Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma

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(Received November 21, 2008/Revised April 10, 2009/Accepted April 19, 2009/Online publication June 2, 2009)

The relationship between overexpression of glypican (GPC)-3 that is specific for hepatocellular carcinoma (HCC) and the prognosis has not yet been clarified. We attempted to determine the expression profile of GPC3 in association with the clinicopathological factors by immunohistochemical analysis in HCC patients and investigated the potential prognostic value of GPC3 by comparing the survival rate between the GPC3-positive and GPC3-negative HCC patients. Primary HCC tissue samples (n = 107) obtained from patients who had undergone hepatectomy between 2000 and 2001 were analyzed. GPC3 expression was less frequently observed in well-differentiated HCC than in moderately and poorly differentiated HCC, the difference in the frequency being statistically significant. GPC3-positive HCC patients had a significantly lower 5-year survival rate than the GPC3-negative HCC patients (54.5 vs 87.7%, P = 0.031). Among 80 of the 107 (74.6%) patients with initial treatment who underwent hepatectomy, none of GPC3-negative HCC patients (n = 16, 20.0%) died during the follow-up period. No deaths were noted in the GPC3-negative HCC patients among the 71 (88.7%) patients with moderately and poorly differentiated HCC. Multivariate analysis identified GPC3 expression (P = 0.034) as an independent prognostic factor for the overall survival. We showed that GPC3 expression is correlated with a poor prognosis in HCC patients. (*Cancer Sci* 2009; 100: 1403–1407)

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is ranked as the third most common cause of cancer-related death worldwide. HCC is generally associated with a poor prognosis, the 5-year survival rate after surgery has been reported to be as low as 25–39%, and systemic therapy with cytotoxic agents provides only marginal benefit.[1] Even in those patients in whom the tumor has been successfully removed, the 2-year recurrence rate can be as high as 50%.[2,3] Several clinicopathological factors including poor levels of differentiation of the cancer cells, large size of the tumor, portal venous invasion, and intrahepatic metastasis have been shown to contribute to the poor prognosis in patients of HCC. Despite the critical need for better methods for the diagnosis and treatment of HCC, the mechanisms underlying the development of HCC remain unclear.

Glypican (GPC)-3 was discovered as a potential serological and histochemical marker that is specific for HCC. GPC3 is a member of the glypican family and belongs to a group of heparan sulfate proteoglycans bound to the outer surface of the cell membrane through a glycosylphosphatidylinositol anchor.[4] In mammals, this family comprises six members, GPC1 to GPC6. GPC are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors, and bone morphogenetic proteins.[5–9] Depending on the context, their functions exerted may either be stimulatory or inhibitory through these pathways. GPC3 has been detected in the placenta and fetal liver, but not in other adult organs. During hepatic carcinogenesis, GPC3 appears in the HCC tissue and is released into the serum.[10–12] In addition, its expression has also been reported in melanoma.[13–15]

A dramatic elevation of GPC3 expression has been reported in a large proportion of HCC, as determined by cDNA microarray analysis, whereas its expression has been shown to be less frequent in preneoplastic or entirely absent in non-neoplastic liver tissue.[16–18] This has led to the notion that GPC3 may have diagnostic usefulness as a marker of differentiation or a specific tumor marker in the case of HCC. However, until now, the relationship between GPC3 overexpression and the prognosis of HCC has not been clarified.

In the present study, we attempted to determine the tumor expression profile of GPC3 in association with clinicopathological factors in HCC patients by immunohistochemical analysis. We also investigated the potential prognostic value of GPC3 by analyzing the survival rate of GPC3-positive and GPC3-negative HCC patients. By elucidating the association between the GPC3 expression level in HCC tumors and the survival rate of the patients, we concluded that the GPC3 expression level is correlated with a poor prognosis in HCC patients.

Materials and Methods

Patients and tumor tissue samples. Primary HCC tissue samples (n = 107) were obtained from patients who underwent hepatectomy at the National Cancer Center Hospital East between 2000 and 2001. The histological types were assigned according to the criteria of the World Health Organization classification. Liver tissue sections prepared from the surgically resected tumors and adjacent parenchyma fixed in 10% formalin and embedded in paraffin were retrieved from the files of the Department of Pathology at our institution.

Immunohistochemical staining. Sections 6 μm thick were prepared from the paraffin-embedded blocks. The sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 20 min. For antigen retrieval, sections were heated in 10 mM citrate buffer (pH 6.0) with microwave at 95°C for 15 min. The slides were then allowed to cool down, and the prediluted primary monoclonal anti-GPC3 antibody (dilution 1 : 300; Biomosaics, Burlington, VT, USA) was added to cover each slide, and the slides were incubated for 2 h at room temperature. Thereafter, the slides were washed three times in TBS–Tween 20 for 5 min each. Mouse Envision Polymer-horseradish

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doi: 10.1111/j.1349-7006.2009.01206.x

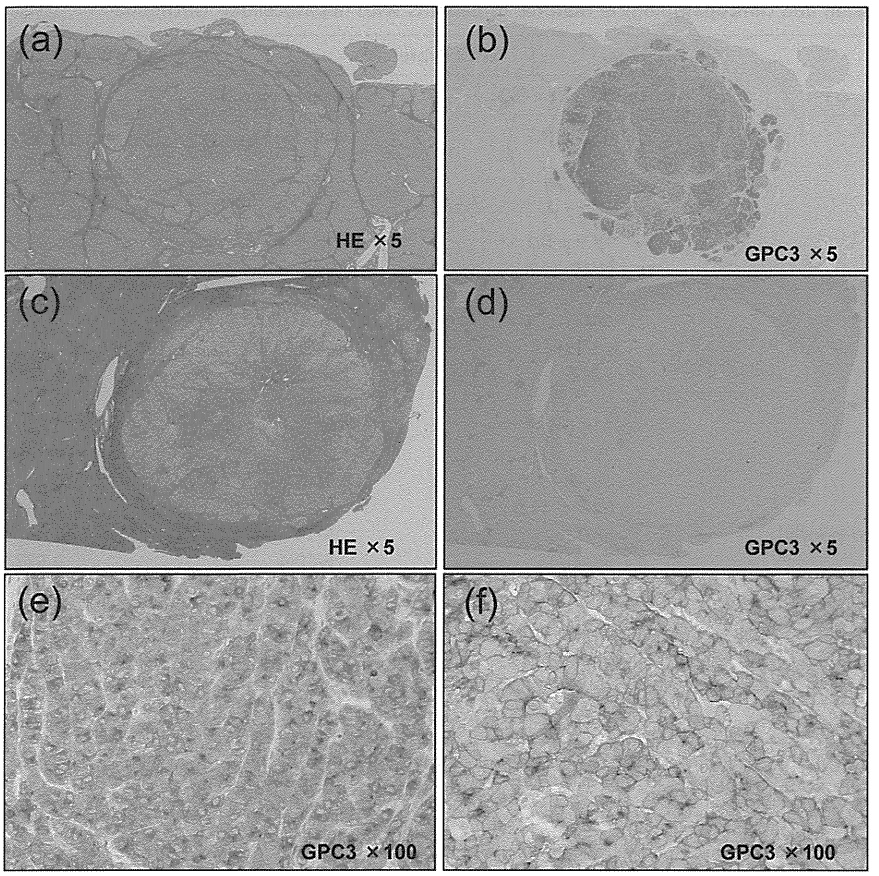

Fig. 1. Glypican (GPC)-3 expression and localization is hepatocellular carcinoma (HCC)-specific. (a,c) Microscopic view of a HE-stained sections of resected HCC. (b,d) HCC sections were stained for GPC3 expression with anti-GPC3 monoclonal antibody. (e) HCC displays prominent bile-canalicular immunostaining. (f) Membranous and cytoplasmic staining of liver tumor cells are shown.

peroxidase (DakoCytomation, Carpenteria, CA, USA), was used as the secondary antibody for 30 min at room temperature followed by three washes in TBS–Tween 20 for 5 min each. Finally, the visualization signal was developed by the addition of 3,3-diaminobenzidine tetrahydrochloride (DakoCytomation) to each slide, followed by incubation for 2 min. Slides were then washed in distilled water, counterstained with hematoxylin, and dehydrated.

For the immunohistochemical analysis of GPC3, we evaluated only the area of GPC3-positive staining in one slide in each patient, including the HCC lesion and adjacent non-cancerous lesion. At first, to analyze GPC3 expression, the results of immunohistochemical staining were classified according to the area of GPC3-positive staining cells as follows: –, negative (<10%); +/–, weakly positive (10–30%); and +, positive (>30%). Finally, in this study, we classified two groups between GPC3-negative (<10%) and GPC3-positive (>10%). The expression of GPC3 was judged to be positive when the percentage of immunoreactive cells was semiquantitatively assessed as being ≥10% in focal lesions. The slides were examined independently by two observers (H. Shirakawa and T. Nakatsura) and then collectively by a pathologist (M. Kojima).

