Table 1. Profiles of 113 Japanese patients with melanoma and quantification of SPARC, GPC3, and 5-*S*-cysteinyldopa in sera of patients

Patient ID	Stage*	Age	Sex	Type	Serum concentrations of tumor markers			
					SPARC staining†	SPARC (μg/mL)‡	GPC3 (units/mL)§	5-*S*-cysteinyldopa (nmol/L)‖
1	0	60	M			4.7	0	4.6
2	0	64	F			1.2	0	2.8
3	0	78	F			3.1	0	2.9
4	0	74	M			3.3	0	6.2
5	0	85	F			0.9	25	3.2
6	0	72	M			3.7	0	3.8
7	0	48	F			2.2	38	3.3
8	0	69	F			1.5	22	2.6
9	0	66	F			2.5	8	4.7
10	0	65	M			3.0	0	6.8
11	0	51	F			1.9	92	1.8
12	0	76	F			3.2	8	5.5
13	0	50	M			3.0	0	6.8
14	0	82	F		++	1.3	39	2.0
15	0	65	M			1.8	0	6.4
16	IA	33	M	Mucous		1.8	103	1.9
17	IA	82	F	LMM		NT	40	5.5
18	IA	75	F	SSM		1.8	25	3.9
19	IA	41	F	SSM		1.8	20	2.3
20	IA	70	F	Mucous		2.3	0	2.4
21	IA	78	M	ALM	++	1.2	0	11.0
22	IA	60	F	ALM		NT	0	3.6
23	IA	61	M	ALM		3.3	0	1.0
24	IA	62	F	ALM		1.2	0	5.9
25	IA	73	M	ALM		0.7	0	3.6
26	IA	70	M	LMM		1.7	20	8.0
27	IA	33	F	Mucous		2.3	10	3.6
28	IA	66	M	ALM		1.2	0	5.9
29	IA	76	M	ALM		1.3	0	5.8
30	IA	58	F	ALM		1.5	0	4.4
31	IA	89	M	LMM		0.5	0	47.6
32	IA	87	F	ALM		1.8	7.4	NT
33	IA	81	F	ALM	++	1.4	0	4.5
34	IA	68	F	LMM	++	1.5	77	1.8
35	IB	58	F	ALM		0.6	61	2.5
36	IB	58	F	Mucous		2.2	0	8.6
37	IB	66	F	Mucous		2.0	23	2.9
38	IB	56	F	SSM		2.0	10	2.3
39	IB	64	F	ALM	+++	2.8	20	7.5
40	IB	84	F	ALM		2.0	0	2.0
41	IB	79	M	ALM		2.9	0	7.3
42	IB	76	F	ALM		2.2	0	5.7
43	IB	74	F	ALM		1.6	0	2.8
44	IB	75	M	LMM	++	3.4	0	6.6
45	I?	82	F	ALM		1.6	0	2.9
46	IIA	74	F	SSM		2.3	106	17.4
47	IIA	75	M	ALM		NT	54	NT
48	IIA	74	F	ALM		1.9	16	3.4
49	IIA	64	M	ALM	++	2.9	0	4.6
50	IIA	47	F	SSM	+++	3.9	0	4.3

(*Continued on the following page*)

Table 1. Profiles of 113 Japanese patients with melanoma and quantification of SPARC,GPC3, and 5-*S*-cysteinyldopa in sera of patients (Cont'd)

Patient ID	Stage*	Age	Sex	Type	Serum concentrations of tumor markers			
					SPARC staining†	SPARC (μg/mL)‡	GPC3 (units/mL)§	5-*S*-cysteinyldopa (nmol/L)‖
51	IIA	77	F	LMM		1.3	34	3.9
52	IIA	83	F	ALM		1.9	0	4.0
53	IIA	71	F	LMM	++	2.0	82	3.4
54	IIA	73	M	ALM	+++	2.3	43	4.0
55	IIB	50	F	SSM	++	1.4	75	6.4
56	IIB	72	M	LMM		2.0	73	7.0
57	IIB	88	M	ALM		1.8	0	1.2
58	IIB	63	M	ALM		1.6	0	3.7
59	IIB	77	M	SSM		3.0	0	NT
60	IIB	69	M	ALM		1.0	15	4.6
61	IIB	57	M	ALM		3.8	0	3.4
62	IIB	69	F	ALM		0.6	0	3.3
63	IIB	71	M	ALM	++	1.7	0	4.7
64	IIB	85	F	LMM		2.6	79	4.8
65	IIB	72	M	ALM		1.8	43	6.3
66	IIB	70	M	ALM		2.6	0	0.4
67	IIB	68	F	ALM	+++	1.2	3.9	4.0
68	IIC	79	F	ALM		NT	25	3.8
69	IIC	42	M	SSM	+++	4.2	0	6.3
70	IIC	72	F	ALM	+	2.2	0	3.6
71	IIC	75	F	Mucous	+	1.8	0	8.7
72	IIC	77	M	ALM	+++	0.6	16	7.4
73	IIC	83	M	SSM		1.4	10	13.3
74	IIC	84	M	LMM		1.6	41	4.2
75	IIC	91	F	ALM		1.1	0	6.0
76	IIIA	83	M	ALM	+++	2.7	0	7.7
77	IIIA	55	M	ALM	+++	1.2	0	8.2
78	IIIA	86	F	ALM	++	1.7	0	9.7
79	IIIA	79	F	ALM	++	0.6	0	6.1
80	IIIA	70	M	ALM		2.1	0	4.0
81	IIIA	63	F	SSM		2.3	10	11.8
82	IIIA	79	M	NM		5.6	10	4.0
83	IIIA	53	F	Mucous		2.4	0	5.2
84	IIIA	53	M	ALM	+	1.6	0	1.9
85	IIIB	85	M	ALM		3.8	140	9.2
86	IIIB	56	M	LMM		2.7	0	15.5
87	IIIB	59	M	Mucous		3.2	0	1.2
88	IIIB	77	M	ALM	+	1.8	67	7.1
89	IIIC	35	F	NM	+++	1.6	132	8.4
90	IIIC	63	F	ALM	+++	2.6	18	4.9
91	IIIC	50	F	Unknown		1.2	0	5.9
92	IIIC	47	M	Mucous		3.9	0	10.3
93	IIIC	70	M	ALM		1.2	22	24.2
94	IIIC	63	M	ALM	+++	2.2	0	14.4
95	IV	47	F	SSM		3.3	35	12.7
96	IV	77	M	ALM	+	2.7	0	748
97	IV	65	M	Unknown		2.4	0	492
98	IV	78	M	Mucous		2.5	0	44.6
99	IV	60	F	SSM	+	1.4	0	32.4
100	IV	76	F	Mucous	+++	1.4	0	1.1

(*Continued on the following page*)

Table 1. Profiles of 113 Japanese patients with melanoma and quantification of SPARC, GPC3, and 5-*S*-cysteinyldopa in sera of patients (Cont'd)

Patient ID	Stage*	Age	Sex	Type	Serum concentrations of tumor markers			
					SPARC staining†	SPARC (μg/mL)‡	GPC3 (units/mL)§	5-*S*-cysteinyldopa (nmol/L)‖
101	IV	72	F	SSM		0.9	0	981
102	IV	73	F	SSM		0.3	0	56.1
103	IV	45	F	Unknown		0.8	10	5.5
104	IV	60	F	Mucous		2.2	8	8.6
105	IV	72	M	NM		0	0	225
106	IV	50	M	SSM		2.7	0	957
107	IV	47	F	NM		0	0	257
108	IV	22	M	Unknown		3.2	57	25.8
109	IV	39	M	NM		0.5	0	170
110	IV	74	F	ALM		2.7	0	395
111	IV	68	M	Unknown		2.1	34	74.2
112	IV	66	F	ALM	+++	2.8	0	246
113	IV	67	F	Unknown		3.5	53	151
Nevus1		32	M	Congenital		3.2	0	3.5
Nevus2		38	M	Congenital		2.1	0	4.1
Nevus3		24	M	Congenital		1.1	0	NT
Nevus4		9	F	Congenital		0.1	0	18
Nevus5		4	M	Congenital		2.9	0	21

NOTE: We could obtain reproducible results in three independent ELISA assays, and the representative results were shown.
Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; Congenital, congenital melanocytic nevus; UICC, Unio Internationale Contra Cancrum; AJCC, American Joint Committee on Cancer; TNM, tumor-node-metastasis; MIA, melanoma-inhibitory activity; NT, not tested.
*Clinical stages are according to the UICC/AJCC TNM classification (11).
†The expression of SPARC protein detected by immunohistochemical analysis. The intensity of staining was classified as +, weaker than adjacent epidermis; ++, same as adjacent epidermis; and +++, stronger than adjacent epidermis.
‡Soluble SPARC in the sera was quantified by ELISA. We fixed the cutoff value at 2.34 μg/mL in this study, and increased values are underlined.
§Soluble GPC3 in the sera was quantified by ELISA. We fixed the cutoff value at 1 unit/mL, and positive values are underlined.
‖We quantified 5-*S*-cysteinyldopa using high-performance liquid chromatography. The cutoff value was fixed at 10 nmol/L (6), and increased values are underlined.

0.43 μg/mL (a mean SPARC concentration plus 2 SD in the healthy donors), 4 of 11 melanoma patients (36.4%) were positive for an increased plasma SPARC as observed in the serum samples of the melanoma patients. In addition, one (4.8%) positive case of 21 healthy donors was observed.

Comparison of serum concentration of SPARC, GPC3, and 5-S-cysteinyldopa in patients with melanoma classified by stage. The above results clearly indicate that SPARC is a novel tumor marker for melanoma. We next compared the serum concentrations of SPARC, GPC3, and 5-*S*-cysteinyldopa in patients with melanoma classified by clinical stage (Tables 1 and 2). We fixed the cutoff level at 1 unit/mL in GPC3 and at 10 nmol/L in 5-*S*-cysteinyldopa as reported (6, 10). Figure 2E shows the serum concentrations of SPARC quantified by ELISA in 109 melanoma patients classified by stage. Although the serum concentrations of 5-*S*-cysteinyldopa increased markedly in patients at stage IV (10), the percentages of serum SPARC positive patients were almost equal among the five clinical stages as seen in GPC3 (10). To our surprise, we detected an increase of SPARC in the sera of patients with very small lesion of melanoma such as stage 0 or I. No significant correlation was observed between the patients positive for each of three markers (Table 1). More importantly, 18 of 36 SPARC-increased patients were negative for both GPC3 and 5-*S*-cysteinyldopa, and many were classified as cases of relatively early Unio Internationale Contra Cancrum stages 0, I, and II (Table 1). The positive rate of these three tumor markers in patients with melanoma, as classified by stage, is shown in Table 2. The total positive rates of increased SPARC (36 of 109, 33.0%) and GPC3 (48 of 113, 42.5%) were significantly higher than the rate for 5-*S*-cysteinyldopa (25 of 110, 22.7%). The positive rates of increased SPARC (8 of 15, 53.3%) and GPC3 (7 of 15, 46.7%) at stage 0 were significantly higher than that for 5-*S*-cysteinyldopa (0 of 15, 0.0%; $P < 0.001$). In addition, when we use SPARC and GPC3 in combination, the positive rates at stage 0 (13 of 15, 86.7%), stage I (14 of 28, 50.0%), stage II (20 of 28, 71.4%), and stage III (12 of 19, 63.2%) were all significantly higher than that of 5-*S*-cysteinyldopa ($P<0.05$). In all, the positive rate of increased SPARC or GPC3 in patients at stages 0 to II (47 of 71, 66.2%) was significantly higher than that of 5-*S*-cysteinyldopa (4 of 71, 5.6%; $P< 0.001$). On the other hand, the positive rate of 5-*S*-cysteinyldopa in stage IV patients (16 of 19, 84.2%) was significantly higher than that of SPARC or GPC3 in combination (12 of 19, 63.2 %). Finally, we were able to detect 78 of 107 (72.9%) cases of preoperative melanoma patients by the combined use of SPARC, GPC3, and 5-*S*-cysteinyldopa. This is an extremely high positive rate.

