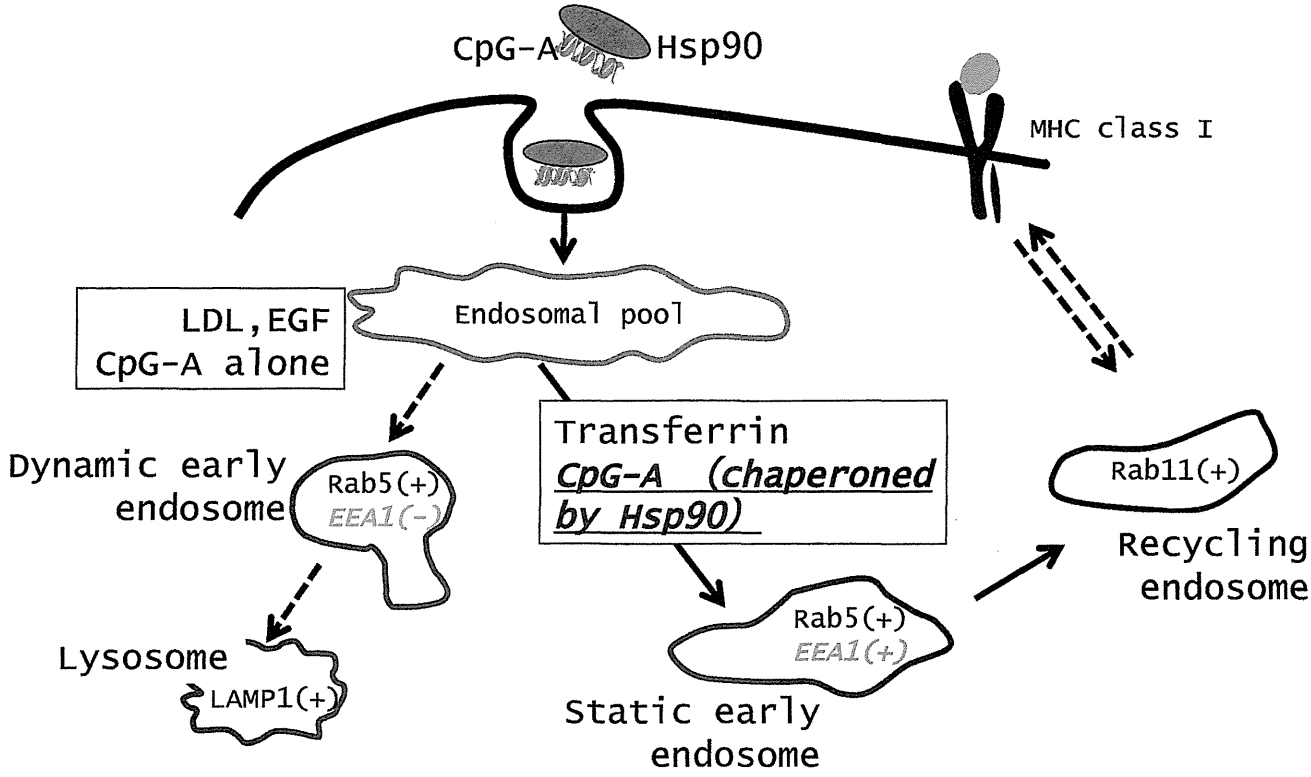

Fig. (2). Extracellular Hsp90 targets chaperoned molecules into static early endosomes. Early endosomes are comprised of static early endosomes (Rab5$^+$ and EEA-1$^+$) and dynamic early endosomes (Rab5$^+$ and EEA-1$^-$). The Hsp90-CpG-A complexes are preferentially targeted and retained in the static early endosomes of DCs, leading to sustained activation of TLR9 and IFN-α production.

in static early endosomes, thus enabling the selective and sustained activation of early endosomal TLR9.

NECROTIC CELL DEATH AS A SOURCE OF ANTIGENS AS WELL AS DANGER SIGNALS

When cells undergo necrotic cell death, it is expected that the release of endogenous danger signals such as DAMPs, including HSPs, occurs. The relationship between danger signals and induction of immunity was originally proposed by Matzinger [74]. It has been demonstrated that the presence of endogenous danger signals released from necrotic cells in the vicinity of exogenous danger signals such as LPS and CpG leads to more vigorous adaptive immune responses. In contrast, apoptotic cells can efficiently inhibit responses of immune cells to exogenous danger signal-induced secretion of proinflammatory cytokines IL-12 or TNF-α by promoting the secretion of TGF-β1 and IL-10. As Hsp90 is one of the most abundant proteins within the cells, it may act as a major endogenous danger signal by itself and/or as an accelerator for innate ligands by associating with them.

HSPS CAN LINK INNATE IMMUNITY AND ADAPTIVE IMMUNITY THROUGH CROSS-PRESENTATION OF HSP-PEPTIDE COMPLEXES

We have shown that both Hsp90-peptide complexes are efficiently cross-presented by DCs and elicit powerful CTLs against the associated peptides [4]. Furthermore, we have recently shown that ER-resident Hsp70 family member, oxygen-regulated protein 150 (ORP150), localized to static early endosome after endocytosis, leading to antigen cross-presentation when pulsed onto DCs [5]. These CTLs could inhibit established tumor growth, indicating that Hsp90/ORP150-peptide complexes act as cancer vaccines. Importantly, the Hsp90/ORP150-mediated cross-presentation pathway for exogenous peptides has been shown to be an endosome-recycling pathway not a conventional TAP-dependent pathway [4, 5]. Targeting to static early endosomes is the key feature of Hsp90 for innate as well as adaptive immune responses.

FUTURE DIRECTIONS

Can all HSPs target chaperoned molecules to static early endosomes? Up to now, it has been shown that the Hsp90 and ORP150 could be targeted to static early endosome after endocytosis, leading to antigen cross-presentation when pulsed onto DCs. For other HSPs, thus remains to be determined, however. More importantly, the HSP receptor responsible for targeting to static early endosomes should be clarified. Elucidation of the molecular basis for sorting HSPs to static endosomes will also be necessary to establish HSP-based cancer vaccines. In particular, for immune system receptors such as those on DCs, a deeper understanding of the factors regulating trafficking of

receptor-ligand complexes should enable the development of innovative cancer vaccines and immunopotentiators.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENT

This work was funded by a grant from Sapporo Medical University to K.H and N.S.

REFERENCES

[1] Udono H, Levey DL, Srivastava PK. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells *in vivo*. Proc Natl Acad Sci U S A 1994; 91: 3077-81.

[2] Srivastava P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol 2002; 20: 395-425.

[3] Binder RJ, Anderson KM, Basu S, Srivastava PK. Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells *in vivo*. J Immunol 2000; 165: 6029-35.

[4] Kurotaki T, Tamura Y, Ueda G, *et al.* Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells *via* an endosomal pathway. J Immunol 2007; 179: 1803-13.

[5] Kutomi G, Tamura Y, Okuya K, *et al.* Targeting to static endosome is required for efficient cross-presentation of endoplasmic reticulum-resident oxygen-regulated protein 150-peptide complexes. J Immunol 2009; 183: 5861-9.

[6] Ueda G, Tamura Y, Hirai I, *et al.* Tumor-derived heat shock protein 70-pulsed dendritic cells elicit tumor-specific cytotoxic T lymphocytes (CTLs) and tumor immunity. Cancer Sci 2004; 95: 248-53.

[7] Bendz H, Ruhland SC, Pandya MJ, *et al.* Human heat shock protein 70 enhances tumor antigen presentation through complex formation and intracellular antigen delivery without innate immune signaling. J Biol Chem 2007; 282: 31688-702.

[8] Oura J, Tamura Y, Kamiguchi K, *et al.* Extracellular heat shock protein 90 plays a role in translocating chaperoned antigen from endosome to proteasome for generating antigenic peptide to be cross-presented by dendritic cells. Int Immunol 2011; 23: 223-37.

[9] Calderwood SK, Mambula SS, Gray PJ, Jr., Theriault JR. Extracellular heat shock proteins in cell signaling. FEBS Lett 2007; 581: 3689-94.

[10] Asea A, Kraeft SK, Kurt-Jones EA, *et al.* HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 2000; 6: 435-42.

[11] Asea A, Kabingu E, Stevenson MA, Calderwood SK. HSP70 peptidembearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperones 2000; 5: 425-31.

[12] Asea A, Rehli M, Kabingu E, *et al.* Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 2002; 277: 15028-34.

[13] Javid B, MacAry PA, Lehner PJ. Structure and function: heat shock proteins and adaptive immunity. J Immunol 2007; 179: 2035-40.

[14] Wheeler DS, Dunsmore KE, Denenberg AG, Muething L, Poynter SE, Wong HR. Biological activity of truncated C-terminus human heat shock protein 72. Immunol Lett 2011; 135: 173-9.

[15] Theriault JR, Mambula SS, Sawamura T, Stevenson MA, Calderwood SK. Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS Lett 2005; 579: 1951-60.

[16] Vabulas RM, Ahmad-Nejad P, da Costa C, *et al.* Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem 2001; 276: 31332-9.

[17] Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol 2000; 12: 1539-46.

[18] Delneste Y, Magistrelli G, Gauchat J, *et al.* Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 2002; 17: 353-62.

[19] Theriault JR, Adachi H, Calderwood SK. Role of scavenger receptors in the binding and internalization of heat shock protein 70. J Immunol 2006; 177: 8604-11.

[20] Theriault JR, Adachi H, Calderwood SK. Role of scavenger receptors in the binding and internalization of heat shock protein 70. J Immunol 2006; 177: 8604-11.

[21] Berwin B, Hart JP, Rice S, *et al.* Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. Embo J 2003; 22: 6127-36.

[22] Berwin B, Delneste Y, Lovingood RV, Post SR, Pizzo SV. SREC-I, a type F scavenger receptor, is an endocytic receptor for calreticulin. J Biol Chem 2004; 279: 51250-7.

[23] Wang XY, Facciponte J, Chen X, Subjeck JR, Repasky EA. Scavenger receptor-A negatively regulates antitumor immunity. Cancer Res 2007; 67: 4996-5002.