Analysis of the correlation of GPC3 expression with various clinicopathological factors. The correlation of GPC3 expression with various clinicopathological factors was analyzed. Overall survival was calculated from the date of surgery to the date of death.

Statistical analysis. The differences in the level of GPC3 expression were tested by the χ^2-test and the means of each subgroup were compared using Student's *t*-test. Survival analyses were carried out according to the Kaplan–Meier method and the differences were assessed using the log-rank test. Follow-up time was censored if the patient was lost to follow up. Cox proportional-hazards analysis was used for univariate and multivariate analyses to explore the effects of the variables on survival. *P*-values of less than 0.05 were considered to be significant.

Results

Glypican-3 expression in HCC. In order to characterize the expression of GPC3 in HCC, 107 surgical specimens were analyzed immunohistochemically. The mean and median follow-up period were 3.4 ± 2.0 years and 3.5 years respectively. GPC3 expression was detected in 87 of the surgically resected tumor specimens (81.3%) (Fig. 1a,b), but not in the remaining 20 specimens (18.7%) (Fig. 1c,d). In most of the GPC3-positive cases, the protein expression was localized mainly in the cellular cytoplasm (Fig. 1e) with some amount detected on the cell membrane (Fig. 1f). The results of the immunohistochemical analysis were evaluated in relation to the pathological findings and follow-up data. There was no correlation between GPC3 expression and any of the clinicopathological features, except that the GPC3 expression increased with increasing degree of dedifferentiation of the cancer cells (Table 1). GPC3 expression was less frequently observed in well-differentiated HCC than in moderately or poorly differentiated HCC; the difference in frequency was statistically significant. Thus, an increase in GPC3 expression was correlated with increasing aggressiveness of the cancer cells, which was accompanied by dedifferentiation of the cells.

Correlation between GPC3 expression and patient survival. In order to determine the prognostic value of GPC3, the overall survival was compared between GPC3-positive and GPC3-negative HCC patients. The GPC3-positive HCC patients had a significantly lower 5-year survival rate than the GPC3-negative HCC patients (54.5 vs 87.7%, $P = 0.031$; Fig. 2a). After surgery,

doi: 10.1111/j.1349-7006.2009.01206.x

Table 1. Correlation between glypican (GPC)-3 expression and clinicopathological features of patients with hepatocellular carcinoma

Variable	GPC3 expression		*P*-value
	Positive (n = 87)	Negative (n = 20)	
Age (years) (mean ± SD)	63.6 ± 9.7	60.2 ± 11.8	0.169
Sex (male/female)	67/20	18/2	0.321
HBsAg status (positive/negative)	26/61	3/17	0.283
HCV status (positive/negative)	50/37	12/8	0.999
ICG R15 (%) (mean ± SD)	15.9 ± 8.1	15.5 ± 7.6	0.823
AFP (ng/mL) (mean)	6710	463	0.198
PIVKA-II (mAU/mL) (mean)	7370	5900	0.823
Tumor occurring (primary/recurrence)	64/23	16/4	0.753
Number of tumor (solitary/multiple)	64/23	11/9	0.172
Resection procedure (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	22/65	7/13	0.378
Operation time (min.) (mean ± SD)	310 ± 165	263 ± 119	0.248
Intraperative blood loss (mL) (mean)	2910	1500	0.356
Perioperative transfusion (present/absent)	45/42	9/11	0.767
Tumor size (mm) (mean ± SD)	54.7 ± 41.9	53.0 ± 31.2	0.861
Histological tumor differentiation (well/moderately and poorly)	6/81	6/14	0.032
pStage (UICC) (I/II/III)	35/41/11	6/10/4	0.577
Portal vein involvement (present/absent)	39/48	8/12	0.885
Hepatic vein involvement (present/absent)	9/78	1/19	0.750
Bile duct involvement (present/absent)	11/76	1/19	0.557
Intrahepatic metastasis (present/absent)	18/69	6/14	0.545
Non cancerous tissue (cirrhosis/non-cirrhosis)	36/51	4/16	0.075
Postoperative recurrence (present/absent)	70/17	16/4	0.963

AFP, alpha-fetoprotein; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; UICC, International Union against Cancer.

HCC recurrence was observed in 86 (80.4%) of the 107 patients. In the majority (97.7%) of patients with recurrence, the recurrence was observed in the residual liver. Among these 86 patients, 43 (50%) and seven (8.1%) developed multinodular and extrahepatic recurrence respectively. Although no correlations were observed between these recurrence patterns and GPC3 expression, GPC3 can only be used as an indicator of poor overall survival in HCC patients.

Among 80 of the 107 (74.6%) patients with initial treatment who underwent hepatectomy, none of the GPC3-negative HCC patients (n = 16, 20.0%) died during the follow-up period (Fig. 2b). The mean and median follow-up periods were 3.7 ± 2.1 and 3.7 years respectively. The 1-, 3-, and 5-year survival rates of the GPC3-positive HCC group were 84.4, 62.5, and 32.8% respectively. With regard to the tumor grade of HCC, 9 (11.3%) of the 80 patients with well-differentiated tumors showed significantly better prognosis without any record of deaths, compared with 71 (88.7%) patients with moderately and poorly differentiated HCC (Fig. 2c).

Further, among the 71 initial treatment patients who underwent hepatectomy and were found on histopathological examination to have moderately and poorly differentiated HCC, there were no deaths during the follow-up period in the GPC3-negative HCC group (Fig. 2d). The mean and median follow-up periods were 3.6 ± 2.0 and 3.6 years respectively.

Univariate and multivariate analyses to identify the prognostic variables in HCC patients. To identify the variables of potential prognostic significance in all the patients with HCC, univariate analysis of each variable was carried out in relation to the survival time. The difference in the prognosis was assessed by examining the relative hazard and *P*-value for each variable. The relative importance of each variable was then determined by multivariate Cox proportional hazards model analysis. Univariate analysis with stepwise inclusion of variables in the model revealed that the significant prognostic factors were GPC3 expression status, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, indocyanine green-retention at 15 min (ICG-R15), serum protein induced by vitamin K absence II (PIVKA-II), tumor occurence, number of tumors, resection volume, pathological bile duct involvement, and pathological intrahepatic metastasis (Table 2). However, the multivariate analysis identified only GPC3 expression (P = 0.034), intrahepatic metastasis (P = 0.027), and multiple tumors (P = 0.006) as the independent prognostic factors related to overall survival (Table 2).

Discussion

In this study, we characterized the association between the expression level of GPC3 and the malignancy grade, and the prognostic value of GPC3 in HCC. Higher levels of GPC3 expression were observed in moderately or poorly differentiated tumor cells, which was in agreement with previous reports.[19] Our contingency table analysis showed that the GPC3 expression level was correlated with the tumor differentiation level. In addition, Kaplan–Meier survival analysis revealed that GPC3 expression was significantly linked to a poor prognosis after surgical resection in HCC patients. Moreover, univariate analysis indicated that GPC3 expression is associated with an increased risk of death from HCC, and this risk factor could still be extracted in a multivariate setting. On the other hand, multivariate analysis did not identify the tumor differentiation level as an independent predictive factor of the prognosis. Among the 80 HCC patients who underwent initial surgical treatment, the GPC3-negative patients showed better prognosis than the GPC3-positive patients. Patients with well-differentiated HCC also showed a better prognosis than those with moderately and poorly differentiated HCC. Furthermore, we confirmed that among the previously treated subjects, the GPC3-negative group had a better prognosis than the GPC3-positive group with moderately and poorly differentiated HCC tumors.