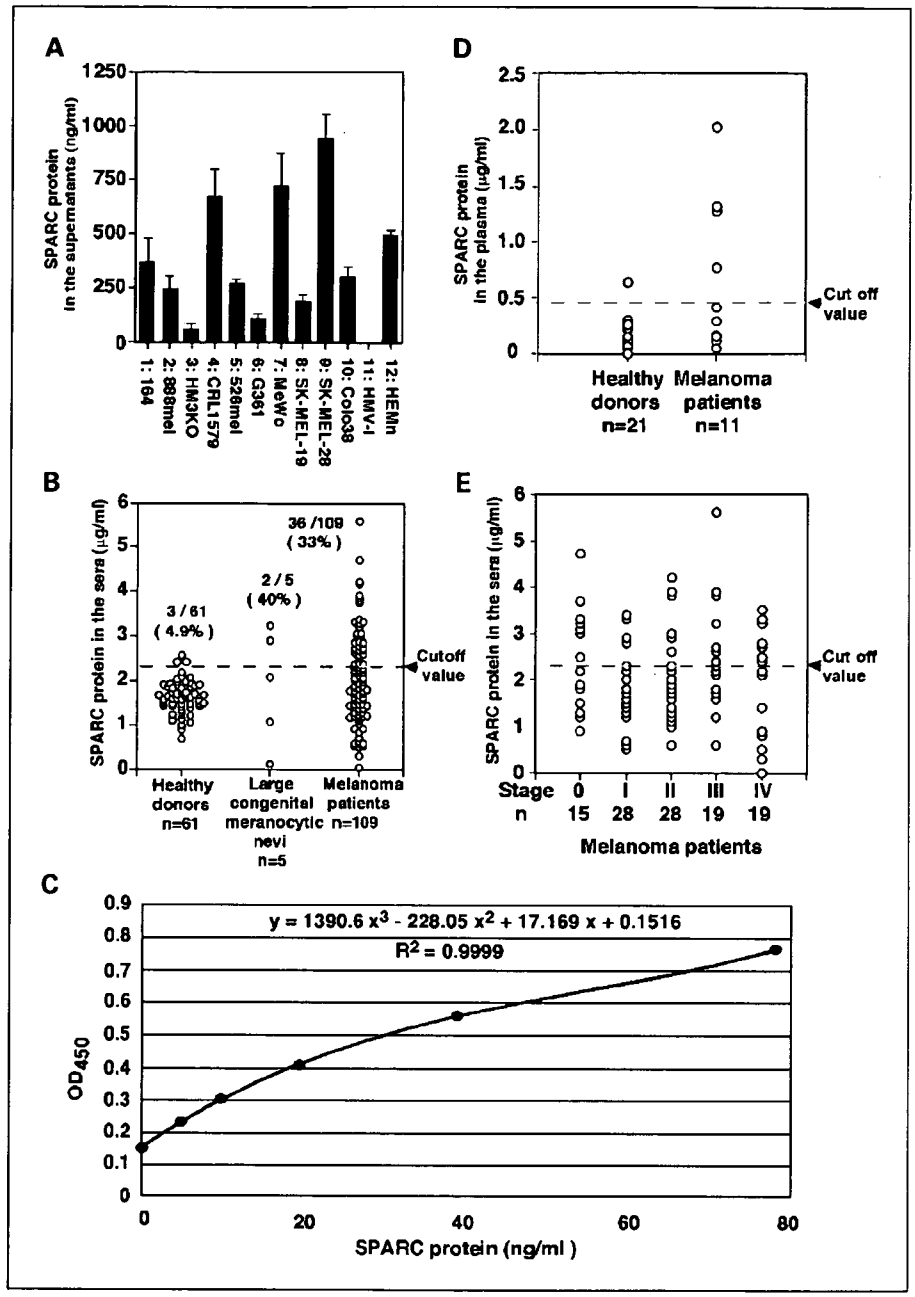

Fig. 2. Quantification of soluble SPARC protein using ELISA. *A*, quantification of SPARC protein secreted in the culture supernatant of melanoma cell lines and HEMn by ELISA. The serum-free culture supernatant was obtained as described in Materials and Methods. Representative of three independent and reproducible experiments with similar results. Columns, mean; bars, SE. *B*, distribution of SPARC protein concentrations estimated by ELISA in sera from 109 patients with melanoma, five patients with melanocytic nevus, and 61 healthy donors. When we fixed the cutoff value at 2.34 μg/mL (the mean SPARC concentration plus 2 SD of the healthy donors), SPARC protein was detected in the sera of 36 of 109 (33.0%) patients with melanoma, 2 of 5 (40%) of patients with melanocytic nevus, and 3 of 61 (4.9%) patients in healthy donors. We could obtain reproducible results thrice. Representative results. *C*, standard curve to quantify the SPARC protein based on absorbance data. Serially diluted human SPARC HON-3030 was used to estimate the standard curve. *D*, the distribution of SPARC protein in plasma from 11 patients with melanoma and 21 healthy donors by ELISA. When we fixed cutoff value at 0.43 μg/mL (mean SPARC concentrations plus 2 SD of the healthy donors), SPARC protein was detected in the plasma of 4 of 11 (36.3%) patients with melanoma and 1 of 21 (4.8%) healthy donors. *E*, comparison of the serum concentration of SPARC in patients with melanoma as classified by stage.

Table 2. Positive rates of increased serum levels of SPARC, GPC3, 5-*S*-cysteinyldopa, SPARC + GPC3, and SPARC + GPC3+5-*S*-cysteinyldopa in patients with melanoma classified by stage

Stage	SPARC (%)	GPC3 (%)	5-*S*-cysteinyldopa (%)	GPC3 + SPARC* (%)	GPC3 + SPARC + 5-*S*-cysteinyldopa† (%)
0	8/15 (53.3)	7/15 (46.7)	0/15 (0.0)	13/15 (86.7)	13/15 (86.7)
I	4/28 (14.3)	12/30 (40.0)	2/29 (6.9)	14/28 (50.0)	15/27 (55.6)
II	7/28 (25.0)	16/30 (53.3)	2/28 (7.1)	20/28 (71.4)	19/27 (70.4)
III	8/19 (42.1)	7/19 (36.8)	5/19 (26.3)	12/19 (63.2)	13/19 (68.4)
IV	9/19 (47.4)	6/19 (31.6)	16/19 (84.2)	12/19 (63.2)	18/19 (94.7)
Total	36/109 (33.0)	48/113 (42.5)	25/110 (22.7)	71/109 (65.1)	78/107 (72.9)

*GPC3 + SPARC: either or both of two markers was positive.
†GPC3 + SPARC + 5-*S*-cysteinyldopa: at least one of three markers was positive.

Decrease of serum SPARC protein in post-operative melanoma patients. The changes in the serum levels of three tumor markers (SPARC, GPC3, and 5-*S*-cysteinyldopa) before and after surgical treatments in SPARC-positive 13 patients are shown in Table 3. In 10 of 13 patients, the serum SPARC levels decreased to below cutoff levels after the surgical treatments, although GPC3 and 5-*S*-cysteinyldopa values were negative before and after the operation in the majority of these patients. It should be noted that SPARC is useful tumor marker to follow the efficacy of surgical treatments. In the case of patients 87 and 92, whose melanoma recurred, the serum SPARC values once decreased to below negative levels and then later increased again, although the serum SPARC level in patient 69 did not increase again when tumor recurrence was identified. The 5-*S*-cysteinyldopa level increased in all patients whose melanoma recurred. Further examinations are needed to elucidate whether the serum SPARC is useful for detecting recurrent tumors.

Table 3. Changes in serum levels of SPARC before and after surgical treatments in the serum SPARC-increased 13 melanoma patients

Patient ID*		SPARC (μg/mL)	GPC3 (units/mL)	5-*S*-cysteinyldopa (nmol/L)
1	Pre ope	4.7	0	4.6
	POD566	1.6	0	4.9
9	Pre ope	2.5	8	4.7
	POD1484	0.5	0	4.1
10	Pre ope	3	0	6.8
	POD15	3.4	0	3.3
12	Pre ope	3.2	7.7	5.5
	POD7	2.3	40	8.8
13	Pre ope	3	0	6.8
	POD37	3.2	0	5
23	Pre ope	3.3	0	1
	POD274	0.9	0	4
44	Pre ope	3.4	0	6.6
	POD140	2	0	3.4
	POD217	2.3	0	4.9
49	Pre ope	2.9	0	4.6
	POD1472	1.6	0	3.7
50	Pre ope	3.9	0	4.3
	POD1008	3.3	0	5.3
	POD1358	2.6	0	2.9
61	Pre ope	3.8	0	3.4
	POD1567	2.3	0	5
69	Pre ope	4.2	0	6.3
	POD329	2	0	4.3
	POD462 (Meta)	1.6	0	3.4
	POD559 (Free)	0.4	0	31
87	Pre ope	3.2	0	8.8
	POD82	2.4	0	5
	POD345	1.4	0	5.1
	POD713 (Meta)	3.5	45	46
92	Pre ope	3.9	0	10.3
	POD465	0.3	0	7.8
	POD678	2.4	0	7.3
	POD762 (Meta)	2.2	0	10

NOTE: Increased values are underlined.
Abbreviations: POD, postoperative days; Meta, metastasis; Free, disease free.
*Patient ID was the same as shown in Table 1.

Discussion

SPARC is a matricellular glycoprotein that modulates cellular interaction with the extracellular matrix during tissue remodeling. Although the specific functions of SPARC still remain unclear, it also plays an important role in wound repair, cell proliferation, cell migration, morphogenesis, cellular differentiation, and angiogenesis (11, 19-21). SPARC was first identified in 1981 as a major noncollagenous constituent of bovine bone (22) and is expressed abundantly in the bone and platelets (23). Recently, many publications have described a high expression of SPARC in a variety of human malignancies (12, 24-35). Tumor-derived SPARC was reported to stimulate tumor progression in many types of cancers. The expression levels of SPARC correlated with the histologic grade of tumor cells (25, 26, 30, 33). A higher SPARC expression was associated with local tumor invasion (24, 29-31, 33); metastasis to the lymph nodes, liver, and bone (24, 28, 32, 33); and poor prognosis and survival (24, 32, 33, 35). Conversely, the expression levels of SPARC was inversely correlated with the degree of malignancy in ovarian cancer (34). In most cancers, SPARC protein is overexpressed in the stromal cells of tumor tissue but is rarely expressed in cancer cells themselves (24, 31). In contrast, melanoma cells by themselves have been shown to express a high level of SPARC, and such increased levels are associated with an invasive phenotype *in vivo* (12, 13).