[24] Barreto A, Gonzalez JM, Kabingu E, Asea A. Fiorentino S. Stress-induced release of HSC70 from human tumors. Cell Immunol 2003; 222: 97-104.

[25] Bausero MA, Gastpar R, Multhoff G, Asea A. Alternative mechanism by which IFN-gamma enhances tumor recognition: active release of heat shock protein 72. J Immunol 2005; 175: 2900-12.

[26] Gastpar R, Gehrmann M, Bausero MA, *et al.* Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. Cancer Res 2005; 65: 5238-47.

[27] Mambula SS, Calderwood SK. Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. J Immunol 2006; 177: 7849-57.

[28] Johnson JD, Campisi J, Sharkey CM, *et al.* Adrenergic receptors mediate stress-induced elevations in extracellular Hsp72. J Appl Physiol 2005: 1789-1995

[29] Vega, VL, Rodriguez-Silva M, Frey T, *et al.* Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. J Immunol 2008; 180: 4299-4307.

[30] De Maio A. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. Cell Stress Chaperone 2011; 16: 235-249.

[31] Vabulas RM, Braedel S, Hilf N, *et al.* The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells *via* the Toll-like receptor 2/4 pathway. J Biol Chem 2002; 277: 20847-53.

[32] Asea A, Rehli M, Kabingu E, *et al.* Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 2002; 277: 15028-34.

[33] Chen X, Tao Q, Yu H, Zhang L, Cao X. Tumor cell membrane-bound heat shock protein 70 elicits antitumor immunity. Immunol Lett 2002; 84: 81-7.

[34] Panjwani NN, Popova L, Srivastava PK. Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. J Immunol 2002; 168: 2997-3003.

[35] Wallin RP, Lundqvist A, More SH, *et al.* Heat-shock proteins as activators of the innate immune system. Trends Immunol 2002; 23: 130-5.

[36] Bausinger H, Lipsker D, Ziylan U, *et al.* Endotoxin-free heat-shock protein 70 fails to induce APC activation. Eur J Immunol 2002; 32: 3708-13.
[37] Gao B, Tsan MF. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. J Biol Chem 2003; 278: 22523-9.
[38] Gao B, Tsan MF. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. J Biol Chem 2003; 278: 174-9.
[39] Gao B, Tsan MF. Induction of cytokines by heat shock proteins and endotoxin in murine macrophages. Biochem Biophys Res Commun 2004; 317: 1149-54.
[40] Habich C, Kempe K, van der Zee R, *et al.* Heat shock protein 60: specific binding of lipopolysaccharide. J Immunol 2005; 174: 1298-305.
[41] Reed RC, Berwin B, Baker JP, Nicchitta CV. GRP94/gp96 elicits ERK activation in murine macrophages. A role for endotoxin contamination in NF-kappa B activation and nitric oxide production. J Biol Chem 2003; 278: 31853-60.
[42] Warger T, Hilf N, Rechtsteiner G, *et al.* Interaction of TLR2 and TLR4 ligands with the N-terminal domain of Gp96 amplifies innate and adaptive immune responses. J Biol Chem 2006; 281: 22545-53.
[43] Zheng H, Dai J, Stoilova D, Li Z. Cell surface targeting of heat shock protein gp96 induces dendritic cell maturation and antitumor immunity. J Immunol 2001; 167: 6731-5.
[44] Liu B, Dai J, Zheng H *et al.* Cell surface expression of an endoplasmic reticulum resident heat shock protein gp96 triggers MyD88-dependent systemic autoimmune diseases. Proc Natl Acad Sci USA 2003; 100: 15824-9.
[45] Dai J, Liu B, Cua DJ, Li Z. Essential roles of IL-12 and dendritic cells but not IL-23 and macrophages in lupus-like diseases initiated by cell surface HSP gp96. Eur J Immunol 2007; 37: 706-15.
[46] Baker-LePain J, C. Sarzotti M, Nicchitta C V. Glucose-regulated protein 94/glycoprotein 96 elicits bystander activation of CD4+ T cell Th1 cytokine production *in vivo*. J Immunol 2004; 172: 4195-203.
[47] Henderson B, Calderwood SK, Coates AR, *et al.* Caught with their PAMPs down? The extracellular signalling actions of molecular chaperones are not due to microbial contaminants. Cell Stress Chaperones 2010; 15: 123-41.
[48] Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 2003; 425: 516-21.
[49] Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011; 29: 139-62.
[50] Yu M, Wang H, Ding A, *et al.* HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock 2006; 26: 174-9.
[51] Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol 2008; 9: 1179-88.
[52] Eigenbrod T, Park JH, Harder J, Iwakura Y, Nunez G. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. J Immunol 2008; 181: 8194-8.
[53] Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*: a novel 'alarmin'? PLoS One 2008; 3: e3331.
[54] Cayrol C, Girard JP. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. Proc Natl Acad Sci U S A 2009; 106: 9021-6.
[55] Hofmann MA, Drury S, Fu C, *et al.* RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 1999; 97: 889-901.
[56] Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol 2010; 10: 826-37.
[57] Tian J, Avalos AM, Mao SY, *et al.* Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol 2007; 8: 487-96.
[58] Wagner H. Bacterial CpG DNA activates immune cells to signal infectious danger. Adv Immunol 1999; 73: 329-68.
[59] Krieg A. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002; 20: 709-60.
[60] Honda K, Yanai H, Negishi H, *et al.* IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 2005; 434: 772-7.
[61] Honda K, Ohba Y, Yanai H, *et al.* Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature 2005; 434: 1035-40.
[62] Guiducci C, Ott G, Chan J, *et al.* Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. J Exp Med 2006; 203: 1999-2008.
[63] Okuya K, Tamura Y, Saito K, *et al.* Spatiotemporal regulation of heat shock protein 90-chaperoned self-DNA and CpG-oligodeoxynucleotide for type I IFN induction *via* targeting to static early endosome. J Immunol 2010; 184: 7092-9.
[64] Lakadamyali M, Rust M, Zhuang X. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 2006; 124: 997-1009.
[65] Yoshida H, Okabe Y, Kawane K, Fukuyama H, Nagata S. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. Nat Immunol 2005; 6: 49-56.
[66] Minota S, Koyasu S, Yahara I, Winfield J. Autoantibodies to the heat-shock protein hsp90 in systemic lupus erythematosus. J Clin Invest 1988; 81: 106-9.
[67] Conroy S, Faulds G, Williams W, Latchman D, Isenberg D. Detection of autoantibodies to the 90 kDa heat shock protein in systemic lupus erythematosus and other autoimmune diseases. Br J Rheumatol 1994; 33: 923-6.
[68] Twomey B, Dhillon V, McCallum S, Isenberg D, Latchman D. Elevated levels of the 90 kD heat shock protein in patients with systemic lupus erythematosus are dependent upon enhanced transcription of the hsp90 beta gene. J Autoimmun 1993; 6: 495-506.
[69] Ripley B, Isenberg D, Latchman D. Elevated levels of the 90 kDa heat shock protein (hsp90) in SLE correlate with levels of IL-6 and autoantibodies to hsp90. J Autoimmun 2001; 17: 341-6.
[70] Perdew G, Hord N, Hollenback C, Welsh M. Localization and characterization of the 86- and 84-kDa heat shock proteins in Hepa 1c1c7 cells. Exp Cell Res 1993; 209: 350-6.
[71] Akner G, Mossberg K, Sundqvist K, Gustafsson J, Wikström A. Evidence for reversible, non-microtubule and non-microfilament-dependent nuclear translocation of hsp90 after heat shock in human fibroblasts. Eur J Cell Biol 1992; 58: 356-64.
[72] Lande R, Gregorio J, Facchinetti V, *et al.* Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 2007; 449: 564-9.
[73] Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest 2005; 115: 407-17.
[74] Matzinger P. The danger model: a renewed sense of self. Science 2002; 296: 301-5.

Received: February 02, 2012 Revised: May 18, 2012 Accepted: July 07, 2012

Pathology International

Pathology International 2012; **62**: 303–308 doi:10.1111/j.1440-1827.2012.02789.x

Rapid Communication

Establishment of a monoclonal anti-pan HLA class I antibody suitable for immunostaining of formalin-fixed tissue: Unusually high frequency of down-regulation in breast cancer tissues

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A novel monoclonal anti-pan human leukocyte antigen (HLA) class I heavy chain antibody, EMR8-5, was established. It could detect HLA-A, -B, and -C antigens in formalin-fixed paraffin embedded tissues. By immunohistochemical staining using the EMR8-5 antibody, various cancer tissues from 246 cases were examined for HLA class I expression. It was found that HLA class I expression was decreased in 20% to 42% of the cases of lung cancer, hepatocellular carcinoma, colon cancer, renal cell carcinoma, and urothelial carcinoma. In contrast, 85% of breast cancer cases had loss of or decreased HLA class I expression. Of the 35 breast cancer cases that had decreased HLA class I heavy chain expression, 33 (94%) also had decreased beta2-microglobulin expression detected by immunohistochemical staining. It was suggested that HLA class I down-regulation might be a common characteristic of breast cancer mostly caused by the down-regulation of beta2-microglobulin expression.

Key words: antigen presentation, beta2-microglobulin, breast cancer, Immune escape, MHC class I

Human leukocyte antigen (HLA) class I molecules have a central role in the cell-mediated immune system, especially as antigen-presenting molecules for cytotoxic T lymphocytes (CTL). The CTL can recognize antigenic peptides presented on the cell surface by HLA class I molecules, and kill the target cells. Recently, a large number of CTL epitope peptides were identified in various tumor antigens, and CTL-based immunotherapy has been widely tested for various cancer patients.[1–4] Favorable outcomes have been obtained in some clinical trials for melanoma patients, however, most of the CTL-based immunotherapies for non-melanoma cancer patients have fallen short of expectations.[4] Precise examination for the insufficiency revealed immune escape of cancer cells, including down-regulation of HLA class I molecules, secretion of immunosuppressive cytokines and infiltration of immunosuppresive cells.[5–8] Though immune escape phenotypes such as tumor HLA class I down-regulation have been demonstrated mostly in primary cultured tumor cells or frozen tumor tissues, they are rarely found in formalin-fixed paraffin-embedded tissues because of the limited availability of anti-HLA class I antibodies that can react to the denatured molecules in the fixed tissues. Since most surgical tumor specimens are examined and stored after formalin fixation, development of novel monoclonal antibodies that are capable of detecting specific denatured antigens in the fixed tissues should contribute to extensive histological examinations using large numbers of archival tumor specimens.

In the present study, we introduce a novel monoclonal anti-pan HLA class I antibody termed EMR8-5, which is suitable for immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections. Immunostaining of various tumor tissues revealed an unusually high frequency of HLA class I down-regulation in breast cancer as compared with lung, colon, kidney, liver, and bladder cancers. Involvement of beta2 microglobulin down-regulation is also addressed as the major cause of the HLA down-regulation in breast cancer.

METHODS

Development of monoclonal anti-pan HLA class I heavy chain antibody

A recombinant His-tagged extracellular domain of HLA-A*2402 heavy chain protein was produced and purified by

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Received 28 August 2011. Accepted for publication 25 December 2011.

using Ni-NTA agarose (QIAGEN, Valencia, CA, USA) as described previously.[9] The purified protein was dialyzed in PBS, and approximately 0.2 mg of protein per 500 μl of PBS was emulsified with 500 μl of either complete Freund's adjuvant (for the first immunization) or incomplete Freund's adjuvant (after the second immunization). BALB/c mice were immunized subcutaneously with the emulsion eight times every week. Four days after the final immunization, spleen cells were fused with NS-1 myeloma cells by using polyethylene glycol #4000 (Kanto Kagaku, Tokyo, Japan) and plated into 96-well plates. Hybridoma supernatants were initially screened using ELISA with a recombinant extracellular domain of HLA-A*2402 heavy chain protein denatured in 8 M urea buffer (8 M urea, 20 mM HEPES [pH 8.0]) as the antigen. The positive supernatants were then screened by Western blotting with cell lysates from OSC20 cells (HLA-A*2402-negative oral cancer cell line),[10] OSC20 transfectants with HLA-A*2402 cDNA, K562 cells, and recombinant HLA-A*2402 protein. The third screening of the supernatants was performed by immunostaining of formalin-fixed paraffin-embedded human tissue sections. The resulting hybridoma EMR8 was cloned by limiting dilution, and finally its subclone EMR8-5, which produced a monoclonal anti-HLA class I heavy chain antibody with the IgG1 subclass and κ chain, was established.

Western blotting

The monoclonal anti-HLA class I antibody HC10 was kindly provided by Dr. S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY, USA).[11,12] Recombinant extracellular domains of various HLA class I allele proteins were kindly provided by MBL Co. Ltd. (Nagoya, Japan). Cultured cells were washed in ice-cold PBS, homogenized in ice-cold RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCL [pH 8.0], and protease inhibitor cocktail [Complete, Roche Diagnostics, Basel, Switzerland]) for 30 min and clarified by centrifugation at 12 000 g for 20 min at 4°C. The lysates and 0.5 μg of recombinant HLA heavy chain proteins were boiled for 5 min with SDS sample buffer and then separated by 7.5% SDS-polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer (5% nonfat dry milk in PBS) at room temperature and then incubated for 60 min with EMR8-5 culture supernatant. After washing three times with PBS-T buffer (0.05% Tween-20 in PBS), the membrane was reacted with a peroxidase-labeled goat anti-mouse IgG antibody (KPL, Gaithersburg, MD, USA) for one hour. Finally, the signals were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Tissue specimens

Formalin-fixed paraffin-embedded tissue specimens of breast cancer (41 cases), lung cancer (35 cases), hepatocellular carcinoma (57 cases), colon cancer (15 cases), renal cell carcinoma (45 cases), and bladder cancer (53 cases) that were resected from patients at the Sapporo Medical University Hospital between 1995 and 2005 were used in the immunohistochemical examination. The study was approved by the Institutional Review Board for Clinical Research of our university.

Immunohistochemistry

Tissue specimens were cut into 5–μm-thick sections and deparaffinized. Antigen retrieval was performed using TT-mega Milestone (ESBE Scientific, Markham, Ontario, Canada) in 10 mM citrate buffer (pH 6.0) at 120°C for 15 min. After incubation in 0.3% hydrogen peroxide for 30 min, tissue slides were reacted with 10 μg/mL purified EMR8-5 antibody or polyclonal anti-beta2-microglobulin antibody (DAKO) for 1 h, washed three times with PBS-T buffer, and then incubated with SimpleStainMax-PO (Nichirei, Tokyo, Japan), followed by reaction with 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide as a chromogen for 1 min. After washing with distilled water, the slides were stained with hematoxylin solution for 1 min, rinsed, dehydrated through graded alcohols into non-aqueous solution, and cover-slipped with mounting media.

Tumor HLA class I expression levels were classified according to the criteria established by the HLA and Cancer component of the 12th International Histocompatibility Workshop.[13] According to these criteria, expression levels were defined as strongly positive (2+) if the cell membrane was stained as strongly as stromal lymphocytes or endothelial cells in more than 75% of the tumor cells. If membrane staining was heterogenous in 25% or more of the constituent tumor cells, it was defined as weak positive (1+). If membrane staining was lost in more than 75% of the tumor cells, it was defined as negative (0). Cases with a level 1+ or less were judged as down-regulated.

RESULTS

Characterization of monoclonal antibody EMR8-5

OSC20 is an HLA-A*2402-negative cell line.[10] HLA-A*2402 cDNA was cloned into plasmid pcDNA3.1 and transfected

into OSC20 cells. The resulting stable transfectant OSC20-A*2402 cells expressed HLA-A24 antigen on the cell surface. K562 is an HLA class I-negative cell line. Western blotting with the EMR8-5 antibody and HC10 antibody indicated that the EMR8-5 antibody could react to both immunized recombinant HLA-A*2402 heavy chain protein and endogenous non-HLA-A*2402 heavy chains expressed in OSC20 cells, whereas the HC10 antibody could react to endogenous non-HLA-A*2402 heavy chains, but not to the HLA-A*2402 heavy chain (Fig. 1).

Western blotting analysis with deletion mutant HLA-A*2402 proteins revealed that the epitope of EMR8-5 was located within a conserved α3 domain (Fig. 2).

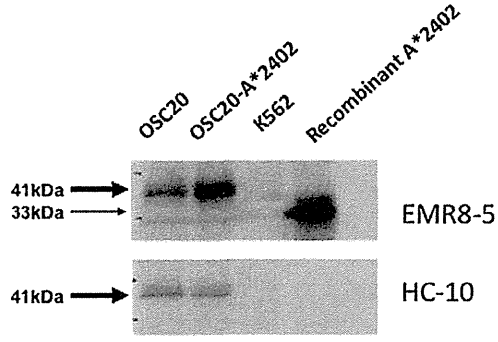


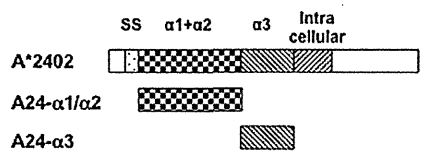
Figure 1 Western blot analysis with antibodies EMR8-5 (upper panel) and HC10 (lower panel). OSC20 cells are HLA-A*2402 negative. OSC20-HLA*2402 cells are OSC20 transfectant cells with pcDNA3.1-HLA-A*2402 cDNA. K562 cells are HLA class I-negative cells. Recombinant A*2402 indicates 0.5 μg of recombinant extracellular domain protein derived from HLA-A*2402 that was used as the immunogen.

To clarify the specificity of these antibodies, reactivity to recombinant extracellular domains of various HLA class I allele proteins was examined by Western blotting (Fig. 3). It was demonstrated that EMR8-5 could react to all the HLA-A, B, and C alleles examined, whereas HC10 reacted preferentially to HLA-B and C alleles as reported previously.[11,12] The data indicate that EMR8-5 was a monoclonal pan-HLA class I heavy chain antibody.

Immunohistochemical staining of formalin-fixed tumor tissues with EMR8-5 antibody

To determine the frequency of down-regulation of HLA class I expression in various tumors, surgically resected tissue specimens from 246 cases were immunostained with the EMR8-5 antibody. Representative immunostaining data of HLA class I-positive cases of colon cancer and breast cancer are shown in Figure 4a,d, respectively, and HLA class I down-regulated cases of colon cancer and breast cancer are shown in Figure 4b,c,e,f, in which stromal lymphocytes and endothelial cells are strongly stained by the antibody, serving as internal positive control. A summary of the frequency of HLA class I down-regulation in each tumor is presented in Table 1. A striking difference in the frequency of HLA class I expression was noted between breast cancer (85%) and other cancers (20% to 42%). HLA class I expression was down-regulated in a larger proportion of breast cancer cases than in other cancers such as lung cancer, colon cancer, hepatocellular carcinoma, renal cell cancer, and bladder cancer.

To determine the mechanism involved in the down-regulation of membrane expression of HLA class I in breast



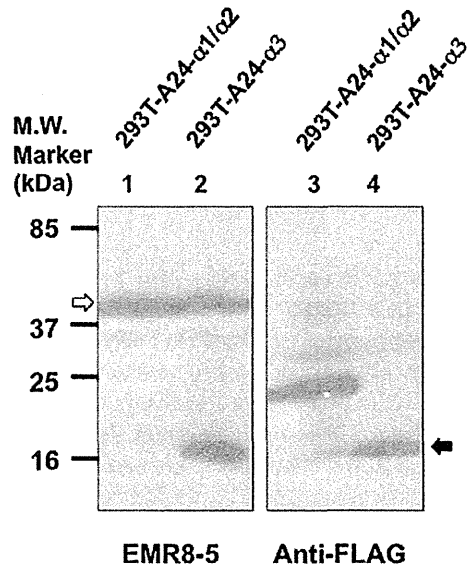


Figure 2 293T cells were transfected with cDNA encoding FLAG-tagged deletion mutant HLA-A*2402 proteins, A24-α1/α2 (lanes 1 and 3) and A24-α3 (lanes 2 and 4). Reactivity of EMR8-5 to the deletion mutant proteins was analyzed by Western blot analysis (lanes 1 and 2). Both of deletion mutant proteins were detected by anti-FLAG antibody (lenes 3 and 4). EMR8-5 reacted only to 16 kDa A24-α3 (lane 2, black arrow). White arrow indicates 41 kDa endogenous HLA class I protein in 293T cells.

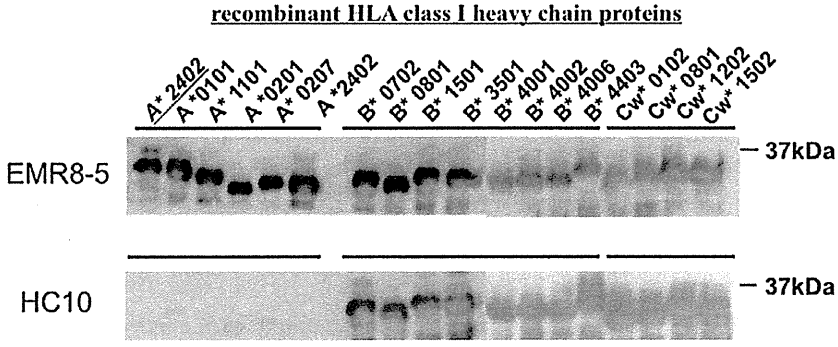


Figure 3 Western blot analysis with antibodies EMR8-5 (upper panel) and HC10 (lower panel). Recombinant extracellular domain proteins (0.5 μg per lane) derived from the indicated HLA class I alleles were run on the SDS-PAGE and immunoblotted with the indicated antibody. Underlined protein in italics indicates the immunogen of our preparation. Other proteins were gifts from Medical & Biological Lab. Co. Ltd. (Nagoya, Japan).

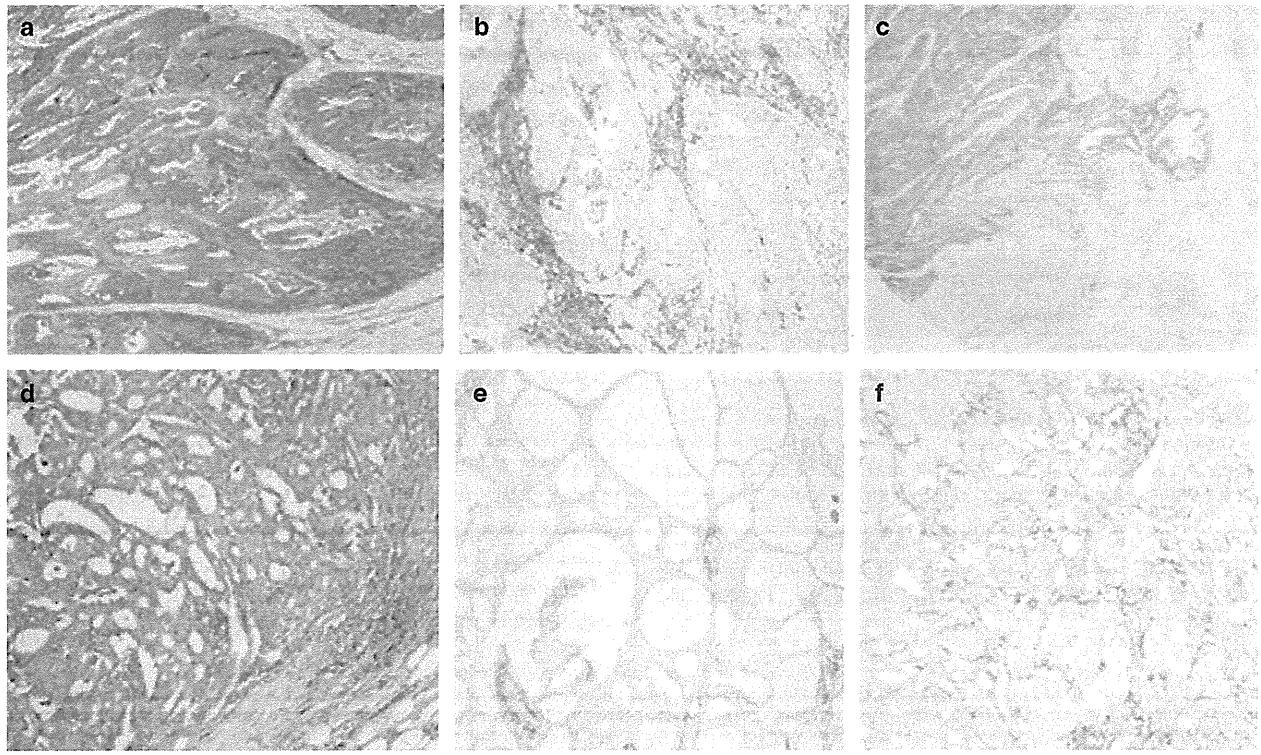


Figure 4 Representative immunostaining pictures with EMR8-5 antibody. Panels **a**, **b** and **c** are colon cancer cases that are HLA class I-positive (2+, Panel **a**), negative (0, Panel **b**) and heterogeneous staining (1+, Panel **c**). Panels **d**, **e** and **f** are breast cancer cases that are HLA class I-positive (2+, Panel **d**), negative (0, Panel **e**) and weak positive staining (1+, Panel **f**). Note that stromal cells such as lymphocytes, endothelial cells and fibroblasts are HLA class I-positive in Panel **b**, **c**, **e** and **f**, serving as internal positive control.

cancers, the same tissue sections were examined for the expression of beta2-microglobulin (HLA class I light chain) by immunostaining. As shown in Table 2, 33 of the 35 cases (94%) of HLA class I weak or negative breast cancer had loss of beta2-microglubulin expression demonstrated by immunostaining. These data indicated that HLA class I down-regulation in breast cancer cells was mostly accompanied by the down-regulation of beta2-microglobulin expression, which might be one of the major causes of the impairment of membrane expression of HLA class I.

Table 1 Human leukocyte antigen (HLA) class I expression levels detected by immunostaining with antibody EMR8-5

Cancer origin	Total cases	2+	1+/0 (%)
Breast cancer	41	6	35 (85%)
Lung cancer	35	28	7 (20%)
Hepatocellular carcinoma	57	33	24 (42%)
Colon cancer	15	11	4 (27%)
Renal cell carcinoma	45	29	16 (35%)
Bladder cancer	53	35	18 (34%)

Expression levels 2+, 1+, and 0 are strongly positive, weakly positive, and negative, respectively.

Table 2 Human leukocyte antigen (HLA) class I heavy chain expression and beta2-microglobulin expression in the breast cancer cases

	Total cases	β2-MG +	β2-MG −
HLA class I 0	15	0	15
HLA class I 1+	20	2	18
HLA class I 2+	6	6	0

Expression levels 2+, 1+, and 0 are strongly positive, weakly positive, and negative, respectively.

DISCUSSION

There are many reports showing the down-regulation of HLA class I and immune escape of cancer cells.[14–22] Since most anti-HLA class I antibodies recognize the allele-specific native structure of cell-surface HLA class I molecules or heterodimeric structure of a heavy chain and beta2-microglobulin, these antibodies cannot react with denatured HLA class I molecules in formalin-fixed paraffin-embedded tissue sections. Two monoclonal anti-HLA class I antibodies, HC10 and HCA2, have been well characterized and are available for the immunostaining of formalin-fixed tissue specimens. However, as shown in our results (Figs 1,3), HC10 can barely react with HLA-A allele proteins, and HCA2 reacts to some HLA-A allele proteins, but not to most HLA-B or C allele proteins.[11,12] In the present study, we demonstrated a novel monoclonal pan-HLA class I heavy chain antibody suitable for the immunostaining of formalin-fixed tissue specimens. The epitope was located within a conserved α3 domain, and the antibody reacted to 17 HLA class I allele proteins examined. Allele frequencies are shown in Table 3. The antibody enabled us to examine HLA class I expression at the tissue level in a large number of archival tissue specimens that were surgically resected and stored for a long time.

By immunostaining of various tumor specimens from 246 cases using EMB8-5, it was found that breast cancer might have a unique immune escape phenotype as compared with other tumors. Our findings are essentially compatible with studies of Ferrone *et al.*[6] and other groups,[16,20] although the frequency was higher in our study. Although we could not find a correlation between the prognoses of the cancer patients and HLA class I expression level, it was noted that all the cases of micropapillary type breast cancer (10 cases) were HLA class I negative, which subtype is known to be extremely metastatic and have a poor prognosis. Of the breast cancer cases with decreased HLA class I heavy chain expression, 94% were accompanied by loss of beta2-microglobulin expression. No beta2-microglobulin gene mutation was detected by sequencing analysis of three breast cancer cases with beta2-microglobulin negative phenotype. It is likely that beta2-microglobulin gene expression may be regulated by epigenetic mechanisms such as gene methylation and histone deacetylation. Actually, we have found that beta2-microglobulin expression of breast cancer cell lines was up-regulated in the presence of a histone deacetylase inhibitor, leading to the restoration of cell surface HLA class I expression (data not shown). Further analysis of the mechanism of HLA class I gene regulation should lead to a novel therapeutic strategy for breast cancer.

Table 3 Human leukocyte antigen (HLA) class I Allele Frequency (Phenotype frequency)

	Allele	Frequency % (Japanese)	Frequency % (Asian in USA)	Frequency % (Caucasoid in USA)
1	A 0101	4	3–10	22–29
2	A 1101	16–22	36–46	12–14
3	A 0201	21–23	18–19	40–48
4	A 0207	4–8	8–13	0
5	A 2402	65–76	36–38	13–26
6	B 0702	10–13	5	20–31
7	B 0801	0	3	13–23
8	B 1501	13–17	7–10	12–16
9	B 3501	4–18	8	9–14
10	B 4001	6–12	16–18	8–14
11	B 4002	6–17	6	2–3
12	B 4006	8–13	4–7	0
13	B 4403	12–24	8–9	6–16
14	Cw 0102	30–40	27	4–7
15	Cw 0801	15–22	22	0
16	Cw 1202	20–24	6	2
17	Cw 1502	3–5	5	3–5

Percentage of individuals who have the allele or gene.