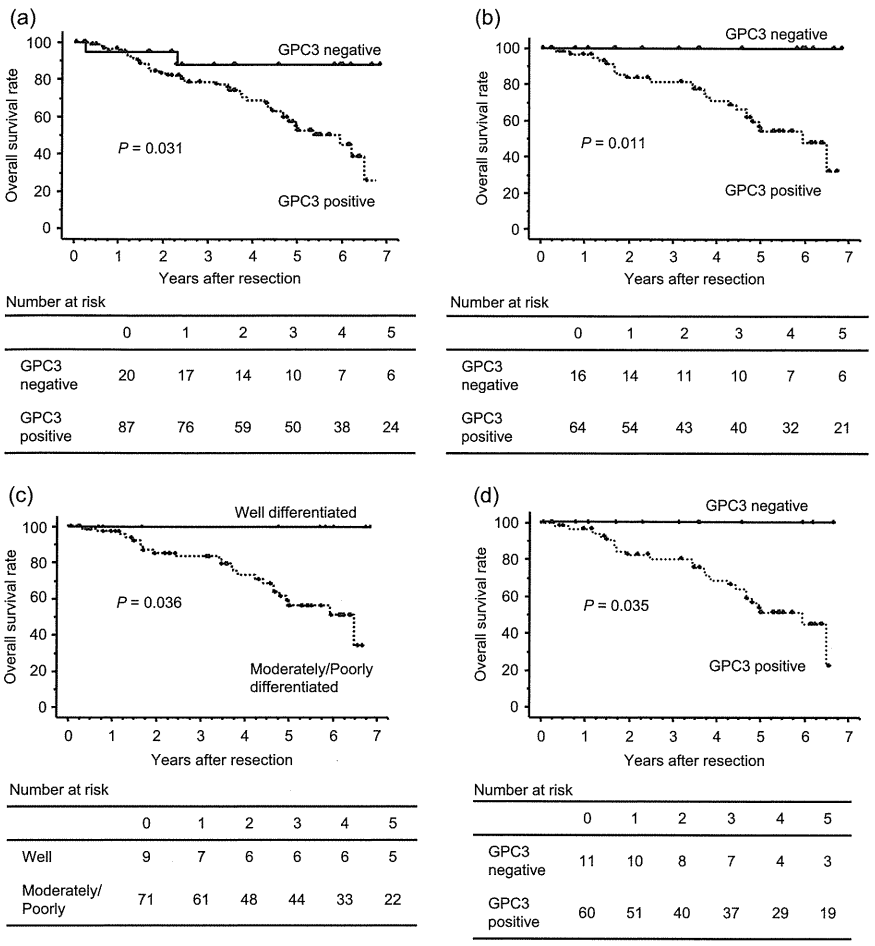

Fig. 2. Overall survival curves for the 107 hepatocellular carcinoma (HCC) patients stratified into those with glypican (GPC)-3-positive and GPC3-negative HCC. (a) Overall survival of patients with GPC3-positive HCC was shorter than those with GPC3-negative HCC ($P = 0.031$). (b) Overall survival curves in 80 of 107 HCC patients with initial treatment who underwent hepatectomy with positive and negative GPC3 expression. Patients with GPC3-positive HCC had a lower 5-year survival than those with GPC3-negative HCC ($P = 0.011$). (c) Overall survival curves in the 71 HCC patients with initial hepatectomy who exhibited well- and moderately and poorly differentiated HCC on histopathological examination. The 5-year survival rate was lower in the moderately and poorly differentiated GPC3-positive HCC than in the corresponding GPC3-negative HCC ($P = 0.036$). (d) Overall survival curves in the 71 initial treatment patients who underwent hepatectomy and exhibited moderately and poorly differentiated HCC on pathological examination with positive and negative GPC3 expression. The 5-year survival rate was lower in the GPC3-positive HCC patients than in the GPC3-negative HCC patients ($P = 0.035$).

Table 2. Prognostic factors for overall survival by univariate and multivariate analyses

Variable	No. patients	Univariate analysis		Multivariate analysis		
		5-year survival rate (%)	*P*-value	RR	95% CI	*P*-value
Age (years) (≥65/<65)	51/56	65.8/53.4	0.531			
Sex (male vs female)	85/22	56.1/72.7	0.403			
HBsAg (positive vs negative)	29/78	51.0/62.3	0.011	1.14	0.31–4.16	0.844
HCV (positive vs negative)	62/45	66.7/46.4	0.004	2.41	0.75–7.69	0.138
ICG R15 (%) (≥15 vs <15)	50/57	70.3/46.8	0.047	0.69	0.31–1.54	0.362
AFP (ng/mL) (≥50 vs <50)	45/62	49.1/65.1	0.132			
PIVKA-II (mAU/mL) (≥700 vs <700)	30/77	35.0/65.6	0.016	1.91	0.730–5.02	0.188
Tumor occurring (first vs recurrence)	80/27	62.8/50.2	0.019	1.83	0.78–4.31	0.167
No. tumors (solitary vs multiple)	75/32	65.7/42.7	0.009	3.53	1.41–8.00	0.006
Resection (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	29/78	36.5/67.1	0.005	1.71	0.52–5.60	0.374
Operation time (min) (>300 vs ≤300)	49/58	43.9/72.3	0.053			
Intraoperative blood loss (mL) (≥1300 vs <1300)	42/65	42.3/68.8	0.097			
Perioperative transfusion (present vs absent)	54/53	49.6/66.5	0.599			
Tumor size (mm) (>50 vs ≤50)	38/69	51.5/62.5	0.154			
Histological differentiation (well vs moderately and poorly)	12/95	77.8/56.4	0.102			
pStage (I vs II/III)	41/66	64.2/56.5	0.071			
Portal vein involvement (present vs absent)	47/60	64.9/58.5	0.369			
Hepatic vein involvement (present vs absent)	10/97	44.4/60.5	0.060			
Bile duct involvement (present vs absent)	12/95	20.0/62.7	0.004	0.94	0.31–2.85	0.912
Intrahepatic metastasis (present vs absent)	24/83	29.0/66.6	0.001	3.57	1.13–10.50	0.027
Non-cancerous lesion (cirrhosis vs non-cirrhosis)	40/67	53.6/61.9	0.232			
GPC3 staining (positive vs negative)	87/20	54.5/87.7	0.025	5.26	1.13–24.39	0.034

AFP, alpha-fetoprotein; CI, confidence interval; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; RR, relative risk; UICC, International Union against Cancer.

doi: 10.1111/j.1349-7006.2009.01206.x

In this study, the patients who were HCV positive, had higher ICG-R15 values, or portal vein involvement showed longer survival times, especially the patients who were HCV-positive or had higher ICG-R15 values, showed statistical significance in the univariate analysis. However, there was no statistical significance in these variables in the multivariate analysis. The reasons for these contradictive results in the univariate analysis are unclear.

In contrast, subgroup analysis did not reveal any significant difference in the disease-free survival rate between the GPC3-positive and GPC3-negative HCC patients (data not shown). The rate of recurrence in patients after surgery was 63.8% within the first 2 years after surgery among the previously treated patients in this study. Tumor recurrence in the GPC3-positive HCC patients occurred earlier than that in the GPC3-negative HCC patients until 9.7 months after the surgery among the patients who had received previous treatment. Two mechanisms of postoperative recurrence of HCC have been suggested: one is intrahepatic metastasis in the residual liver in a metachronous manner, and the other is multicentric hepatocarcinogenesis based on chronic hepatitis.[20–23] Some authors have suggested that early recurrence arises most often from intrahepatic metastases, whereas late recurrence is more likely to be multicentric in origin. Poon *et al.* and Portolani *et al.* reported that tumor factors like neoplastic vascular infiltration, but not host factors, were linked to early recurrence, whereas the risk of late recurrence was dependent on the underlying liver status.[21,22] These results indicate that GPC3 expression may indicate a high risk of intrahepatic recurrence.

Most of the GPC3 expression patterns in HCC cells showed the cytoplasmic pattern. There was no case that showed only the membrane pattern. Almost half of the HCC cases showed the mixed pattern (cytoplasm and membrane) and the other half showed only the cytoplasmic pattern.

There was no statistical significance between the mixed pattern (cytoplasm and membrane) and cytoplasmic pattern ($P = 0.297$) in Kaplan–Meier survival analysis. The functional difference between cytoplasmic GPC3 and membrane GPC3 is unknown, so further investigations are needed to clarify whether the different localization of staining has a different significance.

In addition to the investigation of its role as a prognostic indicator, a phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC is now underway; GPC3 is an ideal target for this therapy because it is more effective in patients with increased expression of GPC3, which is frequently observed in the later stages of HCC, as shown in the present study. The poor prognosis of patients with GPC3-positive HCC also prompted us to develop a strategy of anticancer immunotherapy,[24,25] that is, we may expect the effect of hepatocarcinogenesis prevention after surgery in patients with GPC3-positive HCC.

In summary, our study evaluated the prognostic significance of GPC3 expression at the protein level in clinical tissue specimens of HCC. The overall survival rate was significantly poorer in patients with elevated GPC3 expression in the tumor than in those with lower levels of GPC3 expression. Further functional characterization of GPC3 may be expected to lead to a better understanding of the molecular mechanisms underlying the development and progression of HCC.

Acknowledgments

This work was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan, a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare, Japan, and awardee of research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive 10-Year Strategy for Cancer Control (H.S.).

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Detection of glypican-3-specific CTLs in chronic hepatitis and liver cirrhosis

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Received February 10, 2009; Accepted March 26, 2009

DOI: 10.3892/or_00000418

Abstract. Glypican-3 (GPC3) is one of carcinoembryonic antigens known to be overexpressed in hepatocellular carcinoma (HCC). It has been suggested that GPC3 may be related to the development of HCC in a background of chronic hepatitis (CH) and liver cirrhosis (LC). Therefore, in an attempt to establish an early diagnostic marker of HCC, we quantified the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients. We selected CH and LC patients who were HCV-RNA (+) or HBs antigen (+) within 6 months prior to the study and had no HCC nodules as detected by imaging. A total of 56 patients with CH and LC, and 45 patients with HLA-A24$^+$ or HLA-A2$^+$ were enrolled for this investigation. After isolation of mononuclear cells from each patient's peripheral blood specimens, we performed ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides. In the ELISPOT assay, GPC3-specific CTLs were detected in 10 of the 45 CH and LC cases (22%). In addition, the plasma titers of anti-GPC3 IgG were increased in the CH and LC patients as compared with those in healthy donors. GPC3-specific CTLs were found to be present not only in patients with HCC, but also in patients with CH and LC. This suggests the possibility of GPC3-specific CTLs serving as a marker for the early diagnosis of imaging-invisible HCC.