We confirmed the expression of SPARC at the mRNA and protein levels in human melanoma cell lines and melanoma tissue specimens. In addition, we proved that SPARC was secreted and detected in the culture supernatants of melanoma cell lines and the sera of melanoma patients. In this study, increased serum levels of SPARC were observed in 33% of the melanoma patients. A correlation between the serum levels of tumor markers and tumor progression has been reported (6), as is the case of 5-*S*-cysteinyldopa in their study. However, no significant correlation was observed between the serum SPARC levels and the progression levels of melanoma in this study. We wonder why only 33% of the melanoma patients showed increased levels of SPARC in the sera, and such SPARC concentrations did not correlate with tumor progression, although most melanoma tissues specimens express SPARC protein and almost all examined melanoma cell lines secreted SPARC protein. In melanoma cell lines, the expression level of SPARC did not completely correlate with the levels of soluble SPARC secreted in the culture supernatant (Fig. 1B and Fig. 2A). The same phenomena were also observed in melanoma patients in this study. Sixteen of 24 patients who expressed moderate or strong SPARC protein in melanoma cells did not show elevated SPARC protein in their sera (Table 1). Therefore, not all but some subpopulations of melanoma cells might secrete SPARC protein in the sera of melanoma patients. The concentration of soluble SPARC secreted into the culture supernatant positively correlated with the cell number in the culture dish (data not shown). However, the serum SPARC concentration was not positively associated with the tumor size

or stage. Our evaluation of stage IV individuals revealed the SPARC concentrations to be comparatively low in this group compared with those observed in the early stages. This suggested that SPARC may be a useful tumor marker for detecting relatively early-stage melanoma, although the mechanism of secretion of SPARC from melanoma remains to be elucidated. We are now undertaking further studies to answer these questions.

Positive correlation between the serum SPARC level and platelet count have been reported previously (36). In our work, the serum SPARC concentrations closely correlated with platelet counts in healthy donors (data not shown). In addition, no significant difference was observed between the platelet counts of healthy donors and melanoma patients [213,000 ± 9,000/mm^3 versus 216,000 ± 8,000/mm^3 (mean ± SE)]. In this study of SPARC measurements in sera, there were three (4.9%) false-positive cases, although those increased values were lower than those in most of the positive patients with melanoma. We thought the elevated SPARC concentrations in healthy donors might thus have been due to confounding conditions, such as thrombocytosis; however, no thrombocytosis cases were identified. Although we considered whether the influence of the platelets might be suppressed by measuring SPARC in plasma, we also had one false-positive case (1 of 21, 4.8%). The positive rate of increased plasma SPARC did not significantly differ from that of the increased serum SPARC in melanoma patients (36.4% versus 33.0%). Thereby, it is unlikely that increased SPARC levels in sera of melanoma patients are due to secretion of SPARC from platelets.

Melanocytic nevi tissues expressed both SPARC and GPC3 protein (10). The SPARC levels in sera were increased in two of five (40%) patients with large congenital melanocytic nevus, although the GPC3 level did not increase. We have to thus pay close attention to cases, which are GPC3 negative and SPARC positive, because of the risk of making a false-positive diagnosis.

We used sera from Japanese patients only. The incidence of acral lentiginous melanoma in the Japanese population is much higher than that in Caucasians, whereas superficial spreading melanoma and lentigo maligna melanoma are frequent types observed in Caucasians. Some groups have reported that acral lentiginous melanoma differs from other types of melanomas in its clinical, histopathologic, and genetic characteristics (37–40). We compared the positive rate of increased serum SPARC and GPC3 among patients classified by these clinical types. Thus, no significant correlations were observed between the positive rate and melanoma types (data not shown). Therefore, it seems likely that the usefulness of SPARC and GPC3 for diagnosis of melanoma is not restricted to only Japanese patients.

In conclusion, SPARC was found to be a useful tumor marker for melanoma particularly at the early stage of the disease, and the addition of the other two markers (GPC3 and 5-*S*-cysteinyldopa) had added benefit in diagnosis. On the other hand, the serum levels of these three markers are still unknown in a population of patients with atypical nevi syndrome or other high-risk population in this study. Further investigations are needed to consider future applications of serum SPARC and GPC3 for the mass screening of melanoma.

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Therapeutic effect of α-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells

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The close cooperation of both innate and acquired immunity is essential for the induction of truly effective antitumor immunity. We tested a strategy to enhance the cross-talk between NKT cells and conventional antigen-specific T cells with the use of αGalCer-loaded dendritic cells genetically engineered to express antigen plus chemokine, attracting both conventional T cells and NKT cells. DC genetically engineered to express a model antigen, OVA, along with SLC/CCL21 or monokine induced by IFN-γ/CXCL9, had been generated using a method based on *in vitro* differentiation of DC from mouse ES cells. The ES-DC were loaded with α-GalCer and transferred to mice bearing MO4, an OVA-expressing melanoma, and their capacity to evoke antitumor immunity was evaluated. *In vivo* transfer of either OVA-expressing ES-DC, stimulating OVA-reactive T cells, or α-GalCer-loaded non-transfectant ES-DC, stimulating NKT cells, elicited a significant but limited degree of protection against the i.p. disseminated MO4. A more potent antitumor effect was observed when α-GalCer was loaded to ES-DC expressing OVA before *in vivo* transfer, and the effect was abrogated by the administration of anti-CD8, anti-NK1.1 or anti-asialo GM1 antibody. α-GalCer-loaded double transfectant ES-DC expressing SLC along with OVA induced the most potent antitumor immunity. Thus, α-GalCer-loaded ES-DC expressing tumor-associated antigen along with SLC can stimulate multiple subsets of effector cells to induce a potent therapeutic effect against peritoneally disseminated tumor cells. The present study suggests a novel way to use α-GalCer in immunotherapy for peritoneally disseminated cancer. (*Cancer Sci* 2005; 96: 889–896)

A means to induce the close cooperation of both innate and acquired immunity would be necessary for the induction of efficient antitumor therapy. Recent studies have shown DC to be potent stimulators of both innate and acquired immunity. The *in vivo* transfer of DC presenting tumor-associated antigens has proven to be efficient in the priming of CTL specific to the antigens. α-GalCer presented by DC efficiently stimulates NKT cells,[1–4] a subset of T cells implicated in the innate immunity against infection and cancer.[5–7] In addition, NKT cells stimulated by the *in vivo* administration of α-GalCer secondarily stimulate conventional T cells.[8,9] It is thus presumed that the *in vivo* transfer of DC simultaneously loaded with tumor-associated antigens and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, thus resulting in a potent antitumor immunity.

Chemokines mediate leukocyte adhesion and homing, and the concordant migration of specific leukocyte subsets induced by chemokines is pivotal for the development of proper immune responses. SLC/CCL21 attracts both T cells and DC to lymphoid tissues through its receptor CCR7, and the effect of SLC is essential for the priming of naive T cells in the initiation phase of the immune response. CXCR3 and its ligands, Mig/CXCL9 and IP-10/CXCL10, mediate the migration of effector/memory T cells and NK cells to the site of inflammation. In addition, a recent study revealed that these chemokines and their receptors also mediate the migration of some subpopulations of NKT cells.[10–12]

As a means for loading the tumor-associated antigens to DC, genetic modification to express antigenic proteins has several advantages. The expression of tumor antigens by DC circumvents the need for identifying specific CTL epitopes within the protein, and by that the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[13] For the efficient gene transfer to DC, the use of virus-based vectors is required because DC are not easy to genetically modify. Considering the clinical application, however, there are several problems related to the use of virus vectors. These include the inefficiency of gene transfer, the instability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, in many countries, legal restrictions have been placed on the use of virus vectors outside of carefully isolated laboratories.

We and others have established methods to generate dendritic cells *in vitro* from mouse ES cells.[14,15] ES-DC have the

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Abbreviations: Ab, antibody; BM-DC, bone marrow cell-derived dendritic cell; CBF1, (CBA × C57BL/6) F1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ES cell, embryonic stem cell; ES-DC, ES cell-derived dendritic cell; α-GalCer, α-galactosylceramide; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IFN-γ, interferon-gamma; IL, interleukin; i.p., intraperitoneally; IP-10, IFN-γ-inducible 10 kDa protein; mAb, monoclonal antibody; Mig, monokine induced by IFN-γ; NK, natural killer; OVA, ovalbumin; s.c., subcutaneously; SLC, secondary lymphoid tissue chemokine.

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capacity to stimulate T cells, present antigen and migrate to lymphoid tissues upon *in vivo* administration, and these capacities of ES-DC are comparable to those of BM-DC. The genetic modification of ES-DC can be carried out without the use of virus vectors by introducing exogenous genes by electroporation into undifferentiated ES cells and the subsequent induction of their differentiation into ES-DC. We can generate multiple gene-transfectant ES-DC by the sequential transfection of ES cells with vectors bearing different selection markers.[16,17] In a previous study, we generated double-transfectant ES-DC expressing SLC or Mig along with a model tumor antigen.[16] Using these double-transfectant ES-DC, we demonstrated that the coexpression of SLC or Mig enhanced the capacity of *in vivo*-transferred ES-DC to activate antigen-specific CTL and to protect the recipient mice from a tumor cell challenge.

In the present study, we evaluated the capacity of α-GalCer-loaded ES-DC to stimulate NKT cells both *in vitro* and *in vivo*, in comparison to that of BM-DC. We next evaluated the antitumor effect of simultaneous stimulation of NKT cells and antigen-specific conventional T cells by the *in vivo* administration of α-GalCer-loaded ES-DC expressing a model tumor antigen, namely OVA. Furthermore, we addressed whether coexpression of SLC or Mig with the antigen by ES-DC could enhance the synergic antitumor effect of NKT cells and conventional T cells.

Materials and Methods

Mice

CBA and C57BL/6 mice were obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce CBF1 mice and all *in vivo* experiments were carried out using the CBF1 mice. The animal experiments in this study were approved by the animal experiment committee of Kumamoto University (permission number A16-074).

Reagents

Recombinant mouse GM-CSF was purchased from PeproTech EC (London, UK) and α-GalCer was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Mouse IL-4 and IFN-γ ELISA kits were purchased from eBioscience (San Diego, CA, USA). Polyclonal rabbit anti-asialo GM1 Ab was purchased from Wako Chemicals (Tokyo, Japan).

Cell lines and preparation of DC

The ES cell line TT2, derived from CBF1 blastocysts,[18] was maintained as described previously.[19] MO4[20] was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid. The procedure for inducing the differentiation of ES cells into DC has been reproted previously.[15] ES-DC expressing OVA (ES-DC-OVA) and ES-DC expressing chemokine, SLC or Mig, along with OVA (ES-DC-OVA/SLC or ES-DC-OVA/Mig) were generated as reported previously.[16] ES-DC recovered after a 14-day culture in bacteriological Petri dishes were used for both *in vivo* and *in vitro* assays. To generate BM-DC, bone marrow cells were isolated from CBF1 mice and cultured in RPMI + 10% fetal calf serum + GM-CSF (5 ng/mL) for 7 days, according to the method reported by Lutz *et al.*[21]

Analysis of the activation of NKT cells by DC loaded with α-GalCer

Embryonic stem cell-derived dendritic cells or BM-DC were cultured in the presence of α-GalCer (100 ng/mL) or vehicle (0.00025% Polysorbate-20) alone for 22 h, washed twice, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC $+2.5 \times 10^6$ T cells/well in 24-well culture plates). Splenic T cells were isolated using nylon-wool columns, as described previously.[16] After 24 h or 5 days, the cells were recovered and analyzed on their cytotoxic activity by a 4-h ^{51}Cr-release assay using YAC-1 cells (1×10^4 cells/well) as targets in 96-well round-bottomed culture plates at the effector : target ratio indicated. The amount of IL-4 and IFN-γ in the culture supernatant was measured by ELISA. In the analysis of the stimulation of NKT cells *in vivo*, ES-DC or BM-DC loaded with either α-GalCer (100 ng/mL) or vehicle alone, as described above, were injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed, as described above.

Tumor challenge experiments

Indicated numbers of MO4 cells were injected s.c. into the shaved left flank region, or i.p. on day 0. On day 3, 1×10^5 genetically modified ES-DC preloaded with either α-GalCer or vehicle alone were transferred i.p. into the mice. The survival rate of the mice was monitored and, in s.c. challenge experiments, the tumor sizes were also determined biweekly in a blinded fashion. The tumor index was calculated as:

Tumor index (mm) = square root of (length × width).

In vivo depletion experiments

The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and they were injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given a total of 14 i.p. injections (days −4, −1, 2, 5, 10, 13, 15, 19, 26, 33, 40, 47, 54 and 61) of mAb, ascites (0.1 mL/mouse/injection) from hybridoma-bearing nude mice, or polyclonal rabbit anti-asialo GM1 Ab (50 μg/mouse/injection). The mAbs used were rat antimouse CD4 (clone GK1.5), rat antimouse CD8 (clone 2.43) and mouse anti-NK1.1 (clone PK-136). Normal rat IgG (Sigma, St Louis, MO, USA) was used as a control (200 μg/mouse/injection).

Statistical analysis

Two-tailed Student's *t*-test was used to determine the statistical significance of differences in cytolytic activity and the tumor growth between the treatment groups. A value of $P < 0.05$ was considered to be significant. The Kaplan–Meier plot for survival was used to determine any significant differences in tumor challenge experiments, using the Breslow–Gehan–Wilcoxon test. In some experiments, the difference in the survival rate between treatment groups was assessed for significance using the χ^2-test. Statistical analyses were made using the StatView 5.0 software (Abacus Concepts, Calabasas, CA, USA).

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Results

Activation of NKT cells by ES-DC pulsed with α-GalCer

Mouse splenic DC and BM-DC loaded with α-GalCer have been reported to efficiently stimulate NKT cells, resulting in the rapid induction of NK cell-like cytolytic activity and the production of cytokines such as IL-4 and IFN-γ.[2,3] We examined whether ES-DC loaded with α-GalCer had the capacity to activate NKT cells, as naturally occurring DC do.

TT2 ES cell-derived non-transfectant ES-DC (ES-DC-TT2) or BM-DC were preincubated with α-GalCer, and then cocultured with splenic T cells isolated from syngeneic CBF1 mice. After 24 h, the cultured cells were recovered and their cytolytic activity against YAC-1 target cells was analyzed by a ^{51}Cr-release assay. The results shown in Fig. 1a indicate that a significant cytotoxity against YAC-1 cells was induced in the splenic T cell preparations by coculture with ES-DC loaded with α-GalCer, in comparison to the coculture with ES-DC loaded with vehicle alone. The cytotoxic activity induced by α-GalCer-loaded ES-DC-TT2 was comparable to that induced by α-GalCer-loaded BM-DC (Fig. 1a). As shown in Fig. 1b, IL-4 and IFN-γ were produced by splenic T cells cocultured with α-GalCer-loaded BM-DC or ES-DC, and a similar amount of the cytokines was produced in the culture with BM-DC and ES-DC preloaded with α-GalCer. If the coculture of T cells with α-GalCer-loaded ES-DC was extended to 5 days, the induced killing activity (Fig. 1c) and the amount of IL-4 and IFN-γ produced was increased in parallel (data not shown).

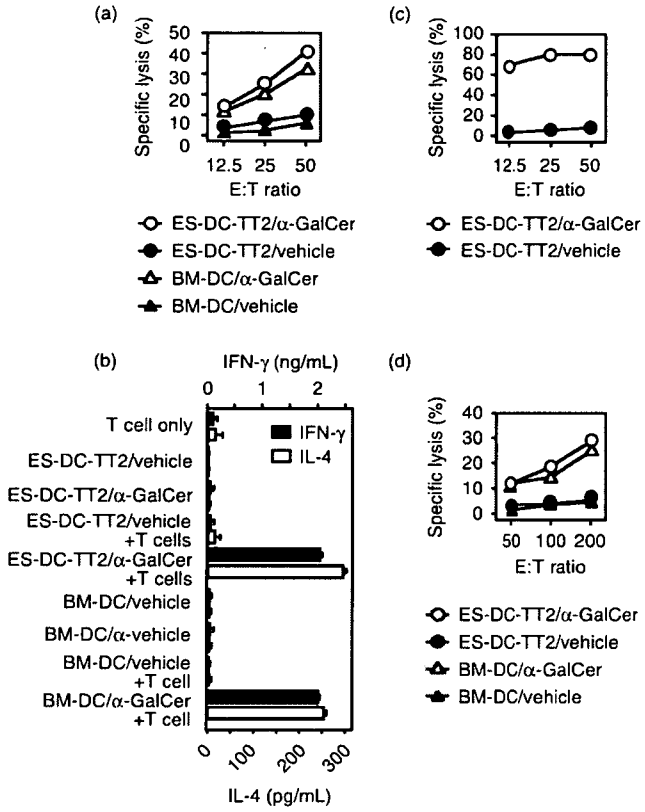

Fig. 1. Activation of NKT cells by the α-GalCer-loaded ES-DC. (a) ES-DC-TT2 or BM-DC were loaded with either α-GalCer (100 ng/mL) or vehicle (Polysorbate-20) alone for 22 h, washed extensively, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC + 2.5×10^5 T cells/well in 24-well culture plates). After 24 h of culture, the cells were recovered and the cytotoxic activity of the harvested cells against YAC-1 cells (1×10^4 cells) was analyzed using a 4-h Cr-release assay at the effector : target (E : T) ratios indicated. (b) Amounts of IL-4 and IFN-γ in the supernatant collected at the end of the 24-h coculture were quantified by ELISA. The results are expressed as the mean cytokine production of triplicate assays + SD. (c) The coculture was extended to 5 days and the killing activity of resultant cells was analyzed as in (a). (d) ES-DC or BM-DC were cultured in the presence of either α-GalCer (100 ng/mL) or vehicle alone for 18 h, washed, and injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, spleen cells were isolated from the mice and their cytotoxic activity against YAC-1 cells was analyzed as in (a). The results are expressed as the mean specific lysis of triplicate assays. The SD of triplicates were less than 2%.

We next analyzed the capacity of α-GalCer-loaded ES-DC to activate NKT cells *in vivo*. ES-DC-TT2 or BM-DC were preloaded with α-GalCer or vehicle alone in the same way as described above and i.p. injected into the syngeneic CBF1 mice. After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed. As shown in Fig. 1d, a significant degree of cytotoxic activity was induced in the spleen cells by transfer of ES-DC loaded with α-GalCer, but it was not induced by the transfer of those loaded with vehicle alone. The capacity to evoke YAC-1 cell-killing activity of ES-DC and that of BM-DC was similar also *in vivo*. The activated NKT cells are known to activate the cytotoxic activity of NK cells.[22] It is therefore possible that the cytotoxic activity observed in these assays were mostly mediated by NK cells secondarily stimulated by NKT cells. Even so, these data collectively demonstrate that ES-DC had the capacity to present α-GalCer to activate NKT cells, and the capacity was similar to that of BM-DC both *in vitro* and *in vivo*.

Anti-tumor effect of α-GalCer-loaded ES-DC

We assessed whether the activation of NKT cells *in vivo* by α-GalCer-loaded ES-DC had any therapeutic effect against the tumor cells growing *in vivo*. MO4, originating from NK-sensitive B16 melanoma cells, were injected s.c. into the left flank region of mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 loaded with α-GalCer or vehicle alone. As shown in Fig. 2a, ES-DC loaded with α-GalCer did not show any therapeutic effect in this s.c. tumor model.

We next investigated the effect of α-GalCer-loaded ES-DC in the peritoneally disseminated tumor model. MO4 cells were injected i.p. into mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC loaded with either α-GalCer or vehicle alone. As shown in Fig. 2b, which indicated the survival rate of the treated mice, the injection of ES-DC-TT2 loaded with α-GalCer elicited a significant ($P < 0.05$) but limited protective effect against the i.p. disseminated tumor cells.

Synergic therapeutic effect of α-GalCer-activated NKT cells and antigen-specific T cells against peritoneally disseminated tumor cells

In a previous study, we demonstrated that the *in vivo* transfer of ES-DC-OVA effectively primed OVA-specific CTL and induced protection against a subsequent challenge with s.c. injected MO4 cells expressing OVA.[16] We investigated

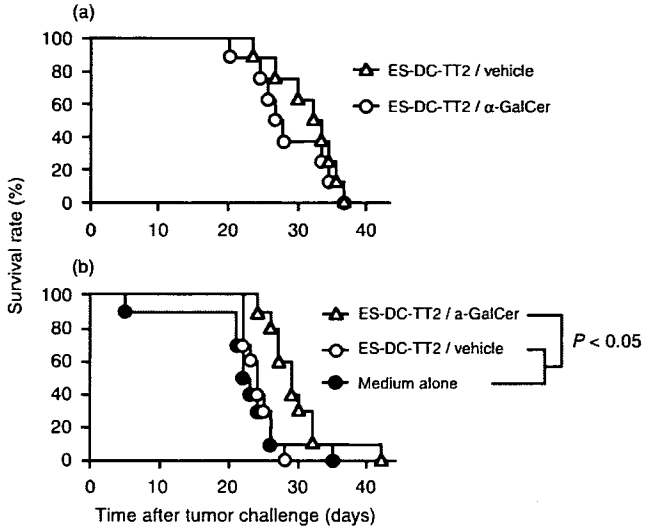

Fig. 2. Anti-tumor effect of *in vivo*-transferred α-GalCer-loaded ES-DC. The mice were (a) inoculated s.c. with MO4 cells (3×10^5 cells/mouse) to the left flank region or (b) inoculated i.p. with MO4 cells (1×10^5 cells/mouse). After 3 days, the mice were treated with i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer, vehicle alone or medium alone, and the mouse survival rate was monitored ($n = 10$ per group). In (b), the survival rate of the α-GalCer-loaded ES-DC-TT2-treated group was higher than that of the other two groups and the difference was statistically significant. Data are representative of four independent and reproducible experiments.

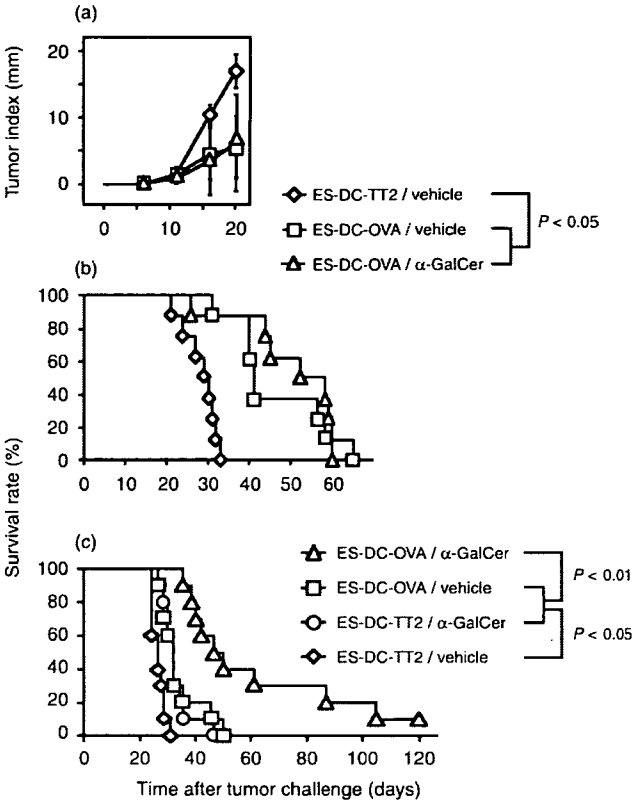

Fig. 3. Synergic effect of α-GalCer loading and the expression of tumor antigen on the protection against tumors induced by ES-DC. MO4 cells (3×10^5 cells/mouse) were injected s.c. into the left flank region of the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with vehicle alone, ES-DC-TT2 loaded with α-GalCer, or ES-DC-OVA loaded with α-GalCer. After that, the tumor sizes were determined (a) and survival rate was monitored (b). Both the differences in tumor index (a) and in mouse survival rate (b) between the vehicle-loaded ES-DC-TT2-treated group and other two groups were statistically significant. (c) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-OVA or ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer or vehicle alone. Thereafter, the mouse survival rate was monitored. The survival rate of the α-GalCer-loaded ES-DC-OVA-treated group was higher than that of the other groups and the difference was statistically significant. The survival rates of the vehicle-loaded ES-DC-OVA-treated group and α-GalCer-loaded ES-DC-TT2-treated group were higher than that of vehicle-loaded ES-DC-TT2-treated group and the difference was statistically significant. Each group included 10 mice. Data are representative of three independent and reproducible experiments.

whether the loading of α-GalCer to ES-DC-OVA before *in vivo* transfer would enhance the therapeutic effect against pre-established MO4 tumor. The mice were challenged s.c. with MO4 cells, and then 3 days later they were treated by i.p. injection of ES-DC-OVA preloaded with α-GalCer or vehicle alone. As shown in Fig. 3a,b, compared to the transfer of ES-DC-TT2, the transfer of ES-DC-OVA, loaded with either α-GalCer or vehicle alone, elicited a significant antitumor effect in this therapeutic model, as observed in the previously reported prevention (prophylactic) model.[16] However, the loading of α-GalCer to ES-DC-OVA did not improve the effect, based on either the tumor growth or the mouse survival time (Fig. 3a,b). These results suggest that the activation of NKT cells by α-GalCer loaded to ES-DC does not enhance the therapeutic effect of antigen-specific T cells against s.c. growing tumor cells.

We next investigated the effect of transfer of α-GalCer-loaded ES-DC-OVA against peritoneally disseminated tumor cells. Mice were i.p. inoculated with MO4 cells and 3 days later they were treated by an i.p. injection of ES-DC-OVA loaded with α-GalCer or vehicle alone, or ES-DC-TT2 loaded with vehicle alone. As shown in Fig. 3c, the therapeutic effect of the transfer of ES-DC-OVA loaded with vehicle alone was significant ($P < 0.05$) in comparison to the treatment with ES-DC-TT2 loaded with vehicle alone, but the effect was less marked than the effect observed in the s.c. growing tumor model (Fig. 3b). In contrast, the treatment with ES-DC-OVA loaded with α-GalCer elicited a potent effect to prolong the survival time of the mice. Given that the antitumor effect elicited by α-GalCer-loaded non-transfectant ES-DC was also limited (Figs 2b,3c), these results indicate that the NKT cells activated by α-GalCer presented by ES-DC and OVA-specific CTL primed by OVA antigen presented by the same ES-DC acted synergistically to protect the mice.

Subsets of effector cells contributing to the antitumor effect induced by α-GalCer-loaded ES-DC that expressed a model tumor antigen

To determine the effector cells exhibiting the observed antitumor effect induced by adoptive transfer of α-GalCer-loaded ES-DC expressing OVA, we carried out depletion experiments by injecting the mice with Abs specific to several subsets of effector cells during the tumor cell challenge and treatment with ES-DC. Figure 4a shows the

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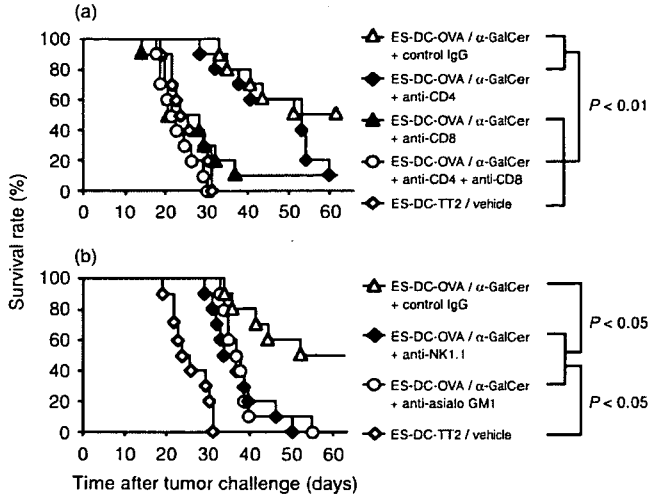

Fig. 4. Effector cells involved in the antitumor effect exerted by adoptive transfer of α-GalCer-loaded, antigen-expressing ES-DC. The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given i.p. injections of mAb or polyclonal rabbit anti-asialo GM1 Ab. The effect of the injection of anti-CD4, anti-CD8, or a combination of these two Abs is shown in (a). The effect of anti-NK1.1 or rabbit anti-asialo GM1 Ab is shown in (b). As a control, the survival of the mice treated with normal rat IgG is shown in both (a) and (b). Each group included 10 mice. In (a), the survival rates of α-GalCer-loaded ES-DC-OVA plus control IgG-treated group and α-GalCer-loaded ES-DC-OVA plus anti-CD4-treated group were higher than those of the other three groups and the difference was statistically significant. In (b), the survival rates of α-GalCer-loaded ES-DC-OVA plus anti-NK1.1 or anti-asialo GM1-treated groups and those of the other two groups were statistically different. The experiment was carried out once.

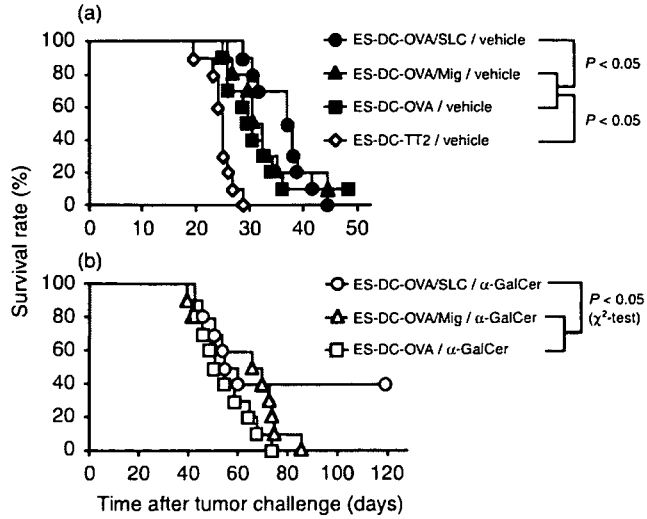

Fig. 5. Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA. (a) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with i.p. injection of ES-DC-TT2, ES-DC-OVA, ES-DC-OVA/Mig, or ES-DC-OVA/SLC (1×10^5 cells/mouse), all loaded with vehicle only. Thereafter, the survival rate of the mice was monitored. The differences in the survival rate between ES-DC-OVA/Mig-treated or ES-DC-OVA-treated group and the other two groups were statistically significant. (b) The mice were challenged with MO4 cells as in (a) and treated with either ES-DC-OVA, ES-DC-OVA/SLC or ES-DC-OVA/Mig, all loaded with α-GalCer. The frequency of mice from the ES-DC-OVA/SLC-treated group surviving for more than 100 days (four out of 10 mice) was significantly higher than that of the other two groups (0 out of 10 mice in each group), according to the χ^2-test. The experiment was carried out once.

effect of the injection of anti-CD4 or anti-CD8 mAbs or a combination of these two mAbs. The injection of anti-CD8 mAb almost totally abrogated the effect of the treatment with the ES-DC, thus suggesting that $CD8^+$ OVA-specific CTL played an important role in protecting the mice from the tumor. Compared to the effect of anti-CD8 mAb, the injection of anti-CD4 mAb had far less influence on the protective effect against the tumor, thus indicating the function of $CD4^+$ helper T cells to be not essential.

Figure 4b shows the effect of the injection of rabbit anti-asialo GM1 Ab, depleting NK cells, and also that of anti-NK1.1 mAb, depleting both NK and NKT cells. Treatment with either of these two kinds of Ab decreased the effect of α-GalCer-loaded ES-DC-OVA to a level similar to that elicited by vehicle-loaded ES-DC-OVA. These results indicate that NK cells played an essential role in the enhanced antitumor effect caused by the activation of NKT cells by α-GalCer.

Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA

We previously found that the coexpression of SLC or Mig, T cell-attracting chemokines that natural DC do not produce, along with OVA by ES-DC significantly enhanced their capacity to prime OVA-specific CTL and also to induce a protective immunity against s.c. injected MO4 cells.[16] A recent study revealed that these two chemokines induced chemotaxis not only of conventional T cells but also of some subpopulations of the NK cells and NKT cells.[10-12,23] We therefore examined whether the coexpression of such chemokine by ES-DC expressing OVA would also have an enhancing effect in protection against the i.p. growing MO4 cells.

We first assessed the effect of the expression of such chemokines by ES-DC without preloading with α-GalCer. We analyzed the capacity of ES-DC-OVA/SLC or ES-DC-OVA/Mig, ES-DC expressing OVA simultaneously with SLC or Mig, to induce protection against i.p. disseminated MO4 cells, comparing the capacity with that of ES-DC-OVA. The effect elicited by ES-DC-OVA/Mig was not higher than that elicited by ES-DC-OVA. Thus, the expression of Mig did not enhance the antitumor effect (Fig. 5a). However, expression of SLC by ES-DC did enhance the protective effect, although the effect of SLC in this i.p. tumor model was less evident than that observed in the s.c. tumor model reported previously.[16]

We next evaluated the effect of the expression of either SLC or Mig on the antitumor effect elicited by α-GalCer-loaded ES-DC-OVA. α-GalCer ES-DC-OVA/Mig and α-GalCer ES-DC-OVA exhibited a similar degree of protection, thus indicating that the coexpression of Mig by α-GalCer-loaded ES-DC-expressing OVA did not have any additive effect (Fig. 5b). In contrast, α-GalCer-loaded ES-DC-OVA/SLC exhibited a far more potent protective effect than α-GalCer ES-DC-OVA/Mig or α-GalCer ES-DC-OVA did. We observed that 40% of the mice treated with α-GalCer-loaded ES-DC-OVA/SLC completely rejected the tumor cells (Fig. 5b). These results suggest that the SLC produced by

ES-DC augmented the synergic effect of antigen-reactive CTL, α-GalCer-activated NKT cells, and probably NK cells.