Phenotype frequencies were calculated from the data of 'The Allele Frequency Net Database' (http://www.allelefrequencies.net/).[23]

ACKNOWLEDGMENTS

We thank Dr S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY) for providing monoclonal antibody HC10. We also thank Medical & Biological Lab. Co. Ltd. (Nagoya, Japan) for providing recombinant HLA class I allele proteins. This study was supported in part by a grant-aid from Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant-aid for Clinical Cancer Research from Ministry of Health, labor and Welfare of Japan.

REFERENCES

1 Sato N, Nabeta Y, Kondo H *et al.* Human CD8 and CD4 T cell epitopes of epithelial cancer antigens. *Cancer Chemother Pharmacol* 2000; **46** Suppl: S86–90.
2 Kawaguchi S, Wada T, Ida K *et al.* Phase I vaccination trial of SYT-SSX junction peptide in patients with disseminated synovial sarcoma. *J Transl Med* 2005; **3**: 1. doi:10.1186/1479-5876-3-1.
3 Tsuruma T, Hata F, Furuhata T *et al.* Peptide-based vaccination for colorectal cancer. *Expert Opin Biol Ther* 2005; **5**: 799–807.
4 Tsuruma T, Hata F, Torigoe T *et al.* 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.

5 Marincola FM, Jaffee EM, Hicklin DJ *et al.* Escape of human solid tumors from T-cell recognition: Molecular mechanisms and functional significance. *Adv Immunol* 2000; **74**: 181–273.
6 Seliger B, Cabrera T, Garrido F *et al.* HLA class I antigen abnormalities and immune escape by malignant cells. *Semin Cancer Biol* 2002; **12**: 3–13.
7 Raffaghello L, Prigione I, Airoldi I *et al.* Mechanisms of immune evasion of human neuroblastoma. *Cancer Lett* 2005; **228**: 155–61.
8 Lopez-Albaitero A, Nayak JV, Ogino T *et al.* Role of antigen-processing machinery in the in vitro resistance of squamous cell carcinoma of the head and neck cells to recognition by CTL. *J Immunol* 2006; **176**: 3402–9.
9 Sato Y, Sahara H, Tsukahara T *et al.* Improved generation of HLA class I/peptide tetramers. *J Immunol Methods* 2002; **271**: 177–84.
10 Miyazaki A, Sato N, Takahashi S *et al.* Cytotoxicity of histocompatibility leukocyte antigen-DR8-restricted CD4 killer T cells against human autologous squamous cell carcinoma. *Jpn J Cancer Res* 1997; **88**: 191–7.
11 Stam NJ, Spits H, Ploegh HL. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J Immunol* 1986; **137**: 2299–306.
12 Stam NJ, Vroom TM, Peters PJ *et al.* HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. *Int Immunol* 1990; **2**: 113–25.
13 Garrido F, Cabrera T, Accolla RS. HLA and cancer: 12th International Histocompatibility Workshop study. In: Charron D, editor Genetic diversity of HLA Functional and medical implication Proc Twelfth International Histocompatibility Workshop and Conference 1997, Vol. II. France: EDK, Sevres: 445-52.
14 Mattijssen V, De Mulder PH, Schalkwijk L *et al.* HLA antigen expression in routinely processed head and neck squamous cell carcinoma primary lesions of different sites. *Int J Cancer Suppl* 1991; **6**: 95–100.
15 Kamarashev J, Ferrone S, Seifert B *et al.* TAP1 down-regulation in primary melanoma lesions: An independent marker of poor prognosis. *Int J Cancer* 2001; **95**: 23–8.
16 Madjd Z, Spendlove I, Pinder SE *et al.* Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. *Int J Cancer* 2005; **117**: 248–55.
17 Kageshita T, Ishihara T, Campoli M *et al.* Selective monomorphic and polymorphic HLA class I antigenic determinant loss in surgically removed melanoma lesions. *Tissue Antigens* 2005; **65**: 419–28.
18 Cabrera T, Lara E, Romero JM *et al.* HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination. *Cancer Immunol Immunother* 2007; **56**: 709–17.
19 Mizukami Y, Kono K, Maruyama T *et al.* Downregulation of HLA Class I molecules in the tumour is associated with a poor prognosis in patients with oesophageal squamous cell carcinoma. *Br J Cancer* 2008; **99**: 1462–7.
20 Morabito A, Dozin B, Salvi S *et al.* Analysis and clinical relevance of human leukocyte antigen class I, heavy chain, and beta2-microglobulin downregulation in breast cancer. *Hum Immunol* 2009; **70**: 492–5.
21 Bandoh N, Ogino T, Katayama A *et al.* HLA class I antigen and transporter associated with antigen processing downregulation in metastatic lesions of head and neck squamous cell carcinoma as a marker of poor prognosis. *Oncol Rep* 2010; **23**: 933–9.
22 Tanaka K, Tsuchikawa T, Miyamoto M *et al.* Down-regulation of Human Leukocyte Antigen class I heavy chain in tumors is associated with a poor prognosis in advanced esophageal cancer patients. *Int J Oncol* 2011; **40**: 965–74.
23 Gonzalez-Galarza FF, Christmas S, Middleton D *et al.* Allele frequency net: A database and online repository for immune gene frequencies in worldwide populations. *Nucleic Acid Res* 2011; **39**: D913–D9.

Tsukahara et al. Diagnostic Pathology 2012, 7:114
http://www.diagnosticpathology.org/content/7/1/114

DIAGNOSTIC PATHOLOGY

CASE REPORT Open Access

NK/T-cell lymphoma of bilateral adrenal glands in a patient with pyothorax

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Abstract

Primary lymphoma of adrenal glands is rare, and non-B-cell lymphoma associated with pyothorax is also very rare. Here we report the first autopsy case of non-B-cell lymphoma in bilateral adrenal glands of a 79-year-old woman with pyothorax who had an aggressive clinical course. Immunohistochemically, tumor cells showed CD3+, CD45RO+, CD5-, CD7-, CD4-, CD8-, CD10-, CD20-, CD30-, CD79a-, CD138-, CD56-, granzyme B-, TIA-1+ and ALK-. In addition, tumor cells were strongly EBER1-positive by in situ hybridization. In genomic DNA of tumor cells, T-cell receptor rearrangements were not detected by southern blotting. We finally diagnosed this case as extranodal NK/T-cell lymphoma (nasal type).

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/8050621197741854.

Keywords: NK/T-cell lymphoma, Adrenal gland, EBV, Pyothorax, Autopsy

Background

Lymphoma in the adrenal gland is rare, accounting for less than 1% of non-Hodgkin lymphomas. The features of lymphoma in adrenal glands are: (i) peak age of onset is elderly (mean age of 68 years), (ii) bilateral adrenal glands are involved in 60% of cases, (iii) adrenal failure occurs in 66% of cases, (iv) B-cell type is predominantly observed in 90% of cases and (v) prognosis is poor [1]. On the other hand, pyothorax-associated lymphoma, which is also rare (only 2% of patients with pyothorax), commonly shows the following features: (i) pathogenesis is related to pyothorax resulting form tuberculosis and artificial pneumothorax, (ii) common site of the tumor is around the pleural cavity, (iii) period of onset is more than 20 years, (iv) B-cell type is typical and strongly positive for Epstein-Barr virus, and (v) prognosis is poor [2]. However, non-B-cell type lymphoma occurring in adrenal glands of a patient with pyothorax has not been reported.

Here we report the first case of NK/T-cell lymphoma in bilateral adrenal glands in a patient with pyothorax.

Case presentation

A 79-year-old Japanese woman presented with cough and bloody sputum in July 2011. She had been followed since 1990 by a pulmonologist under the diagnosis of chronic pyothorax resulting from tuberculosis, and warfarin had been administered under the diagnosis of atrial fibrillation since 2009. There was no obvious evidence of existing immunodeficiency.

At the initial presentation, a large cystic lesion, pleural fluid and bilateral independent adrenal tumors (55 mm x 31 mm on the right side and 57 mm x 32 mm on the left side) were detected by thoracic and abdominal computed tomography (CT) scans, respectively (Figure 1A, 1B). Tuberculosis bacterium was not detected in the sputum using PCR. Serum LDH was elevated to 1,038 U/L. Although use of warfarin was immediately stopped and coagulation therapy was performed using carbazochrome sodium sulfonate hydrate and tranexamic acid, the symptoms were not improved. Nineteen days after initial presentation, bloody sputum was successfully decreased by bronchial artery embolization. The next day, however, hyponatremia (Na: 113 mEq/L) occurred, followed by increases of serum IL-2R (1,185 U/mL) and serum NSE (117.9 ng/mL). An abdominal CT scan showed an increase in sizes of the bilateral adrenal

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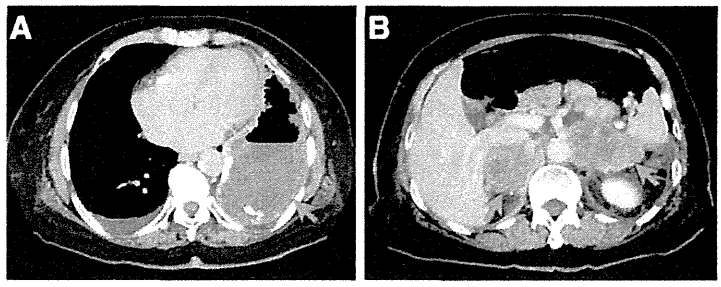

Figure 1 Enhanced computed tomography scan of the chest and abdomen. (**A**) Thoracic CT scan displayed large pyothorax and effusion in the left pleural cavity (indicated by a red arrow). (**B**) Abdominal CT scan displayed bilateral adrenal tumors (indicated by red arrows).

tumor masses (57 mm x 54 mm on the right side and 74 mm x 45 mm on the left side). Two days later, under the diagnosis of adrenal failure caused by the tumors, augmentation of corticosteroid and correction of hyponatremia were started. Serum concentrations of cortisol, sodium and potassium were controlled well. However, seven days after the initiation of treatment, the patient had symptoms of general malaise and chest pain as well as atrial fibrillation with a rapid ventricular response. Serum LDH was increased to 3,650 U/L from 1,038 U/L at the initial presentation. Despite administration of antiarrhythmic agents, blood pressure decreased to less than 70 mmHg, and then cyanosis and hypouresis occurred. Thirty-three days after initial presentation (11 days after initiation of therapy for adrenal failure), the patient died. Autopsy was performed five hours after death.

Materials and methods

The autopsy specimen was fixed with 10% buffered formaldehyde and embedded in paraffin. Sections were cut to 2um in thickness and stained with hematoxylin and eosin. Immunohistochemistry was performed using primary antibodies against cytokeratin, vimentin, CD3, CD45RO, CD5, CD7, CD4, CD8, CD10, CD20, CD79a, CD138, CD56, granzyme B, TIA-1, ALK and Ki-67. Ki-67 index was calculated as the ratio of Ki-67-positive cell number to 1,000 tumor cells. In situ hybridization was performed using an anti-sense probe of Epstain-Barr virus (EBV)-encoded RNA 1 (EBER1). Southern blotting for detection of T cell receptor rearrangement in genome DNA of fresh frozen tumor tissue using probes against Jß1 and Jß2 of TCR ß chain and Jγ of TCR γ chain was performed by BML, Inc (Tokyo, Japan).

Results

Macroscopic findings

In the left pleural cavity, pyothorax as a large cystic lesion (approximately 16 cm x 7 cm) containing necrotic tissues with pyogenic pleural fluid was detected. No tumoral lesion was detected around the left pleural cavity. In both adrenal glands, tumor masses (90 mm x 65 mm on the right and 85 mm x 60 mm on the left) with necrotic change were detected (Figure 2). The right adrenal tumor had infiltrated into the right kidney and inferior vena cava, and the left adrenal tumor had infiltrated into the left kidney and spleen. Enlarged lymph nodes were not detected anywhere.

Histologic and genomic findings

The cystic wall of the pyothorax consisted of fibrous and hyalinizing tissue with a small population of infiltrating lymphocytes without atypia. No EBER1-positive cells were detected around the left pleural cavity by in situ hybridization. The adrenal tumors contained diffuse middle to large-sized neoplastic lymphoid cells with atypia and mitosis (Figure 3A). Both of the adrenal glands had completely disappeared due to invasion of the tumors. Immunohistochemically, the tumor cells were cytokeratin -, vimentin +, CD3+ (Figure 3B), CD45RO+, CD5-, CD7-, CD4-, CD8-, CD10-, CD20- (Figure 3C), CD30-, CD79a-, CD138-, CD56- (Figure 3D), granzyme B-, TIA-1 cytotoxic granule-associated RNA binding protein (TIA-1) + (Figure 3E) and ALK-. As shown in Figure 3F, Ki-67 was strongly positive in tumor cells (Ki-67 index: 77.0%). Results of in situ hybridization showed that EBER1 was strongly positive in most of the tumor cells (Figure 3G). TCR rearrangements in genomic DNA of fresh frozen tumor cells were not detected in TCR ß and TCR γ chains by southern blotting (Figure 3H).

These findings indicated that the tumor had characteristics of peripheral T-cell origin or natural killer (NK)/T-cell origin. The findings of CD3+, CD45RO + and CD56- could indicate peripheral T-cell lymphoma. In contrast, the findings of TIA-1+, EBER1+ and no TCR rearrangement could indicate NK/T-cell lymphoma. Considering the findings of TIA-1+, EBER1+ and no TCR rearrangement, histological type of the tumor was finally diagnosed as CD56-negative extranodal NK/T-cell lymphoma (nasal type).

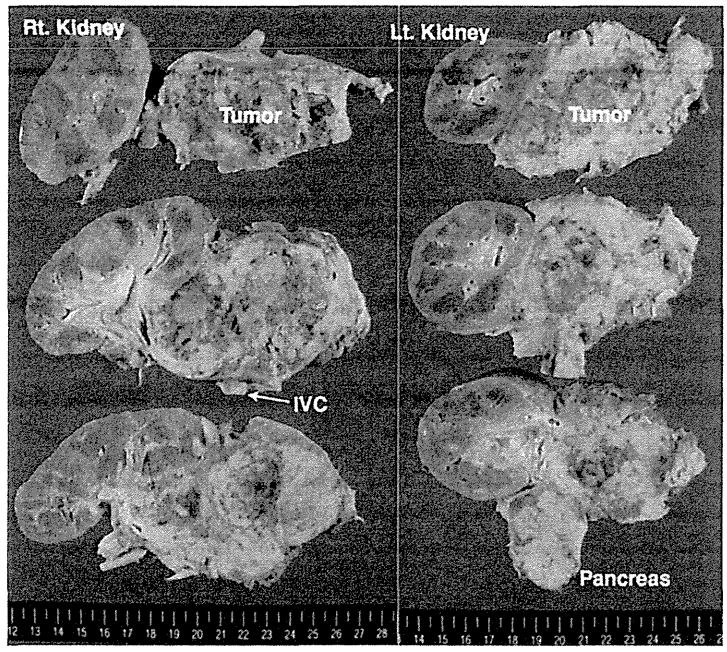

Figure 2 Macroscopic view of bilateral adrenal masses and adjacent organs.

Discussion

This is the first case of non-B-cell lymphoma in bilateral adrenal glands in a patient with pyothorax. Tumor cells had the characteristics of both peripheral T-cell lymphoma and NK/T-cell lymphoma. In addition, no tumor lesions were detected around the pyothorax. The tumor showed highly malignant characteristics and showed an aggressive clinical course.

Commonly, adrenal lymphoma shows histological characteristics of B-cell type, mainly diffuse large B-cell lymphoma (DLBCL). Therefore, this case of non-B-cell lymphoma is a rare case. Six cases of peripheral T-cell lymphoma and five cases of NK/T-cell lymphoma have been reported [3,4]. On the other hand, most pyothorax-associated lymphomas have shown findings of DLBCL occurring in the pleural cavity in addition to strong positive findings of EBV. Nine cases of peripheral T-cell lymphoma associated with pyothorax have been reported [5-13], but no cases of NK/T-cell lymphoma associated with pyothorax have been reported. Only one case of lymphoma occurring in both adrenal glands in a patient with pyothorax has been reported [14]. In that case, a small lesion of lymphoma was also detected around the pyothorax lesion, and the histological type was diagnosed as DLBCL. Thus, non-B-cell lymphoma in adrenal glands in a patient with pyothorax such as the present case is an extremely rare condition.

For differential diagnosis, (i) "peripheral T-cell lymphoma, not otherwise specified", (ii) "extranodal NK/T-cell lymphoma (nasal type)" and (iii) chronic active Epstein-Barr virus infection (CAEBV) were considered. As described above, the tumor had characteristics of both peripheral T-cell and NK/T-cell lymphomas. The findings of CD3 + CD45RO + CD56- could indicate peripheral T-cell lymphoma. On the other hand, the findings of cytotoxicity-associated molecule TIA-1+, no TCR rearrangement and EBV + could indicate NK/T-cell lymphoma. The present case could not strictly be categorized into either. However, considering the findings of TIA-1+, EBER1+ and no TCR rearrangement, we finally diagnosed this lymphoma as extranodal NK/T-cell lymphoma (nasal type). Recently, Miles et al. reported a case of CD56-negative extranodal NK/T-cell lymphoma [15]. In that case, neoplastic lymphoid cells expressed CD3, TIA-1 and EBER1 with an unusual lack of CD56. In addition, the patient had no typical symptoms of infectious mononucleosis-like illness, hypersensitivity to mosquito bites or other symptoms supporting CAEBV [16].

Our patient died only 33 days after initial presentation. Generally, the prognosis of both peripheral T-cell lymphoma and NK/T-cell lymphoma is poor. Nonnasal NK/T-cell lymphoma, such as this case, shows an aggressive clinical course. Chen et al. reported that the mean survival period of nonnasal NK/T-cell lymphoma patients was

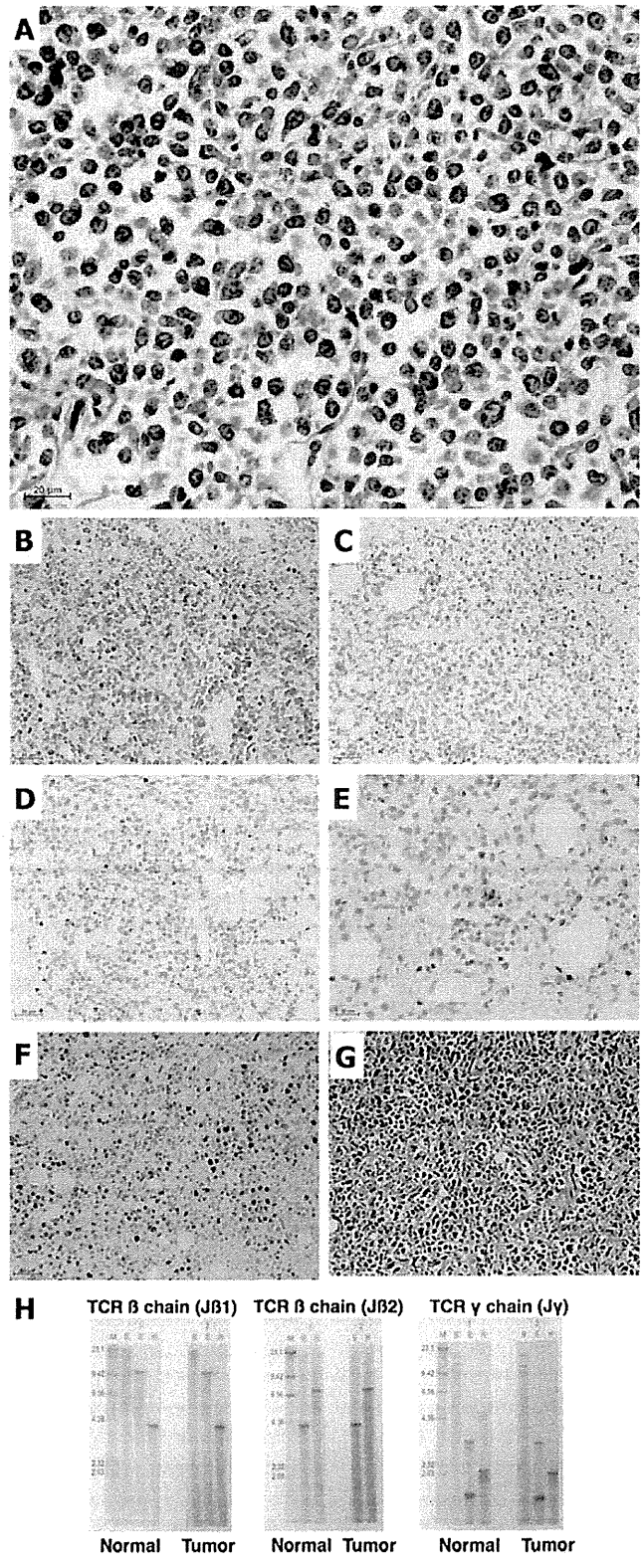

Figure 3 (See legend on next page.)

Figure 3 Microscopic views and T-cell receptor rearrangement of the tumors. HE (**A**) and immunohistochemistry against CD3 (**B**), CD20 (**C**), CD56 (**D**), TIA-1 (**E**) and Ki-67 (**F**). (**G**) In situ hybridization against EBER-1. (H) Southern blotting to detect T-cell receptor rearrangement in fresh frozen tumor tissue. The first column in each blot indicates the size of the DNA fragment (kbp). Restriction enzymes used were as the follows: E, EcoRI; B, BamHI and H, HindIII.

3.5 months (from 1 week to 3 years) after initial presentation [17].

In pyothorax-associated lymphoma, transformation of lymphocytes caused by EBV infection and proliferative stimulation via inflammatory cytokines including interleukin-6 in the microenvironment of chronic pyothorax might be the major cause of tumorigenesis [8]. In a narrow sense, the association between pyothorax and the present lymphoma of adrenal glands is still unknown because no tumor lesions were detected in the pleural cavity. Obviously, there is a possibility that the pyothorax existed incidentally. Nevertheless, the possibility that both the long-standing pyothorax lesion and EBV infection contributed to the tumor-initiating ability of tumor cells in the present case cannot be ruled out. Asakage et al. reported a case of EBV-positive T-cell lymphoma of the stomach in a patient with pyothorax. In that patient, no tumors were detected around the pleural cavity, as in the present case [5].

Conclusion

We have reported the first case of CD56-negative extranodal NK/T-cell lymphoma in bilateral adrenal glands in a patient with pyothorax. The tumor displayed highly malignant characteristics with a distressful clinical course. There is a possibility that chronic inflammation in the microenvironment of the pyothorax and EBV infection contributed to the tumor-initiating ability of tumor cells in the present case.

Consent

Written informed consent was obtained from the family of the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT, AT and MM performed the autopsy and assessed macroscopic and microscopic findings. KO and MN treated the patient and contributed to acquisition of clinical data. TT and AT drafted the manuscript. NS and TH participated in the coordination. All authors read and approved the final manuscript.

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Received: 31 July 2012 Accepted: 26 August 2012
Published: 29 August 2012

References

1. Singh D, Kumar L, Sharma A, Vijayaraghavan M, Thulkar S, Tandon N: **Adrenal Involvement in Non-Hodgkin's Lymphoma: Four Cases and Review of Literature.** *Leuk Lymphoma* 2004, **45**:789–794.
2. Aozasa K: **Pyothrax-associated lymphoma.** *J Clin Exp Hematopathol* 2006, **46**:5–10.
3. Tsai W, Hsieh C, Cheng M, Lin C: **Adrenal insufficiency in T-cell lymphoma.** *Int J Urol* 2006, **13**:794–797.
4. Kang S, Kim W, Lee K, Baek H, Park T, Jin H: **Mainly adrenal gland involving NK/T-cell nasal type lymphoma diagnosed with delay due to mimicking adrenal hemorrhage.** *J Korean Med Sci* 2011, **26**:1386–1390.
5. Asakage N, Yamamoto T, Suzuki T, Haraguch Y, Tsukada K, Kobayashi S, Yamasaki S: **A case of malignant T-cell lymphoma of gastric origin accompanied by pyothrax.** *Case Rep Gastroenterol* 2009, **3**:162–168.
6. Santini M, Fiorello A, Vicidomini G, Busiello L, Baldi A: **A surgical case of pyothorax-associated lymphoma of T-cell origin arising from the chest wall in chronic empyema.** *Ann Thorac Surg* 2009, **88**:642–645.
7. Narimatsu H, Ota Y, Kami M, Takeuchi K, Suzuki R, Matsuo K, Matsumura T, Yuji K, Kishi Y, Hamaki T, Sawada U, Miyata S, Sasaki T, Tobinai K, Kawabata M, Atsuta Y, Tanaka Y, Ueda R, Nakamura S: **Clinicopathological features of pyothorax-associated lymphoma; a retrospective survey involvinv 98 patients.** *Ann Oncol* 2007, **2007**:122–128.
8. Hashizume T, Aozasa K, Tomita Y, Matsushita K: **Pyothorax-associated T-cell lymphoma: a case report.** *Jpn J Clin Oncol* 2003, **33**:145–147.
9. Kamiya I: **A case of T-cell malignant lymphoma developing in the chest wall of chronic pyothorax after artificial pneumothorax.** *Nihon Kyobu Geka Gakkai Zasshi* 1996, **44**:709–713.
10. Nakamura S, Sasajima Y, Koshikawa T, Kitoh K, Kato M, Ueda R, Mori S, Suchi T: **Ki-1 (CD30) positive anaplastic large cell lymphoma of T-cell phenotype developing in association with long-standing tuberculous pyothorax: report of a case with detection of Epstein-Barr virus genome in the tumor cells.** *Hum Pathol* 1995, **26**:1382–1385.
11. Fukino S, Fukata T, Inoue A, Hatazawa Y, Morio S: **A surgical case of T-cell non-Hodgkin's lymphoma originating in the wall affected by chronic tuberculous empyema following artificial pneumothorax.** *Nihon Kyobu Geka Gakkai Zasshi* 1992, **40**:113–117.
12. Iuchi K, Aozasa K, Yamamoto S, Mori T, Tajima K, Minato K, Mukai K, Komatsu H, Tagaki T, Kobashi Y: **Non-Hodgkin's lymphoma of the pleural cavity developing from long-standing pyothorax. Summary of clinical and pathological findings in thirty-seven cases.** *Jpn J Clin Oncol* 1989, **1989**:249–257.
13. Tsuruhara K, Sakaki T, Oyaizu N, Morii S: **An autopsy case of an extranodal malignant lymphoma occurring primarily in the chest wall.** *Gan No Rinsho* 1987, **33**:1287–1293.
14. Nokubi M, Saito K, Kanai N, Fujii T, Nakagami H, Izumi T, Ohkawara K: **An autopsy case of pyothorax-associated pleural lymphoma, initially presenting with bilateral adrenal mass.** *Byori to Rinsyo* 1996, **1996**: 529–533.
15. Miles R, Afify Z, Yaish H, Perkins S: **CD56-negative extranodal nasal type NK/T-cell lymphoma.** *Pediatr Blood Cancer* 2010, **55**:186–189.
16. Kimura H: **Pathogenesis of chronic active Epstein-Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency?** *Rev Med Virol* 2006, **16**:251–261.
17. Chan JK, Sin VC, Wong KF, Ng CS, Tsang WY, Chan CH, Cheung MM, Lau WH: **Nonnasal lymphoma expressing the natural killer cell marker CD56: a clinicopathologic study of 49 cases of an uncommon aggressive neoplasm.** *Blood* 1997, **89**:4501–4513.

doi:10.1186/1746-1596-7-114
Cite this article as: Tsukahara *et al.*: NK/T-cell lymphoma of bilateral adrenal glands in a patient with pyothorax. *Diagnostic Pathology* 2012 7:114.

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Cancer Science
The official journal of the Japanese Cancer Association JCA

Immunotherapeutic benefit of α-interferon (IFNα) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients

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(Received July 3, 2012/Revised October 4, 2012/Accepted October 10, 2012/Accepted manuscript online October 19, 2012/Article first published online December 16, 2012)

Survivin, a member of the inhibitor of apoptosis protein (IAP) family containing a single baculovirus IAP repeat domain, is highly expressed in cancerous tissues but not in normal counterparts. Our group identified an HLA-A24-restricted antigenic peptide, survivin-2B80–88 (AYACNTSTL), that is recognized by CD8 + CTLs and functions as an immunogenic molecule in patients with cancers of various histological origins such as colon, breast, lung, oral, and urogenital malignancies. Subsequent clinical trials with this epitope peptide alone resulted in clinical and immunological responses. However, these were not strong enough for routine clinical use as a therapeutic cancer vaccine, and our previous study of colon cancer patients indicated that treatment with a vaccination protocol of survivin-2B80–88 plus incomplete Freund's adjuvant (IFA) and α-interferon (IFNα) conferred overt clinical improvement and enhanced the immunological responses of patients. In the current study, we further investigated whether this vaccination protocol could efficiently provide not only improved immune responses but also better clinical outcomes for advanced pancreatic cancers. Tetramer and enzyme-linked immunosorbent spot analysis data indicated that more than 50% of the patients had positive clinical and immunological responses. In contrast, assessment of treatment with IFNα only to another group of cancer patients resulted in no obvious increase in the frequency of survivin-2B80-88 peptide-specific CTLs. Taken together, our data clearly indicate that a vaccination protocol of survivin-2B80-88 plus IFA and IFNα is very effective and useful in immunotherapy for this type of poor-prognosis neoplasm. This trial was registered with the UMIN Clinical Trials Registry, no. UMIN000000905. (*Cancer Sci* 2013; 104: 124–129)

Recent progress in human tumor immunology research has presented us with the possibility that immunotherapy could be established as an effective cancer therapy in the very near future.[1–6] Indeed, since the first discovery of a human tumor antigen in 1992,[7] many clinical trials for cancer vaccines have been carried out, and these studies have suggested that active immunization using HLA class I restricted tumor antigenic peptides and the whole or part of the tumor antigenic protein could work as activators of antigen-specific CTLs, at least in some cancer patients.[8–16] However, even in effective cases, vaccination with these molecules alone is not sufficient to evoke a potent and stable immune response and subsequent strong clinical effect. Thus, it is crucial to develop various methods for enhancing the immunological efficacy of tumor antigens.

We have studied how tumor antigenicity can be efficiently enhanced in cancer patients since 2003. In our studies, the HLA-A24-restricted peptide survivin-2B80-88 was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, and urinary bladder cancers, and lymphomas. Clinically, certain patients with colon, lung, and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed by computed tomography (CT).[8–12] These effects, however, were not strong enough for the clinical requirements as decided by the criteria for cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors, which requires more than 30% regression of tumors on CT, only one patient each of 15 with colon cancers and three with urinary bladder cancers had a positive clinical response, indicating that the therapeutic potential was obviously not strong enough for routine clinical use as a cancer treatment.

In a previous study,[8] to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we carried out and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear clinical improvement and enhanced the immunological responses of patients. We also analyzed CTLs of these patients by single-cell sorting, and found that each CTL clone from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Pancreatic cancer is still one of most difficult malignant neoplasms to treat, so in the current study we investigated whether the most effective protocol for colon cancer patients, namely survivin-2B80-88 plus IFA and IFNα, could work similarly in pancreatic cancers as in colon cancers. Furthermore, we carried out frequency monitoring of survivin-2B80-88 peptide-specific CTL in cases of cancer patients treated with IFNα alone, and found no overt increase of these CTLs. Once the survivin-2B80-88 peptide was administered with IFNα, patients showed strong clinical and immunological responses as assessed by tetramer and enzyme-linked immunosorbent spot (ELISPOT) analyses. Taken together, our current data strongly suggest that vaccination using survivin-2B80-88 plus IFA and IFNα is actually very effective in patients with advanced pancreatic cancers from both the clinical and immunological points of view.

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doi: 10.1111/cas.12046

Materials and Methods

Patients. Patient selection was done as reported in our previously published work. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University (Sapporo, Japan).[8–12] All patients gave informed consent before being enrolled. Patients who participated in this study were required to: (i) have histologically confirmed pancreatic cancer; (ii) be HLA-A*2402 positive; (iii) have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) be between 20 and 85 years old; (v) have unresectable advanced cancer or recurrent cancer; and (vi) have Eastern Cooperative Oncology Group performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection, or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, in the Sapporo Medical University Primary Hospital from December 2005 through to November 2010.

Peptide, IFA, and IFNα preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8–10,12] The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by HPLC analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) and stored at −80°C until just before use. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan).

Patient treatment. In this clinical study, we used the protocol illustrated in Fig. 1, with the survivin-2B80-88 peptide plus IFA and IFNα. In this trial, the primary endpoint was safety. The second endpoint was investigation of the antitumor effects and clinical and immunological monitoring.

In this protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, IFNα at a dose of 3 000 000 IU was given s.c. twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination (Fig. 1).

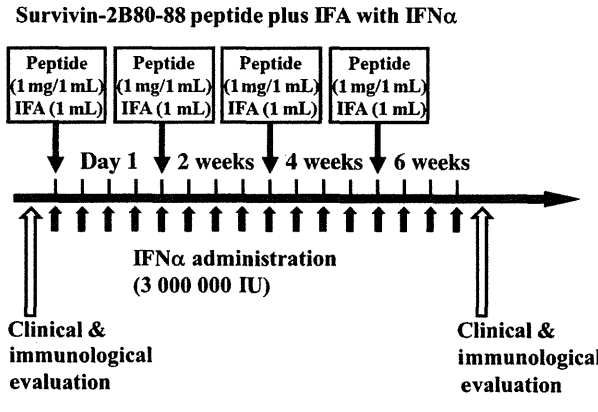

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination.

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.[8–10]

Clinical response evaluation. Physical examinations and hematological examinations were carried out before and after each vaccination.[8–10] A tumor marker (Ca19-9) was examined. Changes in the tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary pancreatic cancer tissues was done with anti-HLA class I heavy chain mAb EMR-8-5[13] (Funakoshi, Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response was defined as a $\geq 30\%$ 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 complete response, partial response, or PD.[8–10] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC, tetramer staining, and ELISPOT assay.** The samples for tetramer analysis and ELISPOT analysis were simultaneously obtained at the time of the hematological examination before and after each vaccination. These experiments were carried out as in our previous report. The 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 μg/mL survivin-2B80-88 in AIM V (Life Technologies Corp, Grand Island, NY, USA) medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 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.

The FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) and phycoerythrin (PE)-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from Medical and Biological Laboratories (MBL) Co., Ltd (Nagoya, Japan). For flow cytometric analysis, PBMCs, stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a PE-Cy5-conjugated anti-CD8 mAb (BD Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was carried out 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.[8,10,12]

The ELISPOT plates were coated overnight in a sterile environment with an IFNγ capture antibody (BD Biosciences) at 4°C. The plates were then washed once and blocked with AIM V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well) that were stimulated *in vitro* as above were then added to each well along with HLA-A24-transfected T2 cells (T2-A24) (5×10^4 cells/well) that had been preincubated with or without survivin-2B80-88 (10 mg/mL) or

with an HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFNγ antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Oberkochen, Germany). In this study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-postive spots as compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTLs. Survivin-2B80-88 peptide tetramer-positive CTLs were sorted and subsequently cloned to single cells using FACS (Aria II Special Order; BD Biosciences). The peptide-specific cytotoxicity of each of these CTLs was determined by pulsing T2A24 cells[8,17] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides, as previously described.

Results

Patient profiles, safety, and clinical responses. In the present protocol with the survivin-2B80-88 peptide plus IFA and IFNα, six patients were enrolled in the study (Table 1). None dropped out because of adverse events due to the vaccination. They consisted of three men and three women, whose age range was 50–80 years.

With respect to the safety, vaccination was well tolerated in all patients. Four patients had fever reaching nearly 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination except for induration at the injection site, which was conduced by IFA.

The clinical outcomes for the six patients treated with survivin-2B80-88 plus IFA and IFNα are summarized in Table 1. In some patients, particularly No. 1, the postvaccination Ca19-9 value was clearly decreased as compared with prevaccination, and was within the normal limit. Other patients (Nos. 2, 4, and 6) also had decreased or stable postvaccination levels of Ca19-9, although not as large. As for tumor size evaluated by CT, four patients (Nos. 1, 2, 4, and 6) were considered to have SD, but the other two patients (Nos. 3 and 5) had PD. Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced or stable Ca19-9 level.

Immune responses, single-cell cloning, and subsequent functional assessment of tetramer-positive CTLs. As in our previous study with colon cancer patients, we determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequencies before the first vaccination (prevaccination) and after the last vaccination (postvaccination) were assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with an HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

As summarized in Table 1, four of the six patients (Nos. 1, 2, 4, and 6) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and all four had SD by CT evaluation, suggesting that immune responses might appropriately reflect clinical responses with the current vaccination protocol.

As in our previous work, we also analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Fig. 2, patient No. 1 (62 years old, female) had a reduced serum Ca19-9 level, and obvious immune responses as assessed by the survivin-2B80-88 ELISPOT and tetramer analyses (Fig. 3) after vaccination.

Subsequently, CD8 T cells of the tetramer-positive fraction were sorted by FACS, then cultured with 1, 3, and 10 cells/well for 7–10 days. Almost all growing T cells were survivin-2B peptide-specific T cells (data not shown), and we next assessed peptide-specific cytotoxicity by using these T cells. As Fig. 4 clearly shows, all T cells had very high peptide-specific cytotoxic potential. Taken together, these data clearly indicated that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα was capable of inducing a strong CTL response and for some pancreatic cancer patients might result in clinical effectiveness.

Assessment of treatment effect with IFNα alone. The above data strongly suggested that the current vaccination protocol

Table 1. Profiles of patients with advanced pancreatic cancer enrolled in the study and their clinical and immunological responses to vaccination with survivin-2B80-88 peptide, incomplete Freund's adjuvant and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre/post (CA19-9 U/mL)	CT eval.	Tetramer staining†		ELISPOT‡	
					Pre/post	% Increase	Pre/post	% Increase
1	62/F	Induration	136.5/31.4	SD	23/246	1069.6	27/294	1088.9
2	61/F	Induration Fever	63.6/60.6	SD	1/157	15700.0	25/71	284.0
3	56/M	Induration Fever Thrombopenia	171.4/978.8	PD	22/19	86.3	19/525	2763.2
4	80/F	Induration Fever	30.0/22.7	SD	9/1030	11444.4	1/101	10100.0
5	58/M	Induration Fever	436.0/2885.0	PD	3/0	0.0	34/20	58.8
6	50/M	Induration	4389.0/4295.0	SD	2/7	350.0	27/85	314.8

†Cytotoxic T-lymphocyte frequency of prevaccinated (pre) and postvaccinated (post) patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The numbers of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 × CD8 T cells are shown. ‡γ-Interferon (IFNγ) secretion of pre- and postvaccinated patients' CD8 T cells was assesed with enzyme-linked immunosorbent spot (ELISPOT) assay using T2-A24 cells pulsed with survivin-2B80-88 peptide. The numbers of spots in 5 × 10^3 CD8 T cells are shown. CT eval., evaluation by computed tomography; IFNα, α-interferon; PD, progressive disease; SD, stable disease.

doi: 10.1111/cas.12046