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Abbreviations: GPC3, glypican-3; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

Key words: glypican-3, CTL, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma

Introduction

The prevalence of hepatocellular carcinoma (HCC) is increasing rapidly in both Asian and Western countries. It is clear that patients with hepatitis B- or C-associated liver cirrhosis are at a higher risk of developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at a higher risk of recurrence (2). Furthermore, the liver function of these patients is often very poor, which restricts further treatment options for recurrence. As a result, the prognosis of HCC remains poor, and the development of new therapies for the prevention of cancer development and recurrence, that is, adjuvant therapy, is urgently needed.

Glypican-3 (GPC3) has been reported to be overexpressed in most types of HCC (3-10) and melanoma in humans (6,8,9). GPC3 belongs to the six-member family of glypicans in mammals (11). GPC3 is a heparan sulfate proteoglycan that is bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor. GPC3 has been shown to regulate the signaling mediated by Wnts (12,13), Hedgehogs (14), fibroblast growth factors (15,16) and bone morphogenetic proteins (15,17). These signaling pathways are only partially dependent on the heparan sulfate chains (11,16,18). However, whether GPC3 plays an oncogenic role in HCC is still controversial.

We recently identified both HLA-A24 (A*2402) and H-2K^d-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL) and HLA-A2 (A*0201)-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We previously reported a preclinical study conducted in a mouse model with a view to designing an optimal schedule for clinical trials of a GPC3-derived peptide vaccine (20). We predicted that overexpression of GPC3 in HCC is related to the development of HCC in a background of chronic hepatitis (CH) and/or liver cirrhosis (LC). Towards establishing the possibility of early diagnosis of imaging-invisible HCC and vaccine therapy, we determined the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients.

Materials and methods

Patients, blood samples and cell lines. Blood samples from patients with CH and LC were collected during routine diagnostic procedures after obtaining their written consent at the Tokyo Rosai Hospital between October 2006 and October 2007. CH and LC patients who were confirmed to be HCV-RNA(+) or HBs antigen(+) within six months prior to registration were eligible for the study. The diagnosis of CH or LC was made clinically by imaging and laboratory data. The patients had no medical history of HCC, and no evidence of HCC on ultrasonography, CT (computed tomography) or MRI (magnetic resonance imaging) conducted prior to the registration.

Human liver cancer cell lines SK-Hep-1/GPC3, HepG2 and K562 were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS. SK-Hep-1/GPC3 has been described previously (19). HepG2 endogenously expressing GPC3 was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). HLA-class I deficient K562 was obtained from Kumamoto University. The origins and HLA genotypes of these cell lines have been described in previous reports (21,22).

Ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay. We isolated peripheral blood mononuclear cells (PBMCs) from the heparinized blood of HLA-A2$^+$ and/or HLA-A24$^+$ Japanese CH, LC or HCC patients and healthy donors by means of Ficoll-Conray density gradient centrifugation. IFN-γ production by the CTLs present in the PBMCs in the presence or absence of the GPC3 peptide was assessed by the ELISPOT assay (BD™ Bioscience, San Diego, CA), as described previously. Briefly, defrosted PBMCs (1x10^6/well) were cultured in 96-well flat-bottomed plates for the ELISPOT assay (BD Bioscience) with HLA-A2-restricted $GPC3_{44-52}$ (A2-1) (RLQPGLKWV), $GPC3_{144-152}$ (A2-3) (FVGEFFTDV), $GPC3_{155-163}$ (A2-4) (YILGSDINV) and HLA-A24-restricted $GPC3_{298-306}$ (A24-8) (EYILSLEEL) (10 μM) with 100 units/ml recombinant human IL-2 overnight *in vitro*. The negative control consisted of medium alone and the positive control included HLA-A24- or -A2-restricted cytomegalovirus. The number and area of the spots were automatically determined and subsequently analyzed with the ELISPOT system (Minerva Tech, Tokyo, Japan).

Induction of GPC3-reactive human CTLs and cytotoxic assay. We evaluated the cytotoxic activity of the CTLs that were induced with the GPC3 A2-3 peptide in the PBMCs isolated from the CH4 patient. PBMCs were isolated from HLA-A2$^+$ CH4 patient, distributed into 4 wells (3x10^5 cells/24-well), and cultured with the GPC3 A2-3 peptide. After culture for 7 and 14 days, the PBMCs cocultured with irradiated autologous monocyte-derived DCs obtained by positive selection with human CD14 Micro Beads (Miltenyi, Bergisch Gladbach, Germany) were pulsed with the GPC3 A2-3 peptide. The CD14$^+$ cells were cultured in the presence of 100 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Inc.) and 100 ng/ml of IL-4 (R&D Systems, Inc.) in RPMI-1640 (Sigma-Aldrich Corp., St. Louis, MO) containing 2% heat-inactivated autologous serum and 1% penicillin-streptomycin-glutamine (Gibco, Invitrogen, Ltd.; Paisley, Scotland, UK). After 5 days, TNFα (PEPRPTECH EC., London, UK) was added at the concentration of 20 ng/ml to induce maturation of the DCs. After 7 days, mature DCs were harvested and pulsed with 10 μM of the candidate peptides for 4 h at room temperature in RPMI. The peptide-pulsed DCs were then irradiated (3500 rads) and mixed at a ratio of 1:20 with autologous PBMCs.

These DCs were set up in 48-well culture plates; each well contained 1.5x10^4 peptide-pulsed DCs, 3x10^5 PBMCs and 5 ng/ml IL-7 (PEPRPTECH EC.) in 0.5 ml of RPMI containing 10% autologous serum. Three days after the start of the incubation, IL-2 (R&D Systems, Inc.) was added to these cultures at a final concentration of 10 U/ml. On days 7 and 14, the T cells were restimulated with the autologous DCs pulsed with the peptide.

After 21 days, the cells were recovered and analyzed for their cytotoxic activity against the target cells with the TERASCAN VPC system (Minerva Tech), as previously described (23). Briefly, SK-Hep-1/GPC3 (GPC3$^+$, A2$^+$, A24$^+$), HepG2 (GPC3$^+$, A2$^+$, A24$^+$) and K562 (HLA-class I$^-$) cells were used as the target cells and labeled with calcein-AM solution for 30 min at 37°C. The labeled cells were washed three times and distributed into a 96-well culture plate (1x10^4 per well) and then incubated with the effector cells for 5 h. The fluorescence intensity was measured before and after 5-h culture, and the Ag-specific cytotoxic activity was calculated using the following formula: cytotoxicity (%) = [(sample release) - (spontaneous release)]/[(maximum release) - (spontaneous release)] x 100.

ELISA for the detection of anti-GPC3 IgG antibodies. Recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) was diluted in 10 x Block Ace (Dainippon Pharmaceutical, Osaka) to a final concentration of 1 μg/ml, dispensed into 96-well plates (100 μl/well) and incubated overnight at 4°C. Then, the plates were blocked with Block Ace for 1 h at room temperature. Plasma samples from CH and LC patients and healthy controls (100 μl, 1:100 dilution) were added to each well, followed by incubation for 2 h at room temperature. After washing three times with PBS containing 0.05% Tween-20 (PBST), Peroxidase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratories, Inc., W. Baltimore, USA) was reacted for 30 min. The plates were washed with PBST and developed with Stable Peroxide Substrate Buffer (Pierce, Rockford, IL) for 20 min. After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 490 nm. All plasma samples were measured in duplicate and were randomly dispensed into the plates.

Statistical analysis. The two-tailed Student's t-test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay. Unpaired Mann-Whitney U tests were used for the evaluation of the significance of differences in the data obtained by ELISA. $P<0.05$ was considered to denote significant difference.

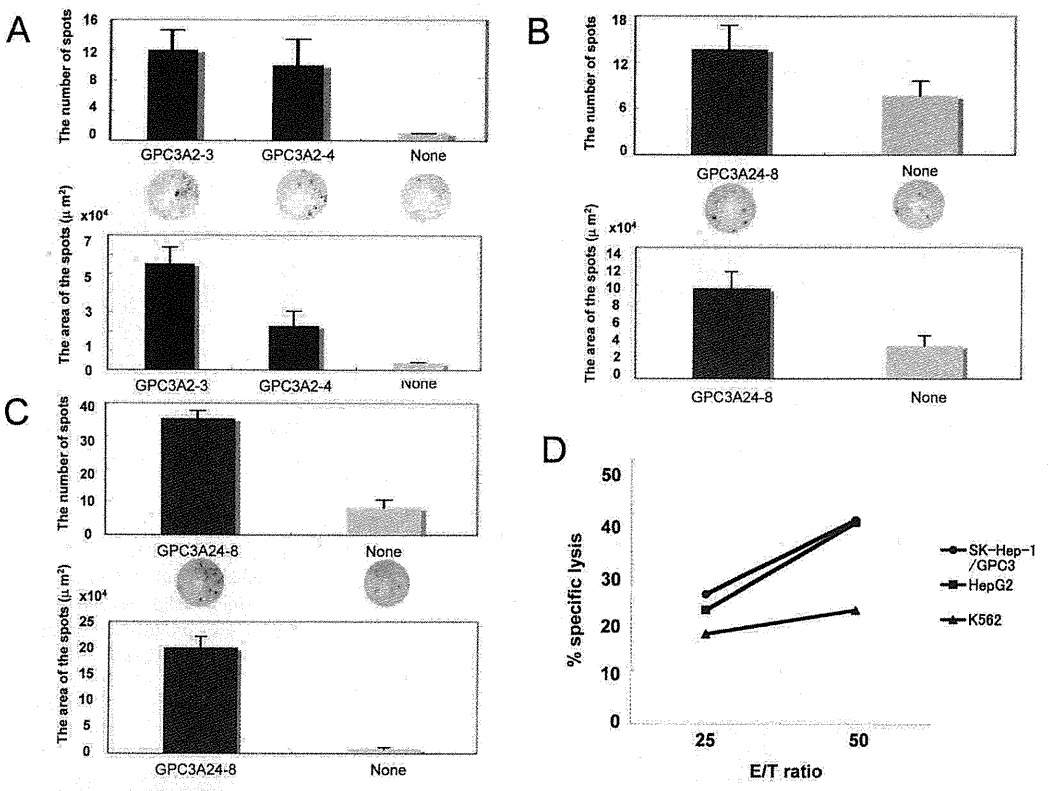

Figure 1. Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH and LC patients and the cytotoxicity of the CTLs induced by stimulation with the GPC3 (A2-3) peptide. GPC3-specific CD8+ T cells were detected in the chronic hepatitis [(A), HLA-A2+ CH4 patient; (B), HLA-A24+ CH5 patient] and liver cirrhosis [(C), HLA-A24+ LC5 patient]. IFN-γ produced by the peptide-specific T cells was measured by the IFN-γ-ELISPOT assay (middle column). The number and area of spots are shown in the upper and lower panels, respectively. Lysis of human hepatoma cell lines SK-Hep-1/GPC3 (circles) and HepG2 (squares) expressing GPC3 and HLA-A2 by GPC3-specific CTLs was observed following stimulation with the GPC3 A2-3 peptide (FVGEFFTDV) [(D), HLA-A2+ CH4 patient]. An HLA-classI- K562 human erythromyeloblastoid leukemia cell line was used as the negative control (triangles).

Results

Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH, LC and HCC patients. We evaluated the frequency of CTLs that recognized the GPC3 A2-1, A2-3, A2-4 or A24-8 peptide in the PBMCs of CH, LC and HCC patients. The CH and LC patients enrolled in this study were 34 male and 22 female patients. The average age of the patients was 64 years. HCV and HBV infection was found in 54 and 2 patients, respectively. The 56 patients were 33 CH and 23 LC cases. Mean serum α-fetoprotein (AFP) was 13.3±21.1 ng/ml (normal <20 ng/ml). In regard to the HLA genotype, 10, 22 and 13 patients, respectively, were HLA-A2+, HLA-A24+ and HLA-A2+/24+. On the other hand, there were 11 patients who were HLA-A2-/A24-. In this investigation, we enrolled the 45 patients who were HLA-A2+ or HLA-A24+.

We determined the presence of CTLs in the PBMCs of the CH and LC patients by ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides (Fig. 1, Table I). The representative data of the ELISPOT assay are highlighted. Interestingly, in the CH4 patient, the spots and areas were highly developed in the GPC3 A2-3 and A2-4 peptide-stimulated PBMCs (Fig. 1A). However, few spots and areas were detected in the negative control (no peptide). In addition, GPC3 A24-8 peptide-restricted CTLs were also detected in the CH5 and LC5 patients (Fig. 1B and C). These results suggest that GPC3-specific CTLs are present in the PBMCs of some of CH and LC patients.

Cytotoxicity of CTLs induced by stimulation with the GPC3 (A2-3) peptide. To clarify the cytotoxic activity of GPC3-specific CTLs induced by stimulation with the GPC3 peptide, the HCC cell line, SK-Hep-1/GPC3, transfected with GPC3 and expressing HLA-A2 and HLA-A24 were used as the target cells (Fig. 1D). The CTLs induced from the PBMCs of CH4 (Table I) patient by stimulation with the GPC3 A2-3 peptide showed specific cytotoxicity against the SK-Hep-1/GPC3 and HepG2 cells. On the other hand, no GPC3-specific cytotoxicity was observed against the HLA-classI- K562 cells. These results indicate that GPC3-peptide-specific CTLs induced from CH4 (Table I) patient are cytotoxic against the GPC3-expressing target HCC cells.

Frequency of HLA-A2+ or HLA-A24+ CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in PBMC The frequency of patients with GPC3-specific CTLs in their PBMCs is shown in Fig. 2, while the clinical backgrounds of the CH, LC and HCC patients are summarized in Table II. CTL positivity was observed in 5 of 26 CH patients (19%), 5 of 19 LC patients (26%), and 21 of 54 HCC patients (39%). In addition, the percentage of CTL-positive patients tended to

Table I. Detection of GPC3-specific CTLs in the PBMCs of chronic hepatitis/liver cirrhosis patients by ELISPOT assay.

	Peptide/Peptide sequence									
	GPC3 A2-1/RLQPGLKWV		GPC3 A2-3/FVGEFFTDV		GPC3 A2-4/YILGSDINV		GPC3 A24-8/RYILSLEEL		No peptide	
	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)
CH[a]1(A*0201)	**1.0±0.0**[c]	**25905.0±8487.8**	**2.0±1.0**	**2826.0±3079.5**	**1.6±1.1**	**13895.0±4486.8**	NT	NT	0.0±0.0	0.0±0.0
CH2 (A*0201)	**1.0±1.7**	**707.0±1223.6**	**1.6±1.1**	**6830.0±6934.2**	**2.6±1.1**	**3297.0±3263.1**	NT	NT	0.0±0.0	0.0±0.0
CH3 (A*0201)	NT[d]	NT	**18.3±5.5**	**85100.0±17050.1**	**15.6±2.5**	**20173.0±4728.4**	NT	NT	8.0±1.7	8045.0±1849.1
CH4 (A*0201)	NT	NT	**12.0±2.6**	**55187.0±8618.4**	**10.0±3.4**	**22832.0±7632.2**	NT	NT	1.0±0.0	3853.0±375.2
CH5 (A*2402)	NT	NT	NT	NT	NT	NT	**13.3±3.7**	**101736.0±54505.9**	7.0±1.0	36502.5±14892.4
LC[b]1 (A*0201)	**1.0±0.0**	**1060.0±815.7**	**2.1±0.2**	**2944.0±815.7**	**6.3±0.5**	**50162.0±4283.0**	NT	NT	0.5±0.0	354.0±0.0
LC2 (A*0201)	**24.0±3.0**	**55891.2±23304.1**	**8.0±2.0**	**45971.9±25440.5**	**8.0±1.0**	**103961.4±13618.6**	NT	NT	4.3±0.5	2098.3±2166.5
LC3 (A*0201)	**1.3±0.5**	**2355.0±2855.2**	**3.6±1.5**	**8007.0±6564.4**	**11.3±5.7**	**100323.0±70946.1**	NT	NT	2.0±3.4	2826.0±4894.7
LC4 (A*2402)	NT	NT	NT	NT	NT	NT	**14.0±8.0**	**41331.0±31472.6**	3.0±0.0	7065.0±3996.5
LC5 (A*2402)	NT	NT	NT	NT	NT	NT	**35.3±2.3**	**200882.0±21210.9**	8.3±2.3	8714.0±2855.5

[a]CH, chronic hepatitis. [b]LC, liver cirrhosis. [c]We show values higher than the value for 'No peptide' by a bold font. [d]NT, not tested.

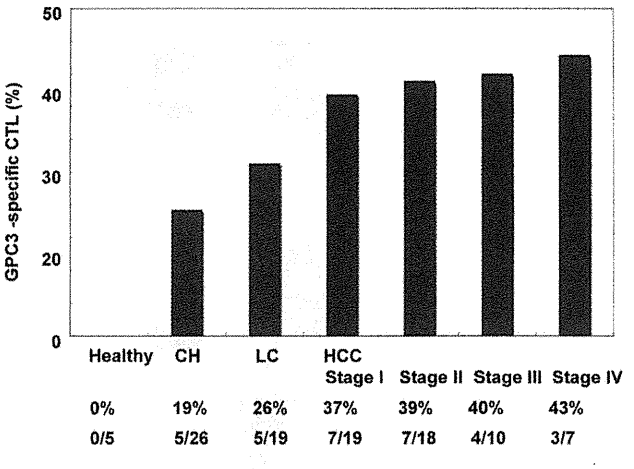

Figure 2. Frequency of HLA-A2+ or HLA-A24+ CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in the PBMCs. GPC3-peptide-specific CTLs were detected in 19 and 26% of the patients with CH and LC, respectively. In the HCC patients, the percentage of these CTLs tended to increase with increasing stage of progression of the disease: 37% (stage I), 39% (stage II), 40% (stage III) and 43% (stage IV).

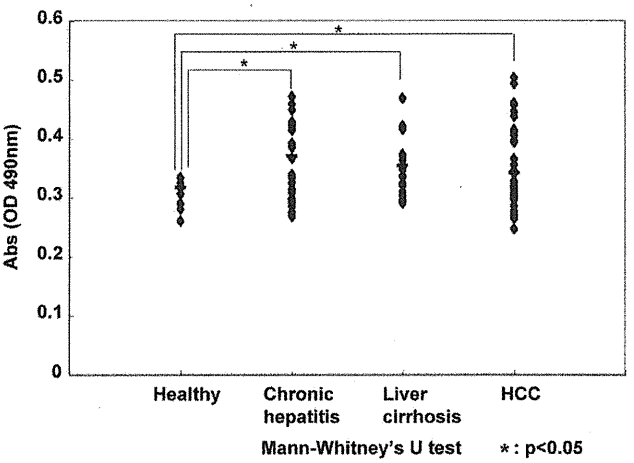

Figure 3. Plasma titers of anti-GPC3 IgG in the CH, LC and HCC patients. Anti-GPC3 IgG was detected by ELISA using recombinant GPC3 protein. A significantly higher titer of IgG to GPC3 was observed in the CH ($p<0.05$), LC ($p<0.05$) and HCC patients ($p<0.05$) as compared with that in healthy donors. *$p<0.05$ (Mann-Whitney U test).

increase with increasing clinical stage of HCC; stage I (7/19, 37%), stage II (7/18, 39%), stage III (4/10, 40%), and stage IV (3/7, 43%) (Table II). There were no CTL-positive cases (0/5, 0%) in healthy donors.

Anti-GPC3 IgG in the plasma in patients with CH, LC and HCC. To examine the quantitative titers of anti-GPC3 IgG in the plasma of patients with CH, LC and HCC, we carried out ELISA using the recombinant GPC3 protein (Fig. 3). The titers in the CH, LC and HCC patients were significantly higher as compared with the peak titer in healthy controls. These results indicate that the GPC3 antigen is expressed not only in HCC patients, but also in CH and LC patients.

Table II. Number of CTL-negative and -positive cases in chronic hepatitis, liver cirrhosis and HCC patients.

	Healthy (n=5)		Chronic hepatitis (n=33)		Liver cirrhosis (n=23)		HCC (n=54)	
Group	Negative (n=5) mean (±SD)	Positive (n=0) mean (±SD)	Negative (n=28) mean (±SD)	Positive (n=5) mean (±SD)	Negative (n=19) mean (±SD)	Positive (n=5) mean (±SD)	Negative (n=33) mean (±SD)	Positive (n=21) mean (±SD)
Age	31.2±7.1	-	61.6±11.2	60.6±12.9	67.3±10.1	71.0±2.7	65.8±7.9	64.0±10.5
Male	4	0	16	3	12	3	28	15
Female	1	0	12	2	6	2	5	6
HCV/HBV								
+/-	ND	ND	5	26	18	5	18	14
-/+	ND	ND	2	0	0	0	4	2
+/+	ND	ND	0	0	0	0	2	2
-/-	ND	ND	0	0	0	0	9	3
AFP (ng/ml)	ND	ND	9.5±18.9	9.6±7.3	21.2±25.4	8.8±7.7	26335.1±143782.5	1431.5±3574.9
HLA-								
$A02^+$	3	0	3	3	2	2	13	8
$A24^+$	2	0	12	1	7	2	18	11
$A02^+/24^+$	0	0	6	1	5	1	2	2
$A02^-/24^-$	0	0	7	0	4	0	0	0

Discussion

The oncofetal antigen GPC3 is known to be overexpressed in HCCs (3-10) and melanomas (6,8,9). We recently identified GPC3-specific peptides restricted to HLA-A24 (A^*2402) and $H\text{-}2K^d$, or HLA-A2 (A^*0201), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We are currently conducting a phase I clinical trial of peptide vaccine prepared using these peptides against advanced HCC. In addition, in the near future, we propose to carry out a phase II clinical trial of the vaccine in HCC patients as well as CH and LC patients to evaluate its efficacy in preventing the onset of HCC. We report the finding of GPC3-specific CTLs in CH and LC patients for the first time in this study. Furthermore, the plasma titers of anti-GPC3 IgG in the CH and LC patients were also found to be significantly increased as compared with those in healthy donors.

It has been suggested that GPC3-specific CTLs may be derived from clinically invisible pre-neoplastic or neoplastic nodular lesions. In previous studies, expression of GPC3 was reported in 2/23 (8%) cirrhotic low-grade dysplastic nodules, and 2/9 (22%) (24), 2/22 (9%) (25) or 6/31 (19%) high-grade dysplastic nodules (26). In one study, among 5 adenomas with malignant characteristics, 3 (60%) showed immunoreactivity for GPC3 in the malignant regions (24). Other studies reported positive staining for GPC3 in 12/20 (60%) (24) and 22/32 (69%) cases (25) of early HCC. Meanwhile, the serum titers of the elevated GPC3 antigen in HCC cases were reported to be correlated with the clinical stage of HCC (19). In our study, we noted an increase of the plasma titers of anti-GPC3 IgG antibody in CH, LC and HCC patients. In addition, the frequency of patients with GPC3-specific CTLs appeared to increase with the stage of progression of the liver disease. These results suggest that GPC3 expression and the appearance of GPC3-specific CTLs may be prediagnostic markers of HCC.

On the other hand, the increase in the frequency of GPC3-specific CTLs and titers of anti-GPC3 IgG in the peripheral blood might be related to the expression of GPC3 in CH with high grade inflammation and LC. In this study, we did not perform immunohistochemical examination for GPC3, because needle biopsy of the liver in our patients was not conducted in our collaborative clinic. Previous studies have demonstrated GPC3 expression by immunohistochemistry in 25/30 (83%) cases of CH with high grade inflammation (27) and 11/95 (12%) cases of LC (26), indicating that GPC3 might be expressed in CH with high-grade inflammation and some LC patients, resulting in the appearance of GPC3-specific CTLs in the PBMCs of these patients.

During the 1-year follow-up of this study, onset of HCC was not observed in any of the 10 CH and LC patients who were positive for GPC3-specific CTLs in the peripheral blood; on the other hand, 2 (1CH and 1LC) patients who were negative for GPC3-specific CTLs showed development of HCC. It would, therefore, seem that the GPC3-specific CTLs might prevent the development of HCC or be predictive of a favorable prognosis of non-neoplastic liver lesions. However, our examination was limited to only HLA-A24- and A2-positive patients, and moreover, we followed up the patients for only one year. Therefore, careful long-term observation of a larger number of CH and LC cases is necessary to determine the role of GPC3-specific CTLs in patients with CH and LC.

In this study, we demonstrated an increase of GPC3-specific CTLs and high titers of anti-GPC3 IgG in CH and LC patients. Thus, GPC3-specific CTLs and anti-GPC3 IgG

may possibly be markers of early imaging-invisible HCC. In addition, active immunotherapy using GPC3 peptides may prevent the development of both non-neoplastic and neoplastic lesions of the liver.

Acknowledgements

We thank Ms. Chinatsu Kojima for her help with the preparation of this manuscript. This study was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, the Foundation for Promotion of Cancer Research in Japan, the Japan Research Foundation for Clinical Pharmacology, and a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan (E.H.).

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Multiple Antigen-targeted Immunotherapy With α-Galactosylceramide–loaded and Genetically Engineered Dendritic Cells Derived From Embryonic Stem Cells

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Summary: Numerous tumor-associated antigens (TAA) have been identified and their use in immunotherapy is considered to be promising. For TAA-based immunotherapy to be broadly applied as standard anticancer medicine, methods for active immunization should be improved. In the present study, we demonstrated the efficacy of multiple TAA-targeted dendritic cell (DC) vaccines and also the additive effects of loading α-galactosylceramide to DC using mouse melanoma models. On the basis of previously established methods to generate DC from mouse embryonic stem cells (ES-DC), 4 kinds of genetically modified ES-DC, which expressed the melanoma-associated antigens, glypican-3, secreted protein acidic and rich in cysteine, tyrosinase-related protein-2, or gp100 were generated. Anticancer effects elicited by immunization with the ES-DC were assessed in preventive and also therapeutic settings in the models of peritoneal dissemination and spontaneous metastasis to lymph node and lung. The in vivo transfer of a mixture of 3 kinds of TAA-expressing ES-DC protected the recipient mice from melanoma cells more effectively than the transfer of ES-DC expressing single TAA, thus demonstrating the advantage of multiple as compared with single TAA-targeted immunotherapy. Loading ES-DC with α-galactosylceramide further enhanced the anticancer effects, suggesting that excellent synergic effects of TAA-specific cytotoxic T lymphocytes and natural killer T cells against metastatic melanoma can be achieved by using genetically modified ES-DC. With the aid of advancing technologies related to pluripotent stem cells, induced pluripotent stem cells, and ES cells, clinical application of DC highly potent in eliciting anticancer immunity will be realized in the near future.

Key Words: cancer immunotherapy, dendritic cells, embryonic stem cells, tumor-associated antigen, α-GalCer

(*J Immunother* 2009;00:000–000)

Received for publication August 9, 2008; accepted November 2, 2008.
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Financial Disclosure: All of authors have declared there are no financial conflicts of interest related to this work.
Supported in part by Grants-in-Aid 18014023, 19591172, and 19059012 from the Ministry of Education, Science, Technology, Sports, and Culture (MEXT), Japan; the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases launched as a project commissioned by MEXT, Japan; Research Grant for Intractable Diseases from Ministry of Health and Welfare, Japan and Uehara Memorial Foundation; and Takeda Science Foundation.
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A number of preclinical studies have demonstrated the efficacy of cancer vaccines using mouse models. However, cancer vaccine trials in humans have yet to demonstrate a sufficient clinical response,[1,2] and at present tumor-associated antigens (TAA)-specific cancer immunotherapies are not regarded as the standard medical technology, except for several antibody therapies. Therefore, there is discrepancy between the promising results obtained in mouse studies and those of clinical trials. Although the reasons for this discrepancy vary, one is the improper selection of mouse models.[3] Most mouse studies use models where the tumor cells are inoculated subcutaneously or intravenously. These are convenient to observe the efficacy of immunotherapy. However, to evaluate the efficacy in clinical medicine, experimental systems should be used that reflect the clinical situations where immunotherapy is actually needed, such as cancers accompanying with multiple metastases or with peritoneally disseminated lesions. The present study evaluated the capacity of genetically engineered dendritic cells (DC) to inhibit peritoneal dissemination and spontaneous metastasis of mouse melanoma to lymph node and lungs.

Another issue to be considered is whether it is appropriate to design clinical strategies that combine multiple agents to modulate the immune response.[2] Clinically manifested cancers are often associated with multiple mechanisms to evade immune attacks, such as antigen loss, active tolerance induction, deficiency in antigen presentation machineries,[4] etc., which are difficult to be addressed successfully with a single agent. To overcome this defense mechanism and to address the low frequency of cytotoxic T lymphocytes (CTL) that recognize single endogenous TAA, the efficacy of multiple TAA-targeted immunotherapies was examined in the present study. DC were generated expressing endogenous TAA, glypican-3 (GPC3), secreted protein acidic and rich in cysteine (SPARC), tyrosinase-related protein-2 (TRP2), and gp100. The oncofetal protein GPC3, glycosylphosphatidylinositol anchored membrane protein, is specifically overexpressed in human melanoma and hepatocellular carcinoma, and GPC3 can be a candidate target for cancer immunotherapy.[5–7] Clinical trials with GPC3 peptide against hepatocellular carcinoma are now ongoing. SPARC, also called osteonectin, is a matricellular glycoprotein that modulates cellular interactions with the extracellular matrix during tissue remodeling.[8] SPARC or its combination with GPC3 is a useful tumor maker for melanoma.[9,10]

The manipulation of DC, specialized antigen-presenting cells, is a promising strategy to improve the efficacy of cancer immunotherapy.[11,12] Genetic modification to express antigenic proteins has several advantages in comparison with using a peptide, protein, or tumor cell lysate as a means for loading TAA to DC.[13] The expression of TAA by DC circumvents the need for identifying specific CTL epitopes within the protein, and the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[14] Furthermore, in vivo transfer of DC transfected with TAA gene are able to prime CTL reactive to multiple TAA-derived epitopes.[15] Others and we have established to generate DC in vitro from mouse embryonic stem (ES) cells (ES-DC).[16,17] ES-DC have the capacity to stimulate T cells, present antigen, and migrate to lymphoid tissues upon in vivo administration, and their capacity is comparable with that of bone marrow-derived DC.[15,17–19] The genetic modification of ES-DC can be carried out without the use of viral vectors by introducing exogenous genes into undifferentiated ES cells by electroporation and the subsequent induction of their differentiation into ES-DC. In a previous study, ES-DC expressing GPC3 showed protective immunity against mouse melanoma, however, the therapeutic effects were insufficient.[15] To counter the cancers with deficiencies in antigen presentation machineries, it is necessary to induce the innate immunity. Alpha-galactosylceramide (α-GalCer) presented by DC efficiently stimulates natural killer T (NKT) cells.[20] Therefore, it is presumed that the in vivo transfer of DC simultaneously loaded with TAA and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, resulting in a potent anticancer immunity. The present study investigated the anticancer effects of multiple TAA-targeted immunotherapies with α-GalCer–loaded and genetically engineered ES-DC against mouse melanoma.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. All studies were performed with C57BL/6 mice syngeneic to the mouse ES cell line B6 at 6 to 8 weeks of age. The mouse experiments were approved by the Animal Research Committee of Kumamoto University.

Cell Lines

The ES cell line B6, derived from C57BL/6 blastocysts, was kindly provided by Drs H. Suemori and N. Nakatsuji (Kyoto University). The method for in vitro induction of differentiation of ES cells into DC was described previously,[17] and ES-DC prepared from a 7-day culture in the presence of granulocyte-macrophage colony-stimulating factor were used for all assays. The C57BL/6-derived tumor cell lines, F10 and BL6 sublines of B16 melanoma, a fibrosarcoma cell line MCA205 (MCA), and a thymoma cell line EL-4 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). The cells were cultured in Roswell Park Memorial Institute-1640 supplemented with 10% horse serum. To produce GPC3-expressing MCA (MCA-GPC3), MCA cells were transfected with pCAGGS-GPC3-internal ribosomal entry site (IRES)–puromycin-resistant (puro-R) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), selected with puromycin, and then subjected to cloning by limiting dilution as described previously.[15,21] To produce EL-4–expressing secreted protein acidic and rich in cysteine (EL-4–SPARC), EL-4 cells were transfected with pCAGGS-SPARC-IRES–puro-R same as above. Plasmid DNA encoding firefly luciferase was kindly provided by Dr M. Nishikawa (Kyoto University), and B16-BL6 cells were transfected with the construct as described above, then the single colonies of G418-resistant cells were picked up, and a clone was selected on the basis of the luciferase activity (B16-BL6/Luc).

Peptides and Cytokines

Known $CD8^+$ T-cell epitope peptides were purchased from AnyGen (Gwangju, Korea) and their amino acid sequences are as follows: mouse $TRP2_{180\text{-}188}$ (SVYDFFVWL, H-$2K^b$ restricted), mouse $gp100_{25\text{-}33}$ (EGSRNQDWL, H-$2D^b$ restricted), and human $gp100_{25\text{-}33}$ (KVPRNQDWL, H-$2D^b$ restricted). A control peptide derived from $OVA_{257\text{-}264}$ (SIINFEKL, H-$2K^b$ restricted) was synthesized by the automatic peptide synthesizer (PSSM8, SHIMADZU, Kyoto, Japan) and subsequently purified by high-performance liquid chromatography. Recombinant mouse granulocyte-macrophage colony-stimulating factor, recombinant human interleukin (IL)-2, recombinant murine interferon (IFN)-γ (PeproTechLondon, UK), and IL-4 (ProSpecTechnoGene, Rehovot, Israel) were purchased. Alpha-GalCer was kindly provided by Kirin Brewery Co (Tokyo, Japan).

Generation of ES-DC expressing GPC3, SPARC, TRP2, or Human gp100

cDNA fragments encoding for total mouse SPARC and TRP2 were obtained by reverse transcription polymerase chain reaction (RT-PCR) from B16-F10. Full-length mouse GPC3 and human gp100 (hgp100) cDNA clones were purchased from Invitrogen. cDNA fragments encoding the whole GPC3 protein and a fragment of hgp100 ($hgp100_{1\text{-}300}$) including the H-$2D^b$–restricted epitope ($hgp100_{25\text{-}33}$) were isolated from those clones. cDNA for GPC3, SPARC, TRP2, or hgp100 was transferred to a mammalian expression vector pCAGGS-IRES–puro-R, containing the CAG promoter and an IRES–puro-R gene cassette,[22,23] to generate an expression vector for GPC3, SPARC, TRP2 and hgp100, pCAGGS-GPC3-IRES–puro-R, pCAGGS-SPARC-FLAG-HA-IRES–puro-R, pCAGGS-TRP2-HA-IRES–puro-R, and pCAGGS-hgp100-HA-IRES–puro-R. All constructs contain HA-tag or FLAG-tag except for that of GPC3 (Fig. 1A). To generate transfectant ES cell clones, B6 ES cells were transfected with the expression vectors by electroporation and selected with puromycin as described previously.[17] Transfectant ES cell clones were subjected to a differentiation culture to generate ES-DC as described previously.[24,25] ES-DC differentiated from GPC3-transfectant, SPARC-transfectant, TRP2-transfectant, or hgp100-transfectant ES cells were designated as ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, or ES-DC–hgp100, respectively. Recombinant mouse IL-4 was given to ES-DC at 20 hours before in vivo transfer.

RT-PCR

Total cellular RNA was extracted by using the RNeasy Mini Kit (QIAGEN, Maryland, MD) and RT-PCR was carried out as previously described.[24] Briefly, total RNA was converted into cDNA and PCR was carried out for 30 cycles for the quantification of *GPC3*, *SPARC*, *TRP2*, *hgp100*, and glyceraldehyde-3-phosphate dehydrogenase

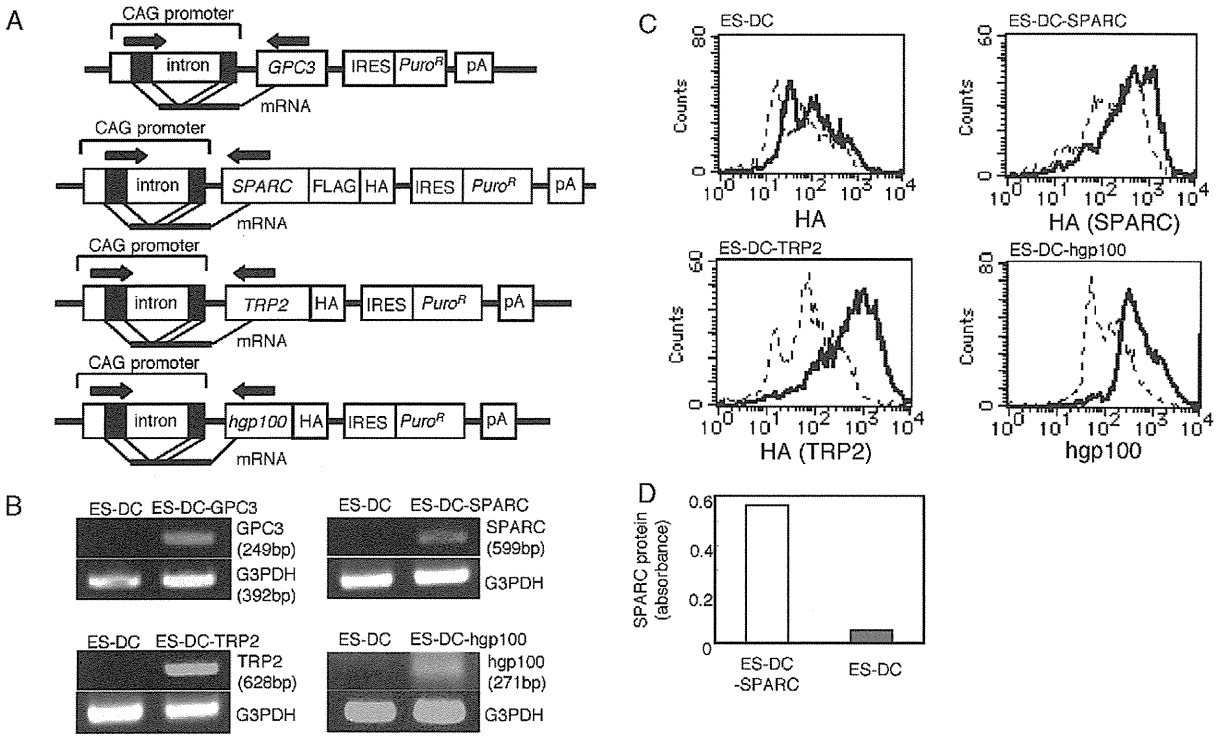

FIGURE 1. The generation of ES-DC expressing melanoma-associated antigens. A, Structure of vectors: pCAGGS-GPC3-IRES–puro-R, pCAGGS-SPARC-FLAG-HA-IRES–puro-R, pCAGGS-TRP2-HA-IRES–puro-R, and pCAGGS-hgp100-HA-IRES–puro-R. To obtain the vectors, each cDNA fragment encoding for a full-length of mouse *GPC3*, *SPARC*, *TRP2*, or $hgp100_{1\text{-}300}$ was inserted into a mammalian expression vector, pCAGGS-IRES–puro-R containing the CAG promoter, and an IRES–puro-R gene cassette. All constructs contain HA-tag or FLAG-tag except for that of GPC3. B, Expression of *GPC3*, *SPARC*, *TRP2*, and *hgp100* mRNA detected by RT-PCR in transfectant ES-DC. Primer sets (arrows in A) were designed to span the intron (917 bp) in the CAG promoter sequence to distinguish the PCR products of mRNA origin from the genome-integrated vector DNA origin. C, The expression of the transfected protein in ES-DC. Transfectant ES-DC were analyzed by flow cytometric analysis using intracellular staining. The staining patterns of specific antibodies (thick line) and negative control (dotted line) are shown. Anti-HA antibodies were used for staining of ES-DC, ES-DC–SPARC, and ES-DC–TRP2 and anti-hgp100 antibodies were used for staining of ES-DC–hgp100. D, SPARC protein secreted from ES-DC–SPARC or ES-DC (negative control) was measured by enzyme-linked immunosorbent assay. ES-DC indicates embryonic stem cell-derived dendritic cells; GPC3, glypican-3; hgp100, human gp100; IRES, internal ribosomal entry site; puro-R, puromycin-resistant; RT-PCR, reverse transcription polymerase chain reaction; SPARC, secreted protein acidic and rich in cysteine; TRP2, tyrosinase-related protein-2.

mRNA. The primer sequences for detection of the transgene in ES-DC and endogenous genes expressed in tumor cells are shown in Table 1. The sense strand primer used for detection of transgene-derived mRNA corresponded to the 5′ untranslated region included in the vector DNA. PCR products were visualized by ethidium bromide staining after separation by electrophoresis in a 1.5% agarose gel.

Flow Cytometric Analysis and Enzyme-linked Immunosorbent Assay

The staining of cells and the analysis on a flow cytometer (FACScan, BD Bioscience, San Jose, CA) were performed as previously described.[24,26] The antibodies and reagents used for staining were: fluorescein isothiocyanate (FITC)-conjugated anti-HA [clone 3F10, rat immunoglobulin (Ig) G1, Fab fragments, Roche, Indianapolis, IN], rabbit anti-gp100 (AnaSpec, San Jose, CA), FITC-conjugated goat antirabbit IgG (clone ALI4408, Biosource, Camarillo, CA), antimouse CD16/CD32 (clone 2.4G2, rat IgG2b BD Bioscience), recombinant soluble dimeric mouse CD1d:Ig (BD Bioscience), phycoerythrin-conjugated antimouse IgG1 (clone A85-1, rat IgG1, BD Bioscience), mouse IgG1 isotype control (clone A111-3, mouse IgG1, BD Bioscience), FITC-conjugated antimouse T-cell receptor (TCR) β (clone H57-597, hamster IgG, BD Bioscience), and IntraPrep permeabilization reagent (Beckman Coulter, Fullerton, CA). Enzyme-linked immunosorbent assay (ELISA) was carried out as described previously.[5,10] The concentrations of HA and FLAG-tagged SPARC proteins in the culture supernatants of ES-DC were measured by ELISA in triplicate wells using anti-FLAG (clone M2; mouse IgG, SIGMA, St Louis, MO) and anti–HA-peroxidase (clone 3F10; rat IgG1, Roche).

Induction of TAA-specific CTL and Cytotoxicity Assay

The mice were immunized intraperitoneally (IP) with each ES-DC twice with a 7-day interval. Seven days after the second immunization, spleen cells were isolated from the mice and cultured (2.5×10^6/well) with ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2 (7×10^4/well), or 0.1 μM hgp100 peptide in 24-well culture plates in Roswell Park Memorial Institute-1640 supplemented with 10% horse serum, recombinant human IL-2, and 2-mercaptoethanol. After culturing for 5 days, the cells were recovered and their cytotoxic activity was analyzed by 4-hour ^{51}Cr release

TABLE 1. Sequences of PCR Primers

	Sense	Antisense
PCR primers used for detection of transgenes expressed in ES-DC		
GPC3	5′-CTGACTGACCGCGTTACTCCCACA-3′*	5′-TAGCAGCATCGCCACCAGCAAGCA-3′
SPARC	5′-CTGACTGACCGCGTTACTCCCACA-3′*	5′-GGCAAAGAAGTGGCAGGAAG-3′
TRP2	5′-CTGACTGACCGCGTTACTCCCACA-3′*	5′-TGGGCAGTCAGGGAATGGAT-3′
hgp100	5′-CTGACTGACCGCGTTACTCCCACA-3′*	5′-GCACCTATCACAGCCAAATG-3′
G3PDH	5′-GGAAAGCTGTGGCGTGATG-3′	5′-CTGTTGCTGTAGCCGTATTC-3′
PCR primers used for detection of endogenous genes expressed in tumor cells		
SPARC	5′-ATGAGