Table 1 Chemotherapy for advanced pancreatic cancer

	Median survival time (months)	Overall response rate (%)	Trial name	References
Gemcitabine	5.65	5.4		J Clin Oncol 1997;15: 2403–13.
Gemcitabine + erlotinib	6.24	8.6	NCIC CTG PA.3	J Clin Oncol 2007;25: 1960–6.
FOLFIRINOX	11.1	31.6	ACCORD 11	N Engl J Med 2011;364: 1817–25.
Nab-paclitaxel + gemcitabine	8.7	29.2	MPACT trial NCT00844649	N Engl J Med 2013;369: 1691–703.
Gemcitabine +TS-1	10.1	29.3	GEST trial	J Clin Oncol 2013; 31:640–8.

treating pancreatic ductal adenocarcinoma (PDAC) [9]. Gemcitabine monotherapy has subsequently become the standard chemotherapy for PDAC, resulting in an MST of 5.65 months (Table 1). Currently, three protocols have proven to be superior to gemcitabine monotherapy. Combining gemcitabine with erlotinib improved the MST of PDAC to 6.24 months in the NCIC CTG PA3 trial [10], while combining gemcitabine with nab-paclitaxel improved the MST to 8.7 months in the MPACT trial [11]. FOLFIRINOX achieved the longest MST for PDAC (11.1 months) in the ACCORD11 trial [12], and the GEST study obtained similar clinical outcomes. S-1 is an oral fluoropyrimidine derivative that has been shown to be effective against various cancers, and a previous study found that it is at least as effective as gemcitabine against PDAC [13]. In addition, treatment with a combination of gemcitabine + S-1 has been demonstrated to result in an MST of 10.1 months [14]. Although these chemotherapies extend the survival period among PDAC patients, they also result in serious adverse events. Therefore, the optimal chemotherapy regimen for PDAC depends on the patient's performance status.

There have been numerous attempts to develop vaccine therapies for cancer over the past century [2, 3]. Although clinical trials of such vaccines have obtained promising results in specific patients, none of the tested vaccines has exhibited significant improvements in efficacy compared with established therapies. In addition, several issues must be resolved before vaccine therapies can be used in the clinical setting. Tumor-associated antigens (TAA) have been demonstrated to recognize specific human leukocyte antigens (HLA) [15]. Theoretically, the tumor lysate contains all of the antigens expressed by the tumor, and cytotoxic T lymphocytes (CTL) are capable of recognizing some of these antigens [16]. All vaccines for pancreatic cancer are based on the fact that CTL recognize TAA expressed on tumor cells and subsequently attack these cells. The question is how strongly and specifically each TAA stimulates CTL in vivo in the clinical setting. Immune tolerance can develop via various mechanisms, including the downregulation of the major histocompatibility complex (MHC) molecule expression, induction of T cell anergy, reductions in the number of immune effectors and increases in the number of regulatory T cells [17, 18], which may explain why no cancer vaccine therapy has been established as a standard treatment for advanced PDAC. Therefore, in this study, we comprehensively reviewed the clinical outcomes of vaccine therapy against advanced PDAC.

Peptide-based vaccines developed within the past few years

MUC1

Mucin 1, cell surface associated, (MUC1) is a type I transmembrane protein containing multiple tandem repeats of a 20-amino acid sequence. Several MUC1 peptides have been tested as vaccines in the clinical setting; however, most of them have failed to activate CTL [19–21]. Ramanathan et al. [22]; Yamamoto et al. [23] injected pancreatic patients with a vaccine containing a 100-mer extracellular tandem repeat domain of MUC1 and Montanide ISA-51, and both studies obtained similar clinical responses; i.e., the authors detected cytokines (interferon (IFN)-γ or interleukin (IL)-4) and anti-MUC1 antibodies in the patients' sera but did not observe any significant clinical effects. Another recent study involving a vaccine based on a different MUC1 epitope showed similar clinical outcomes, i.e., all seven patients had progressive disease (PD), although some of the patients exhibited immunological responses, such as IFN-γ and granzyme B secretion [24].

K-RAS mutants

K-RAS mutations are frequently found in patients with PDAC. Vaccines targeting mutations in codon 12 of the K-RAS gene have been tested as treatments for advanced [25] or postoperative [26] PDAC in the clinical setting. Gjertsen et al. [[21]] investigated the utility of a K-RAS peptide vaccine containing granulocyte–macrophage colony-stimulating factor (GM-CSF) in 10 patients who had undergone potentially curative

resection (CTN RAS 95002) and 38 patients with advanced disease (CTN RAS 97004). In that study, one patient achieved a partial response (PR), which lasted for 28 months, and the MST of the immunological responders was 4.9 months, compared to 2.0 months for the non-responders.

Human telomerase reverse transcriptase (hTERT)

Human telomerase reverse transcriptase (hTERT) is frequently expressed in cancer cells [27]. hTERT maintains functional telomeres at the end of chromosomes, which protect against cell senescence [28]. A vaccine against pancreatic cancer containing the telomerase peptide GV1001: hTERT (611-626) and GM-CSF was examined by Bernhardt et al. [29], who found the MST of the immunological responders and non-responders to be 7.2 and 2.9 months, respectively.

Vascular endothelial growth factor receptor 2 (VEGFR2)

Vascular endothelial growth factor (VEGF) plays an important role in the progression of PDAC. The type 2 VEGF receptor (VEGFR2) is expressed in PDAC and associated with tumor neovascularization. Miyazawa et al. [[30]] investigated the efficacy of combined treatment consisting of PDAC with a VEGFR2-169 peptide-based vaccine and gemcitabine chemotherapy and reported that one patient achieved a PR, while the disease control rate was 67 %. In addition, the MST was 7.7 months, although 15/18 patients were chemotherapy naive.

G17DT (gastrimmune)

Gastrin is expressed in PDAC and plays a role in regulating the autocrine, paracrine and endocrine systems [31]. The administration of the anti-gastrin immunogen G17DT results in increased serum antibody levels and reduced tumor growth in patients with gastrointestinal malignancies [32]. A randomized, double-blind, placebo-controlled multicenter trial of G17DT was also recently performed [33]. Although, among the intention to treat (ITT) population, no significant differences in MST were detected between the PDAC patients treated with G17DT and those given the placebo, the MST of the two groups differed significantly after excluding major protocol violators and censoring for chemotherapy.

Heat shock protein (HSP)

Heat shock protein (HSP) itself is not an immunogen; however, it acts as a chaperone or carrier of antigenic peptides and possesses a repertoire of cellular peptides for pancreatic cancer [34]. Furthermore, HSPPC-96 (Oncophage) has been tested as a vaccine in the adjuvant setting after complete resection of PDAC [35]. In the latter study, the MST of PDAC was reported to be 2.9 months after surgery; however, this did not result in further clinical studies because only two of 10 patients exhibited increased enzyme-linked immunospot (ELISPOT) reactivity.

Biological vaccines

Fowlpox viral vaccine

Carcinoembryonic antigen (CEA) and MUC1 are highly expressed in PDAC [36]. Viral vectors carrying CEA, MUC1 and TRICOM [a triad of costimulatory molecules: B7.1, intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 3 (LFA-3)] have been investigated as vaccines against advanced PDAC [37]. In one study, a vaccinia viral vector was used for the initial T cell priming, and a fowlpox viral vector was used for immune boosting. Although this treatment resulted in an MST of 6.3 months (1.5–21.1 months), the five patients who showed T cell responses achieved a longer survival period than the five patients who did not (15.1 and 3.9 months, respectively; $P = 0.002$) [38]. It should be noted that GM-CSF was used as a vaccine adjuvant in the latter trial (Table 2).

Live-attenuated, double-deleted (LADD) Listeria monocytogene vaccine

ANZ-100 is a live-attenuated double-deleted Listeria monocytogene strain (LADD; Lm ΔactA/ΔinlB) found to induce a local proinflammatory response, resulting in the activation of innate and adaptive effector cells [39]. Mesothelin is expressed in PDAC and plays an important role in tumor progression [40]. CRS-207 is a LADD Lm strain that delivers mesothelin antigens into class I and II antigen-processing pathways [41]. In a study examining the utility of CRS-207 as a treatment for advanced cancer, three of the seven subjects with PDAC were long-term survivors, although the detection of a mesothelin-specific T cell response was not correlated with survival [41].

Recent vaccine therapies

WT1

Kobayashi et al. reported a retrospective analysis of 255 advanced PDAC patients who were treated with dendritic

Table 2 Peptide-based vaccines and biological vaccines for advanced pancreatic cancer

Author	Journal	Antigen peptide	Sequences	Combination	Patients	Outcome/MST
Yamamoto	Anticancer Res. 2005;25:3575–9	MUC1	10-mer extracellular tandem repeat domain: $(GVTSAPDTRPAPGSTAPPAH)_5$	Montanide ISA-51	6	1/6 SD
Rong	Clin Exp Med. 2012;12:173–80	MUC1	PDTRPAPGSTAPPAHGVTSA	DC cells	7	All PD
Gjertsen	Int J Cancer. 2001;92:441–50	K-ras	KLVVVGAGGVGKSALTI Asp: D Arg: R Val: V Cyc: C	GM-CSF	38	1 PR 10 SD (10.2 M; 3-23 M) 27 PD 4.9 M responders 2.0 M non-responders
Abou-Alfa	Am J Clin Oncol. 2011;34:321–5	ras12R ras12 V ras12D Wild-type ras	TEYKLVWGARGVGKSALTIQ TEYKLVWGAVGVGKSALTIQ TEYKLVWGADGVGKSALTIQ TEYKLVWGAGGVGKSALTIQ	hGM-CSF	24	Postoperative adjuvant treatment
Bernhardt	Br J Cancer. 2006;95:1474–82	Telomerase hTERT (611–626)	GV1001; EARPALLTSRLRFIPK	GM-CSF	38	7.2 M (24 responders) 2.9 M (14 non-responders)
Miyazawa	Cancer Sci. 2010;101:433–9	VEGFR2-169	RFVPDGNRI	Gemcitabine	18	7.7 M
Gilliam	Pancreas. 2012;41:374–9	Anti-gastrin G17DT Gastrimmune	EGPWLEEEEEAYGWMDF-DT (diphtheria toxoid)	G17DT vs. placebo	152	5.0 M vs 2.8 M
Maki	Dig Dis Sci. 2007;52:1964–72	HSP HSPPC-96 (gp96, Oncophage)			10	Postoperative adjuvant treatment 2.7 Y
Kaufman	J Transl Med. 2007;5:60	MUC1 and CEA	CEA agonist peptide CAP1-6D (YLSGADLNL) MUC-1 agonist peptide P-93L (ALWGQDVTSV)	B7.1, ICAM-1, LFA-3 (TRICOM) Vaccinia virus: PANVAC-V Fowlpox virus: PANVAC-F GM-CSF	10	6.3 M
Le	Clin Cancer Res. 2012;18:858–68	Listeria vaccine ANZ-100, CRS-207			9 vs. 17	NA

Table 3 Recently developed peptide-based vaccines and multiple vaccines for advanced pancreatic cancer

Author	Journal	Antigen peptide	Sequences	Restricted HLA	Combination	Patients	Outcome/MST
Kobayashi	Cancer Immunol Immunother. 2014;63:797–806	WT1 MUC1	CYTWNQMNL RMFPNAPYL TRPAPGSTAPPAHG-VTSAP DTRPAPGSTAP	A24:02 A02:01/02:06 Any A	DC cells OK432	255	9.9 M 10.4 M (erythema)
Nishida	J Immunother. 2014;37:105–14	WT1	CYTWNQMNL	A24:02	Weekly 1000 mg/m^2 GEM	31	8.1 M 10.9 M (DTH)
Asahara	J Translation Res. 2013;11:291	KIF20A-66	KVYLRVRPLL	A2402	Montanide ISA51VG	31	4.7 M 6.1 M (reaction)
Suzuki	J Immunother. 2014;37:36–42	KIF20A-10-66	KVYLRVRPLL	A2402	Montanide ISA51VG	9	5.8 M
Geynisman	J ImmunoThera Cancer. 2013;1:8	CEA CAP1-6D	YLSGADLNL	A2	Montanide/GM-CSF	19	11.1 M
Kameshima	Cancer Sci. 2013;104:124–9	SVN2B	AYACNTSTL	A2402	Montanide/IFN-oc	6	(9.6 M)
Yutani	Oncology Reports. 2013;30:1094–100	31 vaccine peptides		A2, A24, A3, A26	Mono: 8 Chemo: 33	41	7.9 M 9.6 M (chemo)
Kimura	Pancreas. 2012;41:195–205	WT1, Her2, CEA, MUC1, CA125, autologous tumor lysate			DC cells plus LAK plus GEM and S1 OK432	49	S: 8.0 M G: 12.0 M GS + LAK: 16.9 M
Le	J Clin Oncol. 2014;32(suppl 3):Abstract 177	GVAX pancreas and CRS-207 vs. GVAX pancreas alone	Irradiated GM-CSF-secreting allogeneic pancreatic tumor vaccine (GVAX pancreas)		Cyclophosphamide	90	6.1 M vs. 3.9 M 9.7 M (3 or more rounds of vaccine therapy)

cell (DC) vaccines containing Wilms tumor 1 (WT1) and MUC1 after being recruited from seven institutions that followed a unified standard operating procedure. The MST of these patients was 9.9 months [42]. Nishida et al. also examined the utility of chemo-vaccine therapy in which a WT1-based vaccine was used in combination with the administration of 1,000 mg/m^2 of gemcitabine weekly. The latter regimen resulted in an MST of 8.1 months among 31 advanced PDAC patients [43]. In addition, the MST of the immunological responders in these two studies was very similar (10.4 and 10.9 months, respectively) (Table 3).

KIF20A

Kinesin family member 20A (KIF20A) plays an important role in the trafficking of molecules and organelles [44] and is one of the molecules targeted by vaccines against PDAC. A KIF20A vaccine was recently tested using different regimens, including vaccine monotherapy [45] and chemo-vaccine therapy involving gemcitabine [46], and similar MST values were reported in both studies (4.7 and 5.8 months, respectively).

Carcinoembryonic antigen (CEA)

CEA is a 180-kDa immunoglobulin-like molecule expressed on the surface of 90 % of PDAC tumor cells [47]. CAP1-6D, a modified CEA peptide, was combined with Montanide/GM-CSF to produce a vaccine against pancreatic cancer that was subsequently tested in advanced PDAC patients [48]. The MST of the 19 patients was 11.1 months, and one patient, randomized into the 0.01 mg arm, achieved a complete response (CR).

Survivin2B

Survivin is a member of the inhibitors of apoptosis (IAP) family of proteins that protect apoptotic signals by inhibiting the caspase activity [49, 50]. Hence, survivin-expressing cancer cells escape from apoptosis and do not die. Using a peptide-binding assay, we found that the survivin2B 80–88 peptide induces a strong CTL response [51]. We also examined the effects of a survivin2B 80–88 peptide-based vaccine on various cancers in the clinical setting and obtained promising outcomes. In particular, the anti-tumor effect of the survivin2B 80–88 peptide was enhanced by combining it with incomplete Freud's adjuvant and IFN-α injection. Our preliminary clinical study demonstrated a 66.6 % disease control rate in advanced PDAC patients (four of six patients) [52]. Moreover, the PDAC patients in our recent clinical phase I study exhibited an MST of 9.6 months.

Table 4 Evaluation of therapeutic activity in solid tumors

Method	WHO	RECIST	IrRC
	Sum of the products of the two longest perpendicular dimensions (bidimensional)	Sum of the longest dimensions (unidimensional)	Sum of the products of the two longest perpendicular dimensions (SPD) of all index lesions. (bidimensional)
No. of measured lesions	All lesions	Five per organ, 10 in total	Five per organ, 10 in total, and five cutaneous index lesions
CR	Disappearance of all known disease, confirmed at 4 weeks	Disappearance of all known disease, confirmed at 4 weeks	Disappearance of all known disease, confirmed at 4 weeks apart
PR	>50 % decrease in total tumor size, confirmed at 4 weeks	>30 % decrease in total tumor size, confirmed at 4 weeks	>50 % decrease in tumor burden compared with baseline in two observations at least 4 weeks apart
SD	CR, PR, and PD criteria not met	CR, PR, and PD criteria not met	CR, PR, and PD criteria not met
PD	>25 % increase in total tumor size; no CR, PR, or SD documented before increase in tumor size; new lesion (s); > 25 % increase in size of one lesion	>20 % increase in total tumor size; no CR, PR, or SD documented before increase in tumor burden; new lesion (s)	>25 % increase in tumor burden compared with nadir (at any single time point) in two consecutive observations at least 4 weeks apart

Tumor burden = $SPD_{index\ lesions}$ +SPD new, measurable lesions

Multiple vaccines

Personalized peptides

In a previous study, a set of 31 peptides was used to create personalized vaccines for advanced PDAC [53]. A maximum of four peptides were selected from among the 31-peptide set based on the results of HLA typing and the patients' peptide-specific IgG titers. Eight patients received vaccine monotherapy, and 31 patients received chemo-vaccine therapy. In the chemo-vaccine therapy group, gemcitabine was administered in eight patients, S-1 was administered in six patients and gemcitabine + S-1 was given in eight patients. The overall MST was 9.6 months, although that of the patients who underwent monotherapy was 7.9 months. Yanagimoto et al. reported similar clinical outcomes for chemo-vaccine therapy involving personalized vaccines and gemcitabine based on the same regimen [54]. The MST of the patients in the latter study was 9.0 months, although that of the immunological responders was 15.5 months. None of the patients in either study achieved CR (Table 3).

Autologous tumor lysate combined with lymphokine-activated killer cell therapy

Kimura et al. treated 49 PDAC patients with vaccines based on five different peptides and autologous tumor lysate, although the vaccine preparation regimens and anti-tumor therapies varied in each case [16]. Two patients achieved CR after treatment with a combination of DC cell and lymphokine-activated killer cell (LAK) therapy. The MST of the patients treated with LAK + gemcitabine and S-1 was 16.9 months, whereas that of all patients was 12.0 months. It should be noted that the survival time was calculated from the day after the first vaccination, which may have resulted in a shorter survival time (by a couple of months) than would have been obtained using the methods employed in other studies. It is very difficult to evaluate the clinical results of this study due to the effects of the different therapeutic strategies used in each case. However, the fact that multiple patients achieved CR will encourage researchers to pursue this approach further.

GVAX pancreas with CRS-207

GVAX is a series of irradiated GM-CSF-secreting allogeneic pancreatic cell lines that elicit broad antigenic responses. CRS-207 is a LADD Lm strain (Lm ΔactA/ΔinlB) that expresses mesothelin and stimulates the innate and adaptive immune systems. A phase II randomized control trial of GVAX pancreas combined with CRS-207 versus GVAX pancreas alone was presented at the 2014 American Society of Clinical Oncology (ASCO) Gastrointestinal Cancers Symposium [55]. Interestingly, the clinical results demonstrated that both treatments had dose-dependent survival benefits. The MST of the patients who received three or more rounds of vaccine therapy was 9.7 months, and the MST of the GVAX with CRS-207 arm was longer than that of the GVAX-alone arm (6.1 vs. 3.9 months; $P = 0.01$) [56].

Evaluation of therapeutic activity in solid tumors

The response of solid tumors is evaluated using either the WHO [57] or RECIST criteria [58]. The RECIST criteria were developed because the WHO criteria are quite complex and measuring all visible lesions in two dimensions is both time consuming and subject to measuring bias [59]. However, the use of immunotherapeutic agents in cancer patients is associated with the following problems: (a) The measurable anti-tumor activity can take longer to appear during immunotherapy than during cytotoxic therapy; (b) Responses to immunotherapy can occur after the standard criteria for progressive disease (PD) have been met; (c) Discontinuing immunotherapy may not be appropriate in some cases, unless PD is confirmed; (d) Allowing for "clinically insignificant" PD (e.g., small new lesions developing in the presence of other responsive lesions) is recommended; and (e) Durable stable disease (SD) may represent the anti-tumor activity [60]. Therefore, the immune-related response criteria (irRC) were developed to evaluate the immunotherapeutic activity in solid tumors [61]. The most important aspects of the irRC criteria are that (a) new lesions are not classified as PD and (b) two consecutive observations obtained at least four weeks apart are required to diagnose PD. However, the clinical utility of the irRC remains unclear and these criteria may require further optimization [61] (Table 4).

Future research topics

Initial time point for survival assessments

The initial time point for survival assessments should be unified to allow clinical outcomes to be compared between studies. Most PDAC patients already have advanced disease at the time of diagnosis [6]. In addition, the adverse effects of chemotherapies differ markedly among the various regimens [8]. Therefore, the status of PDAC patients at the time point at which they are registered can differ both within and between clinical studies. Kobayashi et al. reported that the MST from the date of diagnosis and the MST from the first vaccination are very different (16.5 vs.

9.9 months) [42]. Therefore, MST data must be interpreted carefully.

Vaccine therapy and chemotherapy

The goal of vaccine therapy for cancer is to increase the native immunity of cancer patients. However, chemotherapy causes irreversible damage to proliferating cancer cells as well as immune cells, including T and B cells. Therefore, there is a conflict between the fundamental principles of these two treatments. Chemotherapy is currently the gold standard treatment for advanced PDAC. Although the biological mechanisms of vaccine therapy and chemotherapy conflict with each other, the anti-cancer activity of vaccine monotherapy or chemo-vaccine combination therapy should be greater than that of chemotherapy alone.

Slow clinical response to vaccine therapy

It is very hard to achieve a complete response (CR) with vaccine therapy alone. We reviewed 19 studies involving a total of 860 patients and found that CR responses were obtained in only three cases. Although none of these studies involved a large number of patients, the poor reported response rates are a concern. One of the patients who achieved a CR was administered CEA CAP1-6D + Montanide/GM-CSF therapy, while the other two were treated with WT1, Her2, CEA, MUC1, cancer antigen 125 and autologous tumor lysate vaccines combined with DC cell-based LAK therapy and chemotherapy. Immunological responses require a long time to control tumor growth and achieve remission. The primary goal of vaccine therapy is to achieve long-term SD [62]. Most previous clinical studies of PDAC involved patients with advanced disease for whom no other therapies were available. Therefore, vaccine therapy may be suitable for patients in other clinical stages or possibly a useful postoperative adjuvant therapy. The main advantage of vaccine therapy is that it has few adverse effects, although it has also demonstrated minimal clinical effects in previous trials. We are currently conducting a phase II study of the survivin2B 80–88 peptide + Montanide + IFN-β as a treatment for PDAC (SUCCESS, Study of Unresectable CanCEr with Survivin-2B peptide vaccine in Sapporo: UMIN000012146), in which half of the required patients have been recruited. The clinical results of the SUCCESS phase II study will be reported by the end of next year.

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Heat shock protein 90 targets a chaperoned peptide to the static early endosome for efficient cross-presentation by human dendritic cells

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Key words

Antigen presentation/processing, dendritic cells, heat shock protein, T cells, tumor immunity

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The presentation of an exogenous antigen in a major histocompatibility complex class-I- restricted fashion to CD8$^+$ T cells is called cross-presentation. Heat shock proteins (HSPs) such as Hsp70, gp96, and Hsp90 have been shown to elicit efficient CTL responses by cross-presentation through an as-yet entirely unknown mechanism. Hsp90 is the most abundant cytosolic HSP and is known to act as a molecular chaperone. We have shown that a tumor antigen peptide complexed with Hsp90 could be cross-presented by dendritic cells (DCs) through an endosomal pathway in a murine system. However, it has not been determined whether human DCs also cross-present an Hsp90–peptide complex and induce peptide-specific CTLs. In this study, we found that an Hsp90–cancer antigen peptide complex was efficiently cross-presented by human monocyte-derived DCs and induced peptide-specific CTLs. Furthermore, we observed that the internalized Hsp90–peptide complex was strictly sorted to the Rab5$^+$, EEA1$^+$ static early endosome and the Hsp90-chaperoned peptide was processed and bound to MHC class I molecules through an endosome-recycling pathway. Our data indicate that targeting of the antigen to a "static" early endosome by Hsp90 is essential for efficient cross-presentation.

The generation of specific CD8$^+$ CTLs is thought to play a key role in the control of virus-infected cells and tumors. However, immunization with peptides or recombinant proteins generally fails to elicit CTLs because an immunized antigen (Ag) acts as an exogenous Ag. Generally, an exogenous Ag enters the MHC class II pathway and is presented to CD4$^+$ T cells in the context of MHC class II molecules. However, professional Ag-presenting cells, particularly DCs, can take up exogenous Ag and present them on their MHC class I molecules. This process is called cross-presentation and plays an important role in the control of virus-infected cells and tumor growth.[1] There are two pathways of cross-presentation: cytosolic (endoplasmic reticulum–Golgi-dependent) and vacuolar (endosomal) pathways.[2,3] One of the reasons for inefficiency of a vaccine strategy is that the vaccine Ag is usually administered as an exogenous Ag, and it is therefore difficult to introduce the vaccine Ag into the cross-presentation pathway. To overcome this problem, various methods have been developed to target an exogenous Ag into the endogenous MHC class I-restricted pathway. In our previous studies, we showed that extracellular Hsp90–peptide complexes are efficiently cross-presented through the endosome-recycling pathway.[4] In this Hsp90-mediated cross-presentation, the receptor-dependent endocytosed Hsp90–peptide complex was transferred to the early endosome in which a cysteine protease such as cathepsin S processed the precursor peptide. The resulting MHC class I epitope was transferred onto recycling MHC class I molecules, thereby resulting in the expression of an MHC class I–epitope complex on the cell surface. Furthermore, we have shown that immunization with Hsp90–tumor Ag peptide complexes induces Ag-specific CTL responses and strong antitumor immunity *in vivo*. However, how the Hsp90–peptide complex is sorted out after receptor-dependent endocytosis remains unclear. In the present work, we found that Hsp90 complexed with a human tumor Ag peptide derived from survivin-2B[5,6] is cross-presented by human Mo-DCs resulting in the stimulation of peptide-specific CTLs. In addition, we found that Hsp90 targets a chaperoned Ag peptide into the "static" early endosome within Mo-DCs, resulting in cross-presentation of the antigenic peptide through the recycling pathway.

Materials and Methods

The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation,

Sapporo Medical University (Sapporo, Japan). The patients and their families as well as healthy donors gave informed consent for the use of blood samples in our research.

Patient treatment. The patients were vaccinated with survivin-2B$_{80\text{-}88}$ (1 mg) plus Montanide ISA 51 (1 mL; Seppic, Paris, France) s.c. four times at 14-day intervals. In addition, IFN-α (3 000 000 IU; Dainippon-Sumitomo Pharmaceutical Co., Osaka, Japan) was given s.c. twice a week close to the site of vaccination. Hematological examinations were carried out before and after each vaccination.

Induction of human monocyte-derived immature dendritic cells. Autologous monocytes were purified from PBMCs from each patient that were isolated using Lymphoprep (Nycomed, Oslo, Norway). Monocytes (1×10^4/well) in a 24-well plate were cultured in complete RPMI-1640 with 10% FCS and GM-CSF (1000 U/mL) and IL-4 (1000 U/mL) for 7 days. The medium with GM-CSF and IL-4 was gently replaced on day 2 and 4. Human recombinant GM-CSF was a kind gift from Kirin (Tokyo, Japan). Human recombinant IL-4 was purchased from Invitrogen (Carlsbad, CA, USA).

Peptides and proteins. The following peptides were used (underlined sequences representing the precise MHC class I-binding epitope): survivin-2B$_{80\text{-}88}$ (AYACNTSTL); and survivin-2B$_{75\text{-}93}$ (GPGTVAYACNTSTLGGRGG). All peptides were purchased from Sigma-Genosys (Ishikari, Japan). Human Hsp90 was purchased from StressGen (Ann Arbor, MI, USA). Human LDL was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at 20 mg/mL in PBS at −80°C.

Antibodies. Confocal laser microscopy was used to detect organelles with specific antibodies: an anti-Rab5 pAb (MBL, Nagoya, Japan) and EEA1 (Abcam, Cambridge, MA, USA) for early endosomes, and anti-LAMP-1 pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for late endosomes/lysosomes. Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) was used for labeling Hsp90 and LDL.

Generation of Hsp90–peptide complex *in vitro*. As previously described,[4] Hsp90 was mixed with survivin-2B$_{75\text{-}93}$ (GPGTVAYACNTSTLGGRGG) in a 50:1 peptide:protein molar ratio in 0.7 M NaCl containing sodium-phosphate buffer and heated at 45°C for 30 min, then incubated for 30 min at room temperature.

Establishment of survivin-2B$_{80\text{-}88}$-specific CTL clone. We generated survivin-2B$_{80\text{-}88}$-specific CTL clones from a patient with colon cancer (patient 1 in Table 1). After the fourth vaccination, PBMCs were isolated from blood samples by Ficoll–Conray density gradient centrifugation. Phytohemagglutinin blasts were derived from PBMCs by culturing in AIM V medium (Invitrogen) containing 10% human serum, IL-2 (100 U/mL; Takeda Pharmaceutical Co., Osaka, Japan), and PHA (1 mg/mL; Wako Chemicals, Osaka, Japan) for 3 days, followed by washing and cultivation in the presence of IL-2 (100 U/mL) for 4 days. HLA-A*2402-survivin-2B$_{80\text{-}88}$ peptide tetramer-positive (MBL) CTLs were sorted and subsequently cloned to single cells using FACSAria (Becton Dickinson, San Jose, CA, USA). Survivin-2B$_{80\text{-}88}$-specific CTL clones were restimulated with survivin-2B$_{80\text{-}88}$ peptide-pulsed PHA blasts every 7 days in AIM V medium supplemented with 50 U/mL IL-2.

***In vitro* cross-presentation assay.** Human Mo-DCs (1×10^5) were pulsed with Hsp90 (400 μg/mL), survivin-2B$_{75\text{-}93}$ (400 μg/mL) alone, a complex of Hsp90 (100 or 400 μg/mL) and survivin-2B$_{75\text{-}93}$ (100 or 400 μg/mL), a simple mixture of both or survivin-2B$_{80\text{-}88}$ (400 μg/mL) for 2 h at 37°C in 100 μL Opti-MEM and then fixed for 1 min with 0.01% glutaraldehyde. Fixation was stopped by addition of 2 M L-lysine and the cells were washed twice with RPMI-1640 medium and cultured overnight with 1×10^5 survivin-2B peptide-specific CTL clone. Activation of CTLs was measured as IFN-γ production using ELISA. In a dose titration assay, Mo-DCs (1×10^5) were loaded with various doses of survivin-2B$_{80\text{-}88}$ peptide or Hsp90–precursor peptide (survivin-2B$_{75\text{-}93}$) complex for 2 h in 100 μL Opti-MEM and fixed with 0.01% glutaraldehyde. The cells were washed and cultured overnight with 1×10^5 survivin-2B$_{80\text{-}88}$-peptide-specific CTL clone. Interferon-γ in the culture supernatant was measured using ELISA.

***In vitro* stimulation of PBMCs with Mo-DC loaded with Hsp90–precursor peptide complex.** Peripheral blood mononuclear cells were isolated from eight patients suffering from various types of cancer who had been vaccinated with survivin-2B peptide in our clinical study.[7,8] These patients' PBMCs were shown to contain survivin-2B-specific CD8$^+$ T cells. The PBMCs were stimulated with Mo-DCs loaded with survivin-2B$_{80\text{-}88}$ (400 μg/mL), Hsp90 (400 μg/mL), survivin-2B$_{75\text{-}93}$ (400 μg/mL), and Hsp90 (100 or 400 μg/mL)–survivin-2B$_{75\text{-}93}$ (100 or 400 μg/mL) complex in AIM V medium (Life Technologies, Grand Island, NY, USA) containing 10% human serum. Interleukin-2 was added at a final concentration of 50 U/mL on days 2, 4, and 6. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

Assessment of stimulation of Ag-specific CTLs using tetramer assay. The FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) and PE-labeled HLA-A*2402-survivin-2B$_{80\text{-}88}$ peptide tetramers were purchased from MBL. For flow cytometric analysis, PBMCs, which were stimulated *in vitro* as described above, were stained with HIV tetramer or survivin-2B tetramer at 37°C for 20 min. Then a PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, CA, USA) was added at 4°C for 30 min. Cells were washed twice with PBS. After washing, cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry using FACSCalibur and CellQuest software (Becton Dickinson). CD8$^+$ living cells were gated and cells labeled with survivin-2B tetramer were referred to as tetramer-positive cells. The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8$^+$ T cells.

Assessment of stimulation of Ag-specific CTLs using ELISPOT assay. The ELISPOT plates were coated sterilely overnight with anti-IFN-γ capture antibody (BD Biosciences, San Jose, CA, USA) 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), which were stimulated *in vitro* as described above, were then added to each well along with HLA-A24-transfected T2 (T2-A24) cells (5×10^4 cells/well) that had been preincubated with survivin-2B$_{80\text{-}88}$ (10 μg/mL) or HIV 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 IFN-γ antibody (R&D Systems, Minneapolis, MN, USA) and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany).

Immunocytological localization of Hsp90–survivin-2B$_{75\text{-}93}$ peptide complex. Heat shock protein 90 and LDL were conjugated with Alexa Fluor 594 (Molecular Probes) according to the manufacturer's instructions. Monocyte-derived DCs were incubated at 37°C with Alexa Fluor 594-labeled Hsp90 (20 μg) complexed with survivin-2B$_{75\text{-}93}$ peptide (20 μg) for 1 h.

Table 1. Quantitation of survivin-2B-specific CD8$^+$ T cells by tetramer assay

Patient no.	Tumor	Survivin-2B$_{80-88}$-specific CD8$^+$ T cell frequency (tetramer staining)					
		In vitro stimulation	(–)	Survivin-2B$_{80-88}$	Hsp90	Survivin-2B$_{75-93}$	Hsp90–survivin-2B$_{75-93}$	Effect
1	Colon		0.06	4.87	4.47	3.24	8.47	††
2	Colon		0.32	2.87	0.77	4.01	1.30	No
3	Pancreas		0.70	6.27	1.70	1.88	6.64	††
4	Pancreas		0.48	4.56	0.84	1.28	3.02	†
5	Ampulla of Vater		1.27	4.60	0.97	2.24	6.50	††
6	Breast		3.59	3.78	3.00	3.06	3.82	††
7	Breast		3.98	3.91	1.94	2.28	6.19	††
8	Breast		2.76	3.92	2.91	2.08	6.07	††

†Frequency of survivin-2B-specific CD8$^+$ T cells stimulated with heat shock protein 90 (Hsp90)–survivin-2B$_{75-93}$ peptide complex was increased compared with stimulation with survivin-2B$_{75-93}$ precursor peptide. ††Frequency of survivin-2B-specific CD8$^+$ T cells stimulated with Hsp90–survivin-2B$_{75-93}$ peptide was increased compared with stimulation with both survivin-2B$_{80-88}$ peptide and survivin-2B$_{75-93}$ peptide. (–), negative control; No, no effect.

Following incubation, cells were washed twice with ice-cold PBS and fixed with ice-cold acetone for 1 min. Organelles were stained with an anti-Rab5 pAb and EEA1 mAb for early endosomes and anti-LAMP-1 pAb for late endosomes followed by Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) or anti-mouse IgG (Molecular Probes) and then visualized with a Bio-Rad MRC1024ES confocal scanning laser microscope system (Bio-Rad, Richmond, CA, USA). For evaluation of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed.

Inhibition studies. Monocyte-derived DCs were pre-incubated with chloroquine (Sigma-Aldrich) or primaquine (ICN Biomedicals, Irvine, CA, USA) at 37°C for 2 h, and then loaded with survivin-2B$_{80-88}$ peptide alone or Hsp90–precursor peptide (survivin-2B$_{75-93}$) complex for 2 h. The Mo-DCs were then fixed, washed, and cultured overnight with survivin-2B$_{80-88}$-specific CTL clone. Activation of CTLs was measured as IFN-γ production using ELISA.

Statistical analysis. All experiments were independently carried out three times in triplicate. Results are shown as means + SEM. Comparisons between two groups were performed using Student's *t*-test, with a *P*-value < 0.05 considered to be statistically significant.

Results

Heat shock protein 90–survivin-2B$_{75-93}$ peptide complex is *cross-presented* by Mo-DCs *in vitro*. We first examined whether human Hsp90 facilitated cross-presentation of the chaperoned precursor peptide by human Mo-DCs. The Mo-DCs were pulsed with Hsp90 alone, the survivin-2B$_{75-93}$ precursor peptide alone, a simple mixture of both, a complex of them generated *in vitro* at double concentration, or survivin-2B$_{80-88}$ peptide (for positive control) for 2 h at 37°C and then fixed, washed, and cultured with survivin-2B$_{80-88}$-specific CTL clone. The Hsp90–survivin-2B$_{75-93}$ precursor peptide complex elicited a significant amount of IFN-γ production both at 100 and 100 μg/mL, whereas Hsp90 alone, survivin-2B$_{75-93}$ precursor peptide alone, or a simple mixture of both did not induce IFN-γ production by CTLs (Fig. 1a). Strikingly, IFN-γ production induced by Hsp90–survivin-2B precursor peptide complex was much greater than that induced by survivin-2B peptide. These results indicated that cross-presentation of survivin-2B-derived peptide was enhanced when an exogenous precursor peptide was complexed to Hsp90. To confirm these observations, we compared the efficacy of CTL activation between survivin-

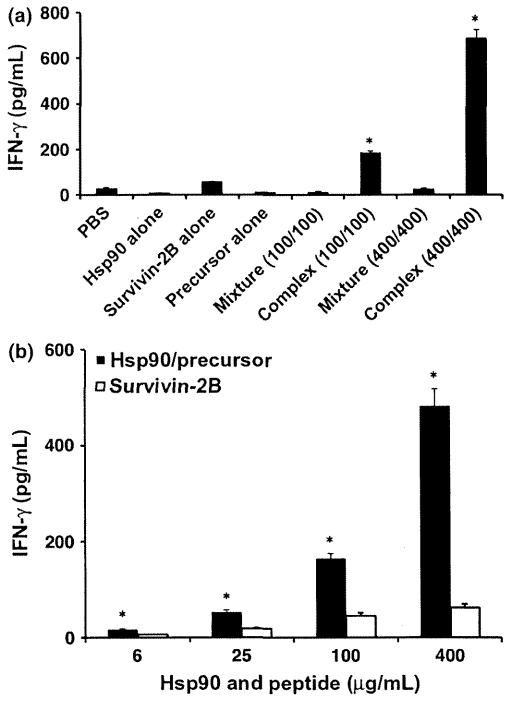

Fig. 1. Cross-presentation of heat shock protein 90 (Hsp90)-chaperoned peptides by human monocyte-derived dendritic cells (Mo-DCs). (a) Human Mo-DCs (1 × 10^5) were pulsed with Hsp90 (400 μg/mL), precursor peptide survivin-2B$_{75-93}$ (400 μg/mL) alone, a complex of Hsp90 (100 or 400 μg/mL) and survivin-2B$_{75-93}$ (100 or 400 μg/mL), a simple mixture of both, or survivin-2B$_{80-88}$ peptide (for positive control) for 2 h at 37°C and then fixed with 0.01% glutaraldehyde, washed, and cultured with survivin-2B$_{80-88}$-specific CTL clone (1 × 10^5/well). Activation of CTLs was measured as γ-interferon (IFN-γ) production using ELISA. (b) Mo-DCs (1 × 10^5) were loaded with various doses of survivin-2B$_{80-88}$ peptide (6, 25, 100, and 400 μg/mL) or Hsp90–survivin-2B$_{75-93}$ precursor peptide complex (6/6, 25/25, 100/100, and 400/400 μg/mL) for 2 h in 100 μL Opti-MEM and fixed with 0.01% glutaraldehyde. The cells were washed and cultured overnight with 1 × 10^5 survivin-2B$_{80-88}$-specific CTL clone. Activation of CTLs was measured as IFN-γ production using ELISA. Data are shown as means + SEM of three independent experiments. *P < 0.01.

$2B_{80-88}$ peptide and Hsp90–survivin-$2B_{75-93}$ precursor peptide complex in a dose titration assay (Fig. 1b). We observed that stimulation of the survivin-$2B_{80-88}$-specific CTL clone with Hsp90–survivin-$2B_{75-93}$ precursor peptide complex was more effective than stimulation with survivin-$2B_{80-88}$ peptide at any dose.

Peptide-specific precursor CTLs are activated by cross-presentation of Hsp90–peptide complex. As we had shown that the Hsp90–survivin-$2B_{75-93}$ precursor peptide complex was efficiently cross-presented, we next examined whether cross-presentation of Hsp90–peptide complex could activate and expand peptide-specific memory $CD8^+$ T cells from patients who had been vaccinated with survivin-2B peptide with incomplete Freund's adjuvant. Activated and expanded survivin-2B-specific $CD8^+$ T cells were detected by tetramer staining. As shown in Figure 2, the survivin-$2B_{75-93}$ precursor peptide chaperoned by Hsp90 was able to activate and expand survivin-2B-specific memory $CD8^+$ T cells more vigorously than was the precursor peptide alone. Interestingly, peptide-specific T-cell frequency was higher when stimulated with Hsp90–survivin-$2B_{75-93}$ precursor peptide complex than that with survivin-$2B_{80-88}$ peptide, indicating that a long peptide chaperoned by Hsp90 was efficiently cross-presented and was able to stimulate peptide-specific $CD8^+$ T cells. To confirm these observations, we compared the efficacy of activation of survivin-2B-specific memory $CD8^+$ T cells by stimulation with survivin-$2B_{80-88}$, survivin-$2B_{75-93}$ precursor peptide, or Hsp90–survivin-$2B_{75-93}$ precursor peptide complex in eight patients. As shown in Table 1, stimulation with Hsp90–survivin-$2B_{75-93}$ complex could expand survivin-2B-specific memory $CD8^+$ T cells from seven out of eight patients compared with stimulation with survivin-$2B_{75-93}$. More importantly, in six out of eight patients, stimulation with Hsp90–survivin-$2B_{75-93}$ complex expanded survivin-2B-specific memory $CD8^+$ T cells more efficiently compared with stimulation with survivin-$2B_{80-88}$.

Memory $CD8^+$ T cells activated by cross-presentation of Hsp90–peptide complex become functional peptide-specific CTLs. To further confirm whether survivin-2B-specific $CD8^+$ T cells activated by Hsp90-mediated cross-presentation were functional or not, we carried out an ELSPOT assay using $CD8^+$ T cells from a patient who had been vaccinated with survivin-2B peptide with incomplete Freund's adjuvant. Figure 3 shows that stimulation of $CD8^+$ T cells from the patient with Hsp90–survivin-$2B_{75-93}$ precursor peptide complex clearly increased functionally positive survivin-2B-specific $CD8^+$ T cells compared with stimulation with survivin-$2B_{75-93}$ precursor peptide or survivin-$2B_{80-88}$ peptide. When $CD8^+$ T cells from the patient were stimulated with Hsp90 (400 μg/mL)–precursor peptide (400 μg/mL) complex, the number of IFN-γ-positive spots was less than that of $CD8^+$ T cells stimulated with Hsp90 (100 μg/mL)–precursor peptide (100 μg/mL) complex. These results were due to the formation of fused large spots that were observed when stimulated with Hsp90 (400 μg/mL)–precursor peptide (400 μg/mL) complex and therefore the number of ELISPOT counted became smaller than that of Hsp90 (100 μg/mL)–precursor peptide (100 μg/mL) complex. These findings indicated that Hsp90–peptide complex is efficiently cross-presented by human Mo-DCs and is capable of stimulating peptide-specific CTLs.

Immunocytological localization of Hsp90–survivin$2B_{75-93}$ peptide complex. For further support of the above-described results, we investigated the intracellular routing of Hsp90 after uptake of it in DCs, using confocal laser microscopy. The Mo-DCs were incubated with Alexa 594-labeled Hsp90–survivin$2B_{75-93}$ peptide complex for 1 h. Following incubation, the cells were fixed and stained with antibodies against markers for organelle structures including EEA1, Rab5, and LAMP-1. Alexa 594-labeled Hsp90–peptide complex was detected in $EEA1^+$ and $Rab5^+$-early endosomes but not in lysosomes (Fig. 4a). Quantitative analysis of the colocalization between the exogenous Hsp90–peptide complex and Rab5, EEA1, and LAMP1 revealed average colocalization incidences of 78.0%, 88.7%, and 7.3%, respectively, providing further evidence that the exogenous Hsp90–peptide complex was delivered to the endosome-recycling pathway (Fig. 4b). We also examined the dynamics of Alexa 594-labeled LDL as a positive control protein for the dynamic early endosomal pathway (Fig. 5). Alexa594-labeled soluble LDL localized to the $Rab5^+$-early endosome as well as the LAMP-1^+-late endosome/lysosome,

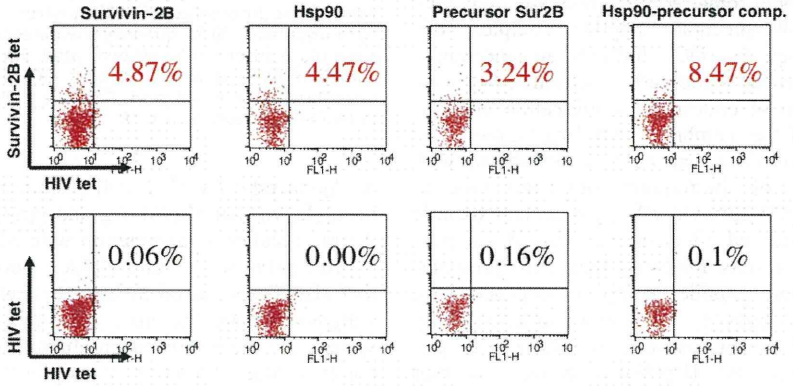

Fig. 2. Peptide-specific precursor CTLs were activated by cross-presentation of heat shock protein 90 (Hsp90)–peptide complex. PBMCs were isolated from patient 1 suffering from colon cancer (Table 1) who had been vaccinated with survivin-$2B_{80-88}$ peptide in our clinical study. The patient's PBMCs were shown to contain the survivin-2B-specific $CD8^+$ T cells. PBMCs were stimulated with human monocyte-derived dendritic cells loaded with survivin-$2B_{80-88}$ (400 μg/mL), Hsp90 (400 μg/mL), survivin-$2B_{75-93}$ precursor peptide (400 μg/mL), and Hsp90 (400 μg/mL)–survivin-$2B_{75-93}$ precursor peptide (400 μg/mL) complex in AIM V medium containing 10% human serum and interleukin-2 (50 U/mL) for 7 days. The stimulated PBMCs were stained with HIV tetramer (tet) or survivin-2B tetramer at 37°C for 20 min. Then a phycoerythrin-Cy5-conjugated anti-CD8 antibody was added at 4°C for 30 min. Cells were washed twice with PBS. After washing, cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry using FACSCalibur and CellQuest software. $CD8^+$ living cells were gated, and cells labeled with survivin-2B tetramer were referred to as tetramer-positive cells. The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of $CD8^+$ cells. Data are shown as means + SEM of three independent experiments. $^*P < 0.01$.

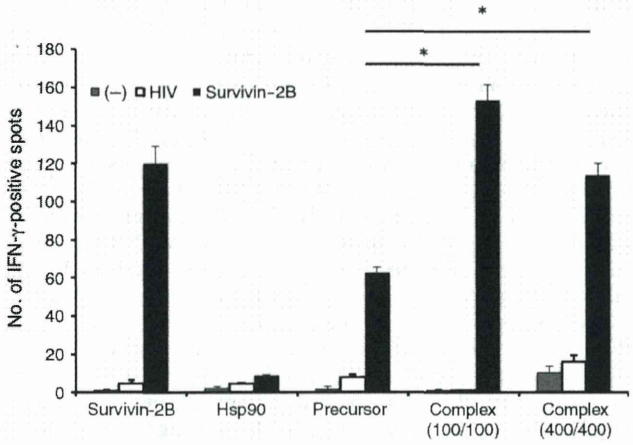

Fig. 3. Memory CD8$^+$ T cells activated by cross-presentation of heat shock protein 90 (Hsp90)–peptide complex became functional peptide-specific CTLs. CD8$^+$ T cells separated from PBMCs (5×10^3 cells/well) from patient 1 (Table 1) were stimulated with human monocyte-derived dendritic cells loaded with survivin-2B$_{80\text{-}88}$ (400 μg/mL), Hsp90 (400 μg/mL), precursor peptide survivin-2B$_{75\text{-}93}$ (400 μg/mL), and Hsp90 (100 or 400 μg/mL)–survivin-2B$_{75\text{-}93}$ (100 or 400 μg/mL) complex, were added to each well along with HLA-A24-transfected T2 (T2-A24) cells (5×10^4 cells/well) that had been preincubated with survivin-2B$_{80\text{-}88}$ (10 μg/mL) or HIV 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 γ-interferon (IFN-γ) antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT. Data are shown as means + SEM of three independent experiments. *$P < 0.01$.

but not to the EEA1$^+$-compartment, thus indicating the dynamic endosomal pathway. These results indicated that the Hsp90–peptide complex was sorted into the static endosomal pathway, not the dynamic endosomal pathway, within human Mo-DCs. In contrast, the soluble LDL protein, which underwent degradation, was translocated to the dynamic endosomal pathway. These results suggested that targeting to the "static" early endosome was required for efficient cross-presentation by Mo-DCs.

Heat shock protein 90–peptide complex is cross-presented by human DCs through an endosome-recycling pathway. We then examined whether Hsp90–precursor peptide complex was cross-presented by human Mo-DCs through an endosomal pathway after targeting to the static early endosome. We used chloroquine for inhibition of endosomal acidification and primaquine for inhibition of the membrane recycling pathway. As shown in Figure 6(a), Mo-DCs that were pre-incubated with increasing concentrations of chloroquine completely blocked cross-presentation of Hsp90–survivin-2B$_{75\text{-}93}$ precursor peptide complex but had no substantial effect on survivin-2B$_{80\text{-}88}$ peptide presentation. These results indicated that cross-presentation of Hsp90–precursor peptide complex depended on endosomal acidification, possibly including proteolysis by endosomal proteases. Moreover, Mo-DC incubated with primaquine could not present the Hsp90-chaperoned precursor peptide-derived survivin-2B$_{80\text{-}88}$ peptide to CTL (Fig. 6b). These results indicated that the Hsp90-chaperoned precursor peptide or processed peptide entered recycling endosomes and were transferred onto recycling MHC class I molecules.

Discussion

It has been shown that immunization with tumor-derived HSPs or HSPs complexed with an Ag peptide/protein elicits tumor-

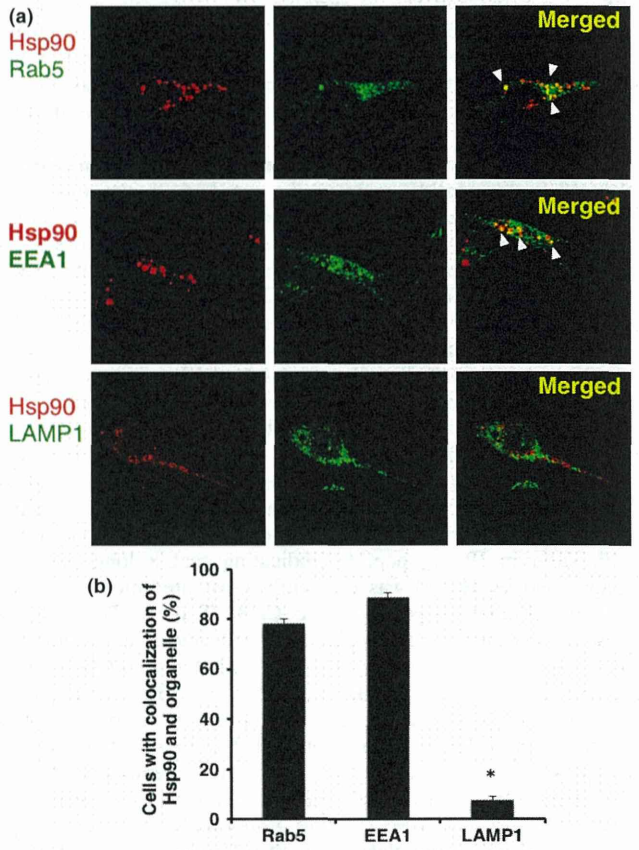

Fig. 4. Heat shock protein 90 (Hsp90)–survivin-2B$_{75\text{-}93}$ precursor peptide complex localized to static early endosomes within human monocyte-derived dendritic cells (Mo-DCs). (a) Human Mo-DCs were incubated at 37°C with Alexa 594-labeled Hsp90–survivin-2B$_{75\text{-}93}$ peptide complex for 1 h and then washed and fixed. Organelles were stained with an anti-EEA1 mAb for early endosomes, anti-Rab5 polyclonal antibody for early endosomes, and anti-LAMP-1 polyclonal antibody for late endosomes/lysosomes followed by Alexa 488-conjugated goat anti-rabbit IgG or anti-mouse IgG and were visualized with confocal laser microscopy. Arrowheads indicate colocalization of the internalized Hsp90–survivin-2B$_{75\text{-}93}$ peptide complex and each organelle. (b) To quantify the percentage of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed. Data are shown as means + SEM of three independent experiments. *$P < 0.01$.

or Ag-specific CD8$^+$ T cell responses.[1,9] Importantly, it has been shown that Hsp70–Ag and gp96–Ag complexes facilitate Ag presentation in association with MHC class I molecules.[10–13] Recently, we[3,4] and Calderwood's group[14] have shown that Hsp90 also acted as an excellent navigator for associated antigens to enter the cross-presentation pathway in the murine system. We here showed that human Hsp90–cancer Ag peptide complex was efficiently cross-presented by human Mo-DCs. These results hold promise for the development of a safe and efficient immunomodulator for cancer immunotherapy. More importantly, we showed that translocation of the Hsp90–Ag complex into the static early endosome after endocytosis was crucial for efficient cross-presentation. It has been shown that the pathway for cross-presentation is comprised of two distinct intracellular routes, a proteasome–TAP-dependent pathway and an endosome-recycling pathway.[2,3] Recent studies have revealed the pathway in which peptide exchange onto recy-

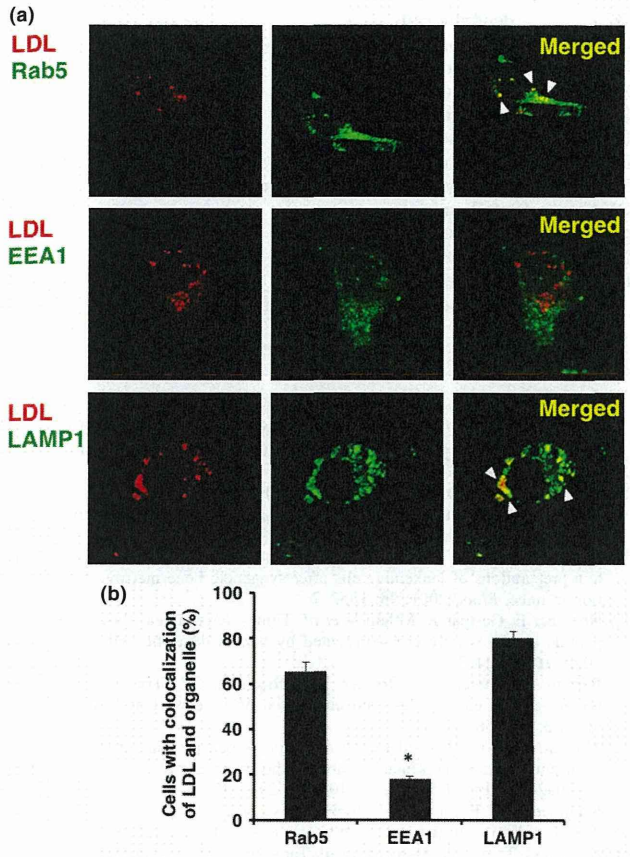

Fig. 5. Low-density lipoprotein (LDL) was targeted to the dynamic early endosome followed by translocation to the late endosome/lysosome for degradation. (a) Human monocyte-derived dendritic cells were incubated at 37°C with Alexa 594-labeled LDL. Organelles were stained with an anti-EEA1 mAb, anti-Rab5 polyclonal antibody, and anti-LAMP-1 polyclonal antibody, followed by Alexa 488-conjugated goat anti-rabbit IgG or anti-mouse IgG. Arrowheads indicate colocalization of internalized LDL and each organelle. (b) To quantify the percentage of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed. Data are shown as means + SEM of three independent experiments. $*P < 0.01$.

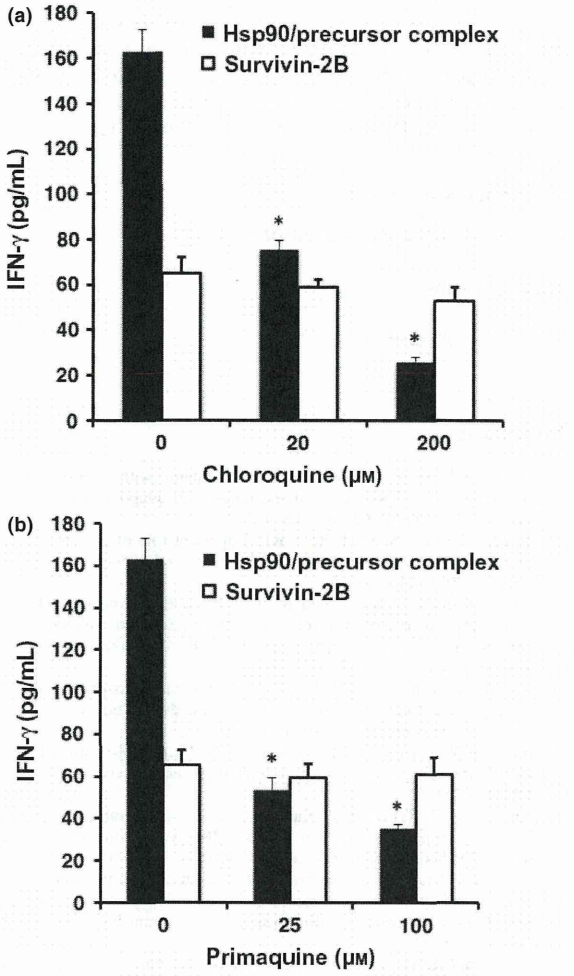

Fig. 6. Heat shock protein 90 (Hsp90)–peptide complex is cross-presented through an endosome-recycling pathway. Human monocyte-derived dendritic cells (Mo-DCs) were pre-incubated with chloroquine (a) or primaquine (b) at 37°C for 2 h and then loaded with survivin-$2B_{80\text{-}88}$ peptide alone or Hsp90–survivin-$2B_{75\text{-}93}$ precursor peptide complex for 2 h. The Mo-DCs were then fixed, washed, and cultured overnight with survivin-$2B_{80\text{-}88}$-specific CTL clone. Activation of CTL was measured as γ-interferon (IFN-γ) production using ELISA.

cling MHC class I molecules occurs within early endosomal compartments.[15] We have shown that Hsp90–peptide complex-mediated[4] and ORP150–peptide complex-mediated[16] cross-presentation was independent of TAP and was sensitive to primaquine, indicating that sorting of peptides onto MHC class I occurs through an endosome-recycling pathway. Lakadamyali *et al.*[17] 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. Cargos destined for degradation, including LDL, epidermal growth factor, and influenza virus, are internalized and targeted to the $Rab5^+$, $EEA1^-$-dynamic population of early endosomes as we have observed using LDL, thereafter trafficking to $Rab7^+$-late endosomes. In contrast, the recycling ligand transferrin is delivered to $Rab5^+$, $EEA1^+$-static early endosomes, followed by translocation to $Rab11^+$-recycling endosomes. Furthermore, Burgdorf *et al.*[18] clearly indicated that a mannose receptor introduced OVA specifically into an $EEA\text{-}1^+$, $Rab5^+$-stable early endosomal compartment for subsequent cross-presentation. In contrast, pinocytosis conveyed OVA to lysosomes for class II presentation. Of interest, OVA endocytosed by a scavenger receptor did not colocalize with EEA1 but colocalized with LAMP-1 in lysosomes, leading to presentation in the context of MHC class II molecules. We showed that the human Hsp90–peptide complex is targeted into $Rab5^+$, $EEA1^+$-early endosomes after internalization by Mo-DCs, suggesting that preferential sorting to the "static" endosome is necessary for cross-presentation of Hsp90–peptide complexes. In contrast, soluble LDL protein was targeted to the $EEA1^-$ and $LAMP\text{-}1^+$-dynamic early endosome–late endosome/lysosome pathway, leading to degradation and presentation in the context of MHC class II molecules. These findings suggested that Hsp90 shuttled the chaperoned precursor peptide into the static endosome-recycling pathway, preventing further degradation, followed by transfer of the peptide onto recycling MHC class I molecules. Together, our findings indicate that the role

of Hsp90 in cross-presentation is to navigate the associated Ag into static early endosomes within human Mo-DCs. Thus, Hsp90 appears to be a promising natural immunoactivator for use of cancer vaccine development due to its excellent ability to target human DCs and to induce specific CTLs.

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

Ag	antigen
DC	dendritic cell
GM-CSF	granulocyte/macrophage colony-stimulating factor
HSP	heat shock protein
Hsp90	heat shock protein 90
IFN	interferon
IL	interleukin
LDL	low-density lipoprotein
Mo-DC	monocyte-derived dendritic cells
OVA	ovalbumin
pAb	polyclonal antibody
PE	phycoerythrin
PHA	phytohemagglutinin
TAP	transporter associated with antigen processing

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ORIGINAL ARTICLE

Tumor-α9β1 integrin-mediated signaling induces breast cancer growth and lymphatic metastasis via the recruitment of cancer-associated fibroblasts

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Abstract
Tumor-derived matricellular proteins such as osteopontin (OPN) and tenascin-C (TN-C) have been implicated in tumor growth and metastasis. However, the molecular basis of how these proteins contribute to tumor progression remains to be elucidated. Importantly, these matricellular proteins are known to interact with α9β1 integrin. Therefore, we hypothesized that tumor-derived α9β1 integrin may contribute to tumor progression. To clarify the roles of α9β1 integrin in tumor growth and lymphatic metastasis, we used an inhibitory anti-human α9β1 integrin antibody (anti-hα9β1 antibody) and a α9β1 integrin-positive human breast cancer cell line, MDA-MB-231 luc-D3H2LN (D3H2LN), in vitro functional assays, and an in vivo orthotopic xenotransplantation model. In this study, we demonstrated that tumor, but not host α9β1 integrin, contributes to tumor growth, lymphatic metastasis, recruitment of cancer-associated fibroblasts (CAFs), and host-derived OPN production. We also found that CAFs contributed to tumor growth, lymphatic metastasis, and host-derived OPN levels. Consistent with those findings, tumor volume was well-correlated with numbers of CAFs and levels of host-derived OPN. Furthermore, it was shown that the inoculation of D3H2LN cells into mammary fat pads with mouse embryonic fibroblasts (MEFs), obtained from wild type, but not OPN knock-out mice, resulted in enhancement of tumor growth, thus indicating that CAF-derived OPN enhanced tumor growth. These results suggested that tumor α9β1-mediated signaling plays a pivotal role in generating unique

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primary tumor tissue microenvironments, which favor lymphatic metastasis and tumor growth.

Key messages

- Tumor α9β1 integrin promotes lymphatic metastasis through enhancing invasion.
- Tumor α9β1 integrin promotes tumor growth through CAFs.
- Tumor α9β1 integrin enhances the recruitment of CAFs into the primary tumor.
- Tumor cells induce the production of OPN by CAFs in the primary tumor.
- CAF-derived OPN promotes tumor growth.

Keywords Osteopontin · α9β1 integrin · Tumor microenvironment · Cancer-associated fibroblasts · Breast cancer

Introduction

Metastasis is a multistage process that includes tumor invasion and colonization in the metastatic site [1]. These steps require a receptive microenvironment constructed by surrounding stromal cells [2]. The most common reactive stromal cells are cancer-associated fibroblasts (CAFs), which enhance tumor growth and metastasis by production of cytokines, growth factors, and extracellular matrix (ECM) proteins [3]. ECM proteins support stable cell adhesion, and transmit signals that are critical for cell growth, migration, and survival. These ECM proteins include collagen, laminin, and fibronectin, which are regarded as classical ECM proteins.

It is now known that there also are nonclassical ECM proteins. These nonclassical ECM proteins are termed "matricellular proteins". Matricellular proteins: (1) induce cell motility, rather than provide scaffolds for stable cell adhesion; (2) are transiently upregulated in pathological conditions rather than constitutively expressed; and (3) can be present as soluble proteins rather than as structural components [4]. Among these matricellular proteins, osteopontin (OPN) and tenascin-C (TN-C) are well characterized and known to be involved in the tumor progression. Breast cancer-derived TN-C enhances the survival of cancer stem cells in metastatic sites [5]. Malignant breast cancer-derived OPN enhances the migration of bone marrow-derived stromal cells into the distant indolent tumor tissue leading to acquisition of malignancy of the indolent tumor cells [6]. Several groups have demonstrated that plasma OPN levels are potential diagnostic and prognostic markers for several human malignancies [7, 8]. Recently, a role of not only tumor-derived but also host-derived OPN and TN-C in tumor biology has been reported. OPN from CAFs enhances tumor growth [9, 10], and TN-C from host fibroblasts can contribute to tumor progression including cancer cell colonization at metastatic sites [11]. However, the molecular basis for the contribution of these matricellular proteins in tumor progression is yet to be determined. Both OPN and TN-C interact with integrins. OPN binds to αvβ1, αvβ3, αvβ5, and α5β1 integrins through its RGD (Arg-Gly-Asp) sequence, or α4β1 and α9β1 integrins through the SVVYGLR sequence, which can be exposed by thrombin cleavage [4]. TN-C also binds to αvβ1, αvβ3, αvβ6, and α8β1 integrins through its RGD sequence, or to α9β1 integrin through the third FN typeIII repeat (Tnfn3) [12]. Importantly, α9β1 integrin is a common receptor of OPN and TN-C. Elevated levels of α9β1 integrin in the primary tumor significantly correlate with poor breast cancer patient survival [13]. Interestingly, a human breast cancer cell line, MDA-MB-468LN with an aggressive lymph node metastatic ability, was shown to express high levels of both α9β1 integrin and OPN, as compared to its parental cell line, MDA-MB-468 [14].

Here, we hypothesized that α9β1 integrin expressed by tumor cells might be involved in tumor progression. We demonstrated that inhibition of human (tumor) α9β1 integrin-mediated signaling reduced cell motility, lymph node metastasis, and in vivo tumor growth through the reduction of recruitment of CAFs into the primary tumor tissue. Furthermore, upon co-inoculation with tumor cells, MEFs produced OPN, which significantly contributed to tumor growth. Thus, our data suggested that tumor α9β1 integrin might be a possible therapeutic target for the treatment of breast cancer.

Materials and methods

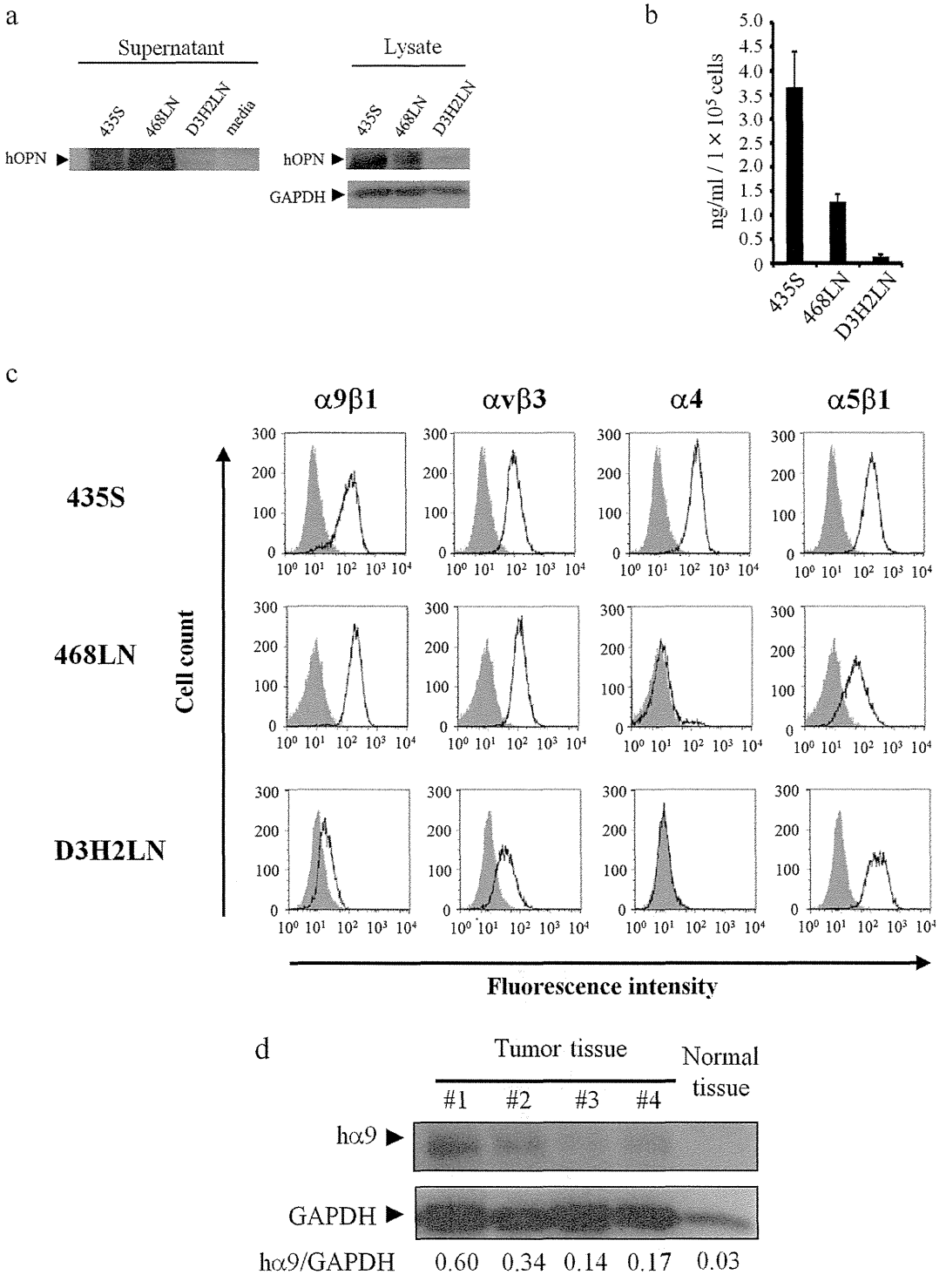
Detailed procedures in this study are described in Supplementary Methods.

Results

Human breast cancer cells express OPN and its receptors

We first found that three human breast cancer cell lines expressed and secreted varied amounts of OPN (Fig. 1a, b).

Fig. 1 Human breast cancer cells express OPN and their receptors. **a** Western blot analysis of OPN from cell culture supernatants of human breast cancer cell lines, 435S, 468LN, and D3H2LN cells and media with no cells as negative control (*left panel*) and lysates of primary tumor tissues obtained from mice xenotransplanted with three cell lines (*right panel*). **b** The secretion of OPN from three human breast cancer cell lines was determined by ELISA. The measured concentration was normalized by the number of cells (1×10^5). **c** The expression of integrins α9β1, αvβ3, α4, and α5β1 in human breast cancer cell lines (435S, 468LN, and D3H2LN cells) was determined by flow cytometry. **d** The expression of α9 integrin protein in breast tumor tissues from four distinct patients ($n=4$) and noncancer breast tissue ($n=1$) was determined by western blot analysis. *Numbers at bottom of figure* indicate the normalized densitometric band densities as determined human α9 integrin/GAPDH



All three cell lines expressed αvβ3, α9β1, and α5β1 integrins (Fig. 1c), to which OPN can bind. We also demonstrated that human breast cancer tissues expressed elevated levels of α9β1 integrin proteins as compared to normal human breast tissue (Fig. 1d). It should be pointed out that MDA-MB-231 luc-D3H2LN (D3H2LN) cells secrete very little if any human OPN as compared to other two cell lines (Fig. 1b), allowing us to examine the role of tumor-derived

versus host-derived OPN in various aspects of tumor biology. Therefore, we used D3H2LN cells in the following experiments.

In vitro human breast cancer cell invasion is regulated by tumor α9β1 integrin

To evaluate the role of tumor α9β1 integrin in progression of human breast cancer, we generated an inhibitory monoclonal antibody that specifically reacts to the human α9β1 integrin (anti-hα9β1 antibody). Anti-hα9β1 antibody reacts with CHO cells transfected with human α9 integrin (hα9/CHO), but not with control CHO cells, CHO cells transfected with the human α4 integrin (hα4/CHO), or murine fibroblasts transfected with murine α9 integrin (mα9/NIH). In addition, this antibody could detect endogenous α9β1 integrin expressed by D3H2LN cells (Supplementary Fig. S1a). Anti-hα9β1 antibody could inhibit the binding of hα9/CHO cells to the synthetic peptides SVVYGLR and AEIDGIEL (which are internal sequences within OPN and TN-C, respectively, and are ligands for α9 integrin), but not to the GRGDS peptide (Supplementary Fig. S1b). We analyzed whether tumor α9β1 integrin-mediated signaling had any effect on tumor cell invasion and proliferation in vitro. Invasion was significantly reduced by anti-hα9β1 antibody (Fig. 2a), indicating that tumor α9β1 integrins are involved in the invasion process. In sharp contrast to cell invasion, proliferation of D3H2LN cells was not α9β1 integrin-dependent (Fig. 2b).

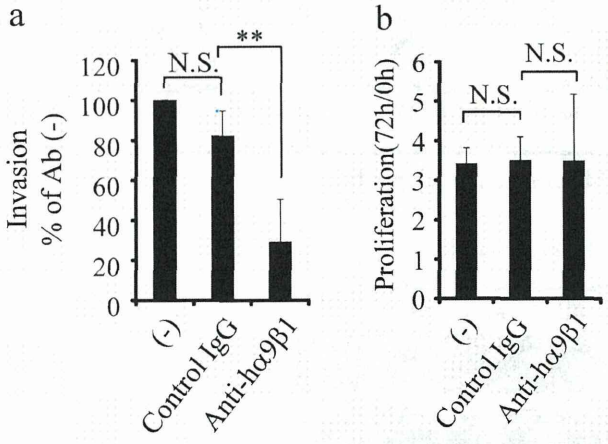


Fig. 2 Human breast cancer cell migration and invasion in vitro are regulated by tumor α9β1 integrin. **a** Matrigel invasion assay using D3H2LN cells treated with 20 μg/ml of anti-hα9β1 or control IgG, was performed. *Bars* indicate mean values±S.D. of three independent experiments. *N.S.* No significant difference, $^{**}p<0.01$, ANOVA with post test analysis (PLSD). **b** The proliferation of D3H2LN cells was examined in the presence or absence of 20 μg/ml of anti-hα9β1 antibody or control IgG. *Column* indicates the fold increase of OD.450 nm at 72 h as compared to that at the time of culture initiation (0 h). *Bars* indicate mean values±S.D. of three independent experiments. *N.S.* No significant difference, ANOVA with post test analysis (PLSD)

Thus, our in vitro study demonstrated that invasion, but not proliferation, is regulated by tumor α9β1 integrin.

In vivo human breast cancer cell growth and metastasis are regulated by tumor α9β1 integrin

We next analyzed the in vivo role of α9β1 integrin on tumor growth and metastasis using orthotopically xenografted human breast cancer cells in nude mice as shown in Fig. 3a. Primary tumor growth of D3H2LN cells implanted into the mammary fat pad was significantly reduced by anti-hα9β1 antibody treatment (Fig. 3b), which was well-correlated with the reduction of Ki-67 positive cells in primary tumor tissues (Fig. 3c). It is well known that angiogenesis is important for tumor growth and metastasis [15]. However, here, we found that angiogenesis as judged by CD31 staining in primary tumor was not significantly affected by the anti-hα9β1 antibody treatment (Fig. 3d). The incidence of lymph node metastasis at day 30 after inoculation was significantly inhibited by anti-hα9β1 antibody treatment as assessed by two different assays, in vivo imaging of luciferase and counting numbers of metastasis positive lymph nodes in histological sections (Fig. 4a, Supplementary Table). It should be pointed out that the inhibition of tumor metastasis incidence was not significant after day 40. However, importantly, the volumes of metastatic foci at lymph nodes in the anti-hα9β1 antibody-treated group were significantly smaller than those of the control IgG group at day 50 (Fig. 4b). The reduction of metastatic lymph node number and metastatic burden per lymph node may not be due to the inhibition of tumor cell proliferation at lymph nodes since Ki-67 positive cell numbers within human vimentin-positive breast cancer cells were not significantly different between control IgG and anti-hα9β1 antibody-treated group (Supplementary Fig. S2). Thus, it is likely that tumor metastasis was reduced due to the inhibition of cell invasion. Thus, these results suggest that tumor α9β1 integrin contributed to lymphatic metastasis in this model. It has been reported that α9β1 integrin expressed by lymph vessels is a receptor for VEGF-C and -D and involved in lymphangiogenesis, thus lymph node metastasis [16]. Therefore, we asked whether endogenous (murine) α9β1 integrin plays a role in xenografted human breast cancer cell growth and metastasis. To explore this aim, we injected a blocking monoclonal antibody against murine α9β1 (anti-mα9β1 antibody) in tumor-bearing mice (Supplementary Fig. S3a). We found that murine α9β1 integrin plays a little role if any on tumor growth and metastasis (Supplementary Fig. S3b, c). Consistently, anti-mα9β1 antibody did not reduce lymphangiogenesis, induced by tumor inoculation as judged by LYVE-1 staining in the primary tumor tissue and axillary lymph node (Supplementary Fig. S3d). Thus, our data demonstrated that in vivo primary tumor growth and metastasis were regulated by tumor α9β1 integrin.