As we reported previously,[16] coexpression of SLC along with OVA in ES-DC enhanced the capacity of ES-DC to prime OVA-specific CTL upon *in vivo* transfer. The data shown in the Fig. 5a also indicate that coexpression of SLC enhanced the capacity of ES-DC to induce antitumor immunity mediated by OVA-specific CTL in the absence of α-GalCer. To assess the effect of SLC produced by ES-DC on the activation of NKT or NK cells, we compared the capacity of α-GalCer-loaded ES-DC-OVA and α-GalCer-loaded ES-DC-OVA/SLC to stimulate NKT cells by an experiment similar to that shown in Fig. 1d. As a result, we observed that the capacity of α-GalCer-loaded ES-DC to induce YAC-1 cell-killing activity was not enhanced by expression of SLC (data not shown). Thus, effect of SLC produced by ES-DC to enhance the activation of NKT and NK cells was not detected at least by this short-term (24 h) assay. Based on these observations, it may be considered that the expression of SLC by ES-DC dominantly enhanced the activation of antigen-specific CTL rather than NKT or NK cells.

Discussion

In the present study, we evaluated the effect of loading α-GalCer to ES-DC expressing a model tumor antigen on their capacity to induce antitumor immunity. Upon loading with α-GalCer, ES-DC had a capacity comparable to that of BM-DC to stimulate NKT cells (Fig. 1). The *in vivo* administration α-GalCer-loaded non-transfectant ES-DC had some antitumor effect in an i.p. disseminated tumor model but not in an s.c. growing tumor model (Fig. 2). The difference in the effect of loading of α-GalCer to ES-DC in between the two models may be accounted for by the tissue distribution of NKT cells. NKT cells localize mainly in the liver, lung, spleen, bone marrow and peritoneal cavity.[6,11,24,25] In parallel with these observations, the loading of α-GalCer to ES-DC-OVA enhanced their antitumor effect against i.p. disseminated but not s.c. growing MO4 tumor cells (Fig. 3).

In a previous study, we observed that the protective effect against s.c. growing MO4 cells by transfer of ES-DC-OVA was almost totally abrogated by the depletion of either of CD4 or CD8 T cells.[16] In contrast, in the present study, the depletion of $CD8^+$ T cells but not $CD4^+$ T cells diminished the antitumor effect against i.p. MO4 cells elicited by α-GalCer-loaded ES-DC-OVA (Fig. 4a). These results indicate that CTL play a pivotal role in both conditions, and that $CD4^+$ T helper cells were not essential in the protective immunity against i.p. tumor cells on the occasion of simultaneous activation of NKT cells. The reason for the dispensability of $CD4^+$ T helper cells may be that NKT cells and NK cells, secondarily activated by NKT cells, provide help to OVA-specific CTL.[26] The data shown in Fig. 4b revealed that the depletion of NK cells decreased the effect of α-GalCer-loaded ES-DC-OVA to a degree similar to that elicited by vehicle-loaded ES-DC-OVA, indicating that NK cells played an essential role in the enhancement of the antitumor effect obtained by loading α-GalCer to ES-DC-OVA. Collectively, $CD8^+$ CTL, NKT cells and NK cells played essential roles in the antitumor effect obtained by α-GalCer to ES-DC expressing

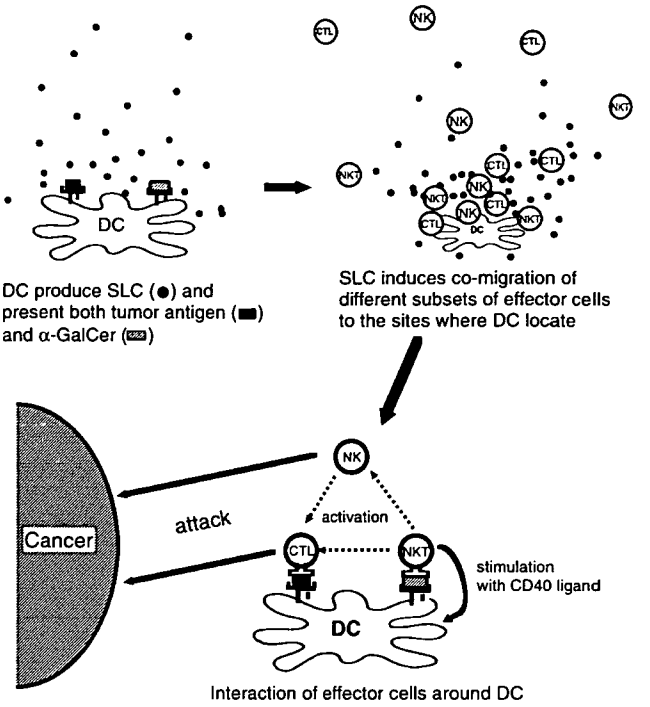

Fig. 6. A schematic depiction of the enhanced cross-talk of different subsets of effector cells induced by α-GalCer-loaded ES-DC expressing OVA plus SLC. SLC secreted by ES-DC induces the comigration of different subsets of effector cells, including NKT cells, NK cells and antigen-specific T cells, to the sites where the injected ES-DC are located. The effector cells of both innate and acquired immunity gathered around ES-DC, which present both α-GalCer and tumor antigen, thus closely interacting to develop a potent antitumor immunity.

the antigen (Fig. 6). Presumably, the sequential stimulation of NKT cells and NK cells augmented the antitumor effect of OVA-specific CTL[9] and probably the interactions of effector cells were mediated by IFN-γ and IL-2.[22,27–31]

The data shown in Fig. 5b indicate that the expression of SLC by ES-DC enhanced the antitumor effect induced by the transfer of α-GalCer-loaded ES-DC expressing OVA. SLC has been reported to attract not only conventional T cells and DC but also NKT cells.[10,23] SLC also induces chemotaxis of $CD56^{bright}$ $CD16^-$ NK cells and has a costimulatory effect on the proliferation of NK cells.[32] Thus, SLC probably induced the comigration of conventional T cells, NKT cells and NK cells to the sites where ES-DC were located, and, as a result, the close interaction of such multiple subsets of effector cells may have occurred (Fig. 6).

In the past decade, α-GalCer has been attracting attention as a novel immunostimulatory reagent for antitumor therapy. Based on the promising results of preclinical studies demonstrating antitumor effects of α-GalCer,[2,25,33] several phase I clinical studies on anticancer immunotherapy by the direct intravenous administration of α-GalCer or the administration of α-GalCer-loaded DC have been carried out.[34–37] Although the activation and expansion of NKT cells by the administration of α-GalCer has been observed, the results seemed to be unsatisfactory from the viewpoint of the clinical effect. The present study demonstrated that α-GalCer is useful for induction

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of immunity against peritoneally disseminated tumor cells, especially when it is loaded to DC genetically engineered to express tumor antigen. Although metastasis of melanoma to visceral organ sites is observed frequently in patients with advanced (stage IV) malignant melanoma, peritoneal dissemination of melanoma is very rare. Thus, we are planning another study with more clinical relevance, using models of cancer with a high tendency to peritoneal dissemination.

In recent years, a number of tumor-associated antigens have been identified. These antigens are potentially good targets for immunotherapy. To establish truly effective anticancer immunotherapy, a method for potently polarizing the immune system toward these antigens is essential. Antitumor immunotherapy with DC loaded with HLA-binding peptides derived from tumor antigens has been tested clinically in many institutions. In most cases, the DC are generated by culture of monocytes obtained from peripheral blood of the patients. To generate a sufficient number of DC for treatment, apheresis, a procedure that is sometimes invasive for patients with advanced stages of cancer, is necessary to obtain a sufficient number of monocytes as a source for DC. In addition, the culture to generate DC should be done separately for each patient and for each treatment, and thus the procedure used at present may be too labor-intensive and expensive to be applied broadly in a practical setting. Alternately, the source of ES-DC, ES cells, have the capacity to propagate infinitely. We would thus be able to use human ES cells as an infinite source of DC. In addition, we will be able to generate genetically engineered DC without the need to use virus vectors, as mentioned above. We may thus be able to generate multiple gene-transfectant ES-DC expressing tumor antigen plus immunostimulating molecules, which could be more potent in stimulating antitumor immunity than monocyte-derived DC are.

Regarding the future clinical application of ES-DC, we recently established a method for generating ES-DC from ES cells of a non-human primate, namely the cynomolgus monkey, and also for their genetic modification (unpublished data). We believe that this method would be applicable to human ES cells, although some modifications may be necessary. Considering the future clinical application of ES-DC technology, allogenicity (i.e. differences in the genetic background) between patients to be treated and ES cells as a source for DC may cause problems. However, it is expected that human ES cells sharing some HLA alleles with patients will be available for most cases. We recently found that antigen-expressing ES-DC potently primed antigen-specific CTL after the transfer to semiallogeneic mice sharing some MHC alleles with the ES-DC, and protected the recipient mice from subsequent challenge with tumor cells bearing the antigen.[38] CD1d is monomorphic and thus a CD1d-α-GalCer-complex on ES-DC can stimulate the NKT cells of any recipients. α-GalCer would thus be an ideal adjuvant to enhance the immune response toward the tumor antigens presented by ES-DC.

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DNA vaccination of HSP105 leads to tumor rejection of colorectal cancer and melanoma in mice through activation of both CD4+ T cells and CD8+ T cells

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We report that HSP105, identified by serological identification of antigens by recombinant expression cloning (SEREX), is overexpressed in a variety of human cancers, including colorectal, pancreatic, thyroid, esophageal, and breast carcinoma, but is not expressed in normal tissues except for the testis. The amino acid sequences and expression patterns of HSP105 are very similar in humans and mice. In this study, we set up a preclinical study to investigate the usefulness of a DNA vaccine producing mouse HSP105 whole protein for cancer immunotherapy *in vivo* using BALB/c and C57BL/6 mice, Colon26, a syngeneic endogenously HSP105-expressing colorectal cancer cell line, and B16.F10, a melanoma cell line. The DNA vaccine was used to stimulate HSP105-specific T-cell responses. Fifty percent of mice immunized with the *HSP105* DNA vaccine completely suppressed the growth of subcutaneous Colon26 or B16.F10 cells accompanied by massive infiltration of both CD4+ T cells and CD8+ T cells into tumors. In cell transfer or depletion experiments we proved that both CD4+ T cells and CD8+ T cells induced by these vaccines play critical roles in the activation of antitumor immunity. Evidence of autoimmune reactions was not present in surviving mice that had rejected tumor cell challenges. We found that HSP105 was highly immunogenic in mice and that the *HSP105* DNA vaccination induced antitumor immunity without causing autoimmunity. Therefore, HSP105 is an ideal tumor antigen that could be useful for immunotherapy or the prevention of various human tumors that overexpress HSP105, including colorectal cancer and melanoma. (*Cancer Sci* 2005; 96: 695–705)

Colorectal cancer (CRC) and melanoma are common and serious malignancies, for which surgery remains the main treatment, although the success of the treatment depends on the stage of the disease. Although adjuvant systemic chemotherapy or chemoradiation can confer a limited but significant survival advantage, novel and more effective therapies are needed. Identification of tumor associated antigens (TAA) expressed by CRC or melanomas remains one of the goals for designing novel immunological treatments for these tumors. Ideal targets for immunotherapy are gene products that are silenced in normal tissues except immune privilege tissue such as testis tissue, and that are overexpressed in cancer cells.

More than 2000 candidate TAA have been identified by using the serological identification of antigens by recombinant expression cloning (SEREX) method. We have also reported TAA identified by using this method.[1–4] We earlier found that HSP105 (often called HSP110), as identified by SEREX was overexpressed specifically in a variety of human cancers, including colorectal, pancreatic, thyroid, esophageal, and breast carcinoma, but was not expressed in normal tissues except for testis tissue.[1,5] We recently found that HSP105 was also overexpressed in melanoma (unpublished data). If HSP105 can induce strong antitumor immunity, it may be a potential candidate as a target antigen for cancer immunotherapy. In the present study, we set up a preclinical study to investigate the usefulness of a *HSP105*-DNA vaccine, using BALB/c and C57BL/6 mice, the syngeneic endogenously HSP105-expressing CRC cell line Colon26, and the melanoma cell line B16.F10. Using these models, we analyzed both the antitumor effects and side-effects, including autoimmunity of the *HSP105* DNA vaccination.

The pioneering studies of Srivastava and colleagues led to the proposal that several HSP, including HSP70, HSP90 and gp96, bind antigenic peptides and deliver these peptides (through receptor-mediated endocytosis of the HSP) into the antigen-processing pathway of the antigen presenting cell (APC) for presentation on major histocompatibility complex (MHC) class I molecules. This HSP-involved pathway has been demonstrated to evoke potent antiviral and antitumor immune responses.[6] However, many researchers have identified MHC class I-presented peptide epitopes derived from HSP. HSP are

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Abbreviations: C26 (C20), Colon26 clone 20; CRC, colorectal cancer; CTL, cytotoxic T lymphocytes; HE, hematoxylin and eosin; HSP105, heat shock protein 105; APC, antigen presenting cell; mAb, monoclonal antibody; MHC, major histocompatibility complex; SEREX, serological identification of antigens by recombinant expression cloning; TAA, tumor associated antigens.

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rich sources of MHC-bound peptides, and the expression of these peptides increases as a result of cellular stresses.[7]

Recently, Subjeck and colleagues tested a vaccine using the chaperoning properties of HSP110 as Srivastava and colleagues had done before them.[8,9] They reported that HSP110 overexpression increases the immunogenicity of murine CT26 colon tumors.[10] *HSP110* cloned from CHO cells[11] and *HSP105* cloned from mice[12] and humans[13] are homologs. We show here that this HSP105 is highly immunogenic for stimulating tumor immunity against mouse CRC and melanoma. Furthermore, both $CD4^+$ T cells and $CD8^+$ T cells induced by the *HSP105* DNA vaccination play critical roles in the activation of antitumor immunity. These findings indicate that HSP105 itself could be considered a valuable TAA for the immune-based therapy of various tumors overexpressing HSP105, including CRC and melanoma.

Materials and Methods

Cell lines and mice

A subline of the BALB/c-derived CRC cell line Colon26, C26 (C20),[14] was provided by Dr Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Japan). B16.F10 was kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). These cell lines were maintained *in vitro* in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. Female 7-week-old BALB/c mice ($H\text{-}2^d$) and C57BL/6 mice ($H\text{-}2^b$), purchased from Charles River Japan (Yokohama, Japan), were kept in the Center for Animal Resources and Development (CARD) of Kumamoto University, and handled in accordance with the animal care policy of Kumamoto University.

Histological and immunohistochemical analysis

Immunohistochemical detections of HSP105, CD8 and CD4 were carried out as described elsewhere.[1,5,15–18] The primary antibody used in this study, rabbit polyclonal antihuman HSP105 was purchased from Santa Cruz (Santa Cruz, CA, USA). Hematoxylin and eosin (HE) staining and standard methods were used for histological analysis. We purchased Human Normal Organs and Cancer Multi Tissue Slide, BC4, from SuperBioChips Laboratories (Seoul, Korea) for immunohistochemical analysis.

Construction of a mouse *HSP105* expression plasmid DNA

Plasmid pcDNA105, which expresses mouse *HSP105* whole protein was generated as described elsewhere.[12] To construct this plasmid, the mouse *HSP105* full-length cDNA derived from the pB105-1 plasmid was subcloned into *Eco*RV–*Xba*I sites of the mammalian expression vector pcDNA3 (Invitrogen, Osaka, Japan). The pCAGGS expression vector was kindly provided by Dr Junichi Miyazaki (Osaka University, Japan) and this vector induces strong gene expression when injected into muscle.[19] We constructed a pCAGGS-*HSP105* plasmid by inserting mouse *HSP105* cDNA into the *Eco*RI site of the pCAGGS expression vector, which carries the CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter, and prepared the plasmid using a Qiagen EndoFree plasmid Mega kit (Qiagen GmbH, Hilden, Germany). We used the empty pCAGGS plasmid as a control.

DNA vaccination

We immunized mice twice by intramuscular injection into the anterior tibialis muscle. Booster immunization was carried out at 7 days after the primer immunization. The groups of mice were given the following vaccines: (i) saline group: given with 100 μL saline; (ii) control vector group: given 50 μg pCAGGS plasmids lacking inserts and diluted in 100 μL saline; (iii) *HSP105* DNA vaccine group: given 50 μg of pCAGGS-*HSP105* plasmid diluted in 100 μL saline.

In vivo tumor challenge

Subcutaneous tumors were established by the injection of 3×10^4 C26 (C20) cells or 1×10^4 B16.F10 cells suspended in 100 μL Hanks' Balanced Salt Solution (Gibco, Grand Island, NY, USA) medium into the right flank of BALB/c or C57BL/6 mice 7 days after the last vaccination. Tumor incidence and volume were assessed twice weekly using calipers until the mice died. Tumor area was calculated as a product of width and length. The results are presented as mean area of tumor ± SE; however, individual tumor area is presented for some experiments.

In vivo depletion of $CD4^+$ T cells and $CD8^+$ T cells

Each mouse was given a total of six intraperitoneal transfers (days −18, −15, −11, −8, −4, −1) of ascites (0.1 mL per mouse per transfer) from hybridoma-bearing nude mice. The mAbs used were rat antimouse CD4 (clone GK1.5) and rat antimouse CD8 (clone 2.43). Normal rat IgG (Sigma, St. Louis, MO, USA; 200 μg per mouse per transfer) was used as a control. The depletion of T cell subsets by treatment with mAbs was confirmed by flow cytometric analysis of spleen cells, which showed a > 90% specific depletion.

Cell transfer *in vivo*

We purified $CD8^+$ T cells, $CD4^+$ T cells, and natural killer (NK) cells from spleen cells using the magnetic cell sorting system with antimouse CD8α (Ly-2) mAb, antimouse CD4 (L3T4) mAb, antimouse NK (DX5) mAb, and these $CD8^+$ T cells, $CD4^+$ T cells, and NK cells were used for adoptive transfer into BALB/c mice. To investigate tumor growth in a homeostatic lymphocyte proliferation model, we intravenously injected 1.5×10^7 whole spleen cells or 3×10^6 $CD8^+$ T cells, $CD4^+$ T cells, NK cells, or $CD8^-$ $CD4^-$ NK^- cells 3 days after sublethal irradiation (5 Gy). Subsequently, we subcutaneously inoculated BALB/c mice with C26 cells (3×10^4) 3 days after irradiated mice inoculated with cells.

Statistical analysis

We analyzed all data using the StatView statistical program for Macintosh (SAS, Cary, NC, USA) and evaluated statistical significance using the unpaired *t*-test. The overall survival rate was calculated using the Kaplan–Meier method, and statistical significance was evaluated using Wilcoxon's test.

Results

Similar tissue and cancer-specific expression of HSP105 in mice and humans

We have previously reported that HSP105 is overexpressed in a variety of human cancers, including colorectal, pancreatic, esophageal, thyroid, and breast cancer, whereas HSP105 is

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expressed at low levels in many normal tissues, except for testis tissue.[1,5] In the present study, we carried out an immunohistochemical analysis of HSP105 using various human and mouse tissues (Fig. 1). Human HSP105 is overexpressed in almost all CRC cells, melanoma cells (unpublished data), and normal testis tissue, but there is no expression or only a low-level expression of HSP105 in normal liver, brain, spleen, lung, and kidney tissue (Fig. 1a). Mouse HSP105 is also overexpressed in liver metastasis of the murine colorectal adenocarcinoma cell line C26 (C20), lung metastasis of the murine melanoma cell line B16.F10 and normal testis tissue, but there is no expression or only low-level expression in normal liver, cerebrum, cerebellum, spleen, lung, and kidney tissue (Fig. 1b). Another group reported that HSP105/110 is expressed in neurons in the cerebrum and Purkinje cells in the cerebellum,[20] we found the same pattern in the present study, but the level of expression in the neurons and Purkinje cells was much weaker than that in CRC and testis tissue (Fig. 1a,b). As a result, the expression levels of HSP105 protein in human colorectal, pancreatic, esophageal, thyroid, and breast cancers, melanoma, C26 tumors, and B16.F10 tumors were evidently much higher than those in all normal adult tissues, including brain, but not testis in both humans and mice. Because the expression pattern of HSP105 is very similar in humans and mice, we are able to analyze both the antitumor effects and side-effects (including autoimmunity) of HSP105 vaccination using this mouse model of CRC and melanoma.

HSP105 DNA induced rejection of C26 and B16.F10 tumor challenge in mice

We investigated the effects of *HSP105* DNA vaccination using a subcutaneously injected C26 (Fig. 2a–d) and B16.F10 (Fig. 2e–h) tumor model. Mice were divided into three groups: mice inoculated with (i) saline; (ii) pCAGGS, and (iii) pCAGGS-*HSP105*. No mice died during the vaccination period.

Subcutaneous inoculation of C26 cells (3×10^4) into the right flank was given 7 days after the last vaccination (Fig. 2a–d). In groups (i) and (ii), subcutaneous tumors appeared in some mice 10 days after inoculation. Measurement of tumor size was continued until 24 days after inoculation with the tumor cells, when one mouse died. The mean tumor size on day 24 in group (iii) mice (26.4 ± 10.8 mm^2) was significantly smaller than that in the other two groups (105.0 ± 15.7, and 86.0 ± 8.3 mm^2, respectively; $P < 0.05$; Fig. 2a). Six of the 10 mice (60%) in group (iii) did not have subcutaneous tumors on day 24 (Fig. 2b). All mice in groups (i) and (ii) had subcutaneous tumors within 13 days, and died within 41 days of inoculation with the tumor cells (Fig. 2c,d). Five of the 10 mice (50%) in group (iii) completely rejected the 3×10^4 C26 cells during the 108 days after the inoculation (Fig. 2c,d). A statistically significant difference in survival time was found between group (iii) and groups (i) and (ii) ($P < 0.05$).

Subcutaneous inoculation of B16.F10 cells (1×10^4) into the right flank was carried out 7 days after the last vaccination (Fig. 2e–h). Measurement of tumor size was continued until 30 days after inoculation with the tumor cells, when one mouse died. Mean tumor size on day 30 in group (iii) mice (103.9 ± 49.8 mm^2) was significantly smaller than that in the other two groups (272.1 ± 69.7, and 361.6 ± 50.3 mm^2, respectively; $P < 0.05$; Fig. 2e). Six of eight mice (75%) in group (iii) did not have subcutaneous tumors on day 30 (Fig. 2f). All mice in groups (i) and (ii) had subcutaneous tumors within 41 days, and died within 65 days of inoculation with the tumor cells (Fig. 2g,h). Four of eight mice (50%) in group (iii) completely rejected the 1×10^4 B16.F10 cells during the 100 days after the inoculation (Fig. 2g,h). A statistically significant difference in survival time was found between group (iii) and groups (i) and (ii) ($P < 0.05$). Therefore, the *HSP105* DNA vaccine has the potential to prevent the growth of tumors expressing HSP105.

We also subcutaneously inoculated five surviving group (iii) mice that completely rejected the first challenges with C26 cells with further (3×10^4) C26 cells. These mice also rejected the second challenge with C26 cells, even at 108 days after the first challenge (data not shown). These results demonstrate that the effects of vaccination in group (iii) continued for a long time, and that the vaccination prevented the recurrence of HSP105-expressing tumors.

Expression of HSP105 protein and infiltration of CD4$^+$ T cells and CD8$^+$ T cells in the injection sites

To observe HSP105 expression and infiltrating cells in muscles injected with the *HSP105* DNA vaccine, we carried out intramuscular immunizations with pCAGGS DNA into the right anterior tibialis muscle, and with pCAGGS-*HSP105* DNA into the left anterior tibialis muscle of four mice. After 48 h, we killed the mice and evaluated the muscles by histological and immunohistochemical analysis (Fig. 3). In HE-stained sections, there were some transverse sections of injection sites that included many cells in both the pCAGGS- and pCAGGS-*HSP105*-immunized muscles. But only in the transverse sections of the injection sites in pCAGGS-*HSP105*-immunized muscles could we observe many cells expressing HSP105 at a high level, and also a considerable number of both CD4$^+$ T cells and CD8$^+$ T cells. Although we did not immunohistochemically stain the dendritic cells in these traverse sections, we did find some dendritic cell-like large cells.

Infiltration of CD4$^+$ T cells and CD8$^+$ T cells into the C26 tumor after vaccination

To observe the antitumor effects of *HSP105 DNA*-vaccination, we evaluated the tumor using immunohistochemical staining of CD8 and CD4. Figure 4a shows the tumor inoculation sites from two *HSP105* DNA-immunized mice, a saline-inoculated mouse, and a pCAGGS-immunized mouse that did not reject the tumor challenge. There were few lymphocytes in the tumors removed from both the saline-inoculated mouse and the pCAGGS immunized mouse, but there were many CD4$^+$ T cells and considerable numbers of CD8$^+$ T cells making contact with the tumor cells and surrounding the tumors removed from the two *HSP105* DNA-immunized mice. These layers of CD4$^+$ T cells surrounding the tumor were thick in the case of *HSP105 DNA* vaccinated mice. Furthermore, there were a considerable number of CD8$^+$ T cells and CD4$^+$ T cells that had infiltrated into the tumor (Fig. 4a).

Vaccination with *HSP105* DNA did not induce damage of normal tissues

HSP105 expression in normal adult mice is limited to several tissues, and HSP105 expression levels in these tissues are

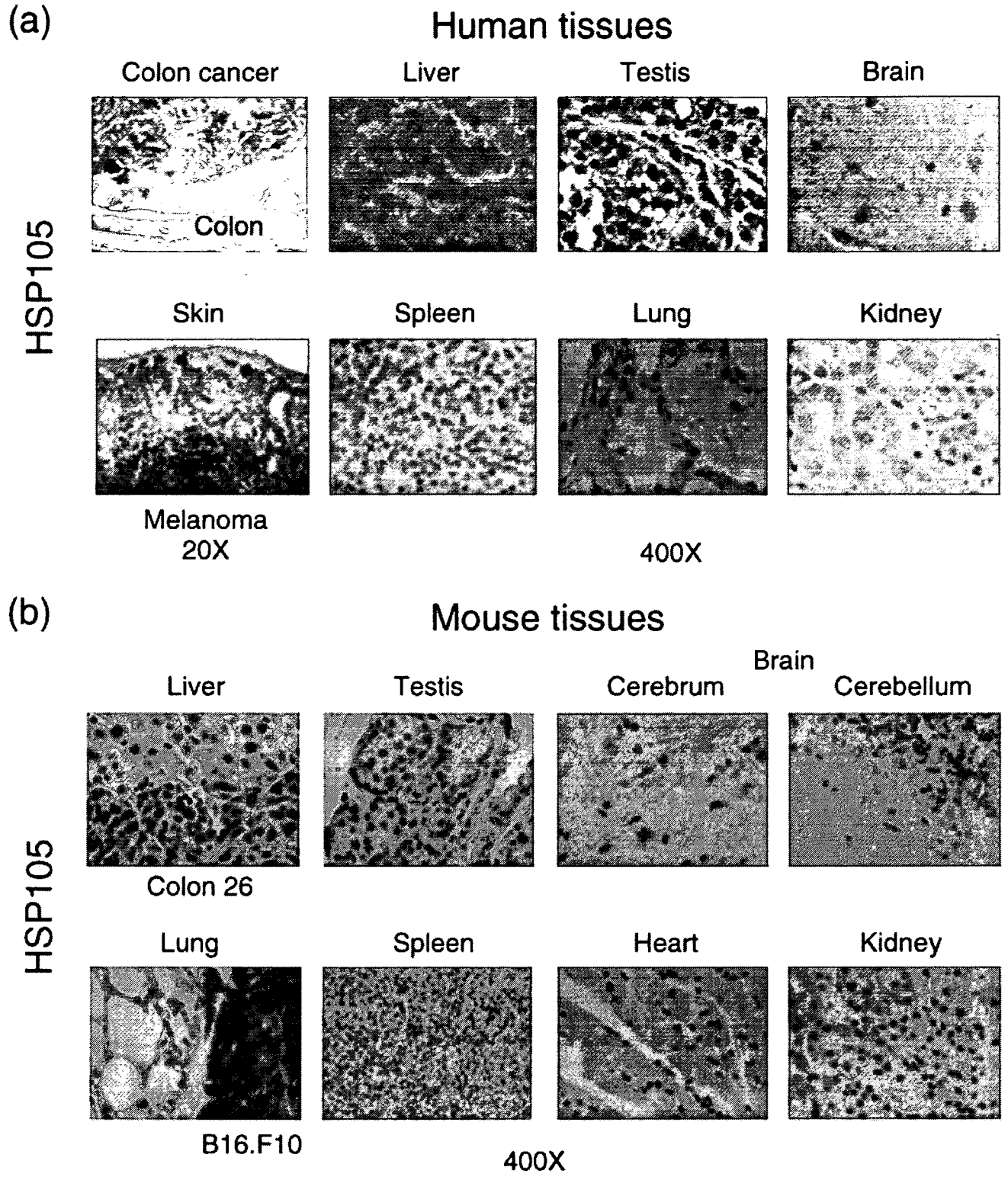

Fig. 1. Expression of the HSP105 protein, a candidate for immunotherapy for CRC and melanoma, in human and mouse tissues and cells. Expression of HSP105 protein detected by immunohistochemical analysis in various (a) human and (b) mouse tissues. Objective magnification was 400× or 20×.

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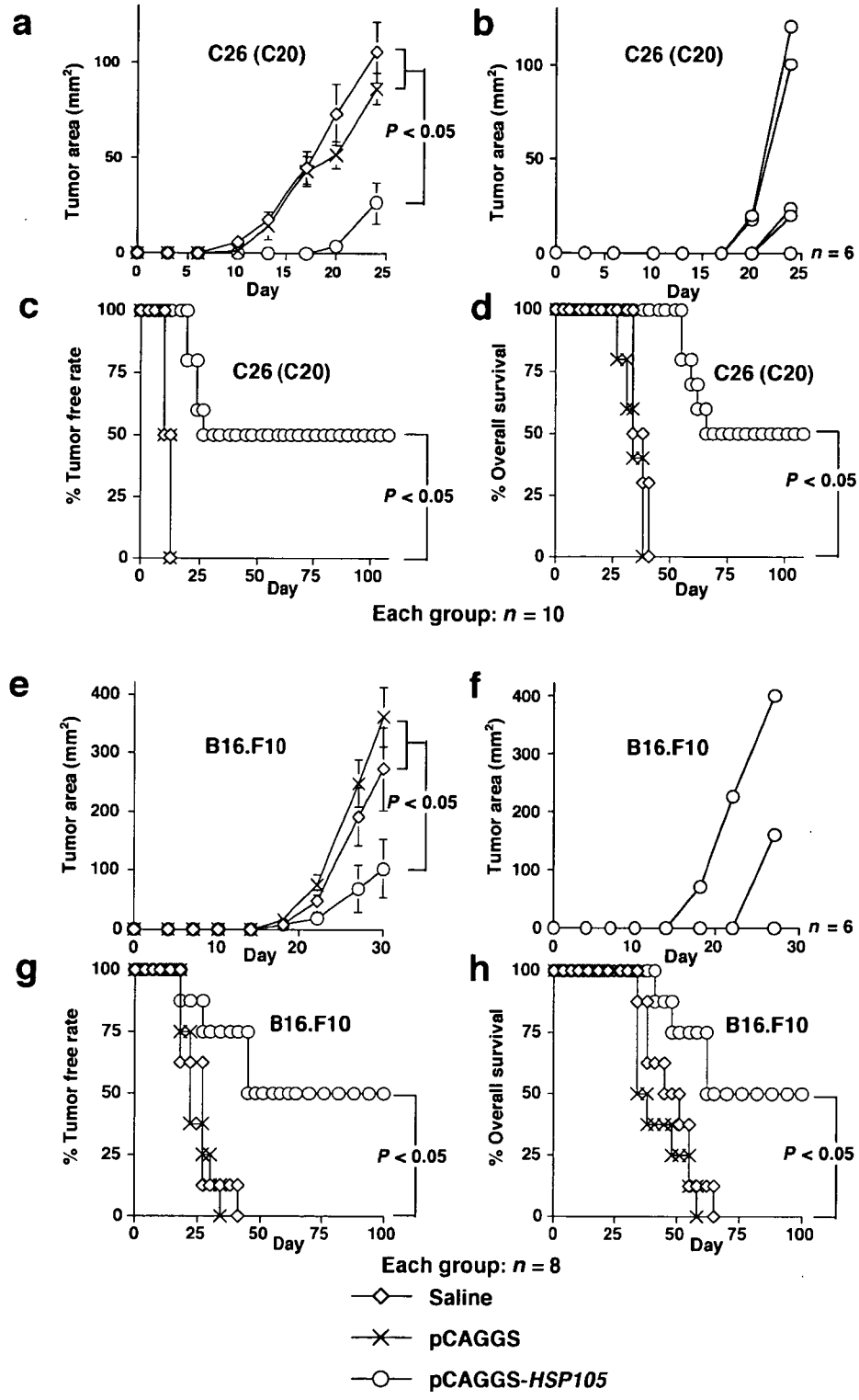

Fig. 2. Vaccination with *HSP105* DNA suppressed the growth of (a–d) C26 and (e–h) B16.F10 tumors in mice. Each group consisted of 10 (a–d) or eight (e–h) mice. (a,b,e,f) Suppression of the growth of HSP105-expressing C26 (a,b) or B16.F10 (e,f) tumors inoculated subcutaneously into mice vaccinated with *HSP105* DNA. The tumor area was calculated as the product of width and length. The result is presented as mean area of tumor ± SE, and we evaluated statistical significance using the unpaired *t*-test (a,e). Growth curves of 10 and eight individual tumors in the mouse group treated with pCAGGS-*HSP105* are presented in (b) and (f), respectively. (c,d,g,h) Percentage tumor free rate (c,g) and percentage overall survival (d,h) were calculated using the Kaplan–Meier method, and the statistical significance of differences between groups was evaluated using Wilcoxon's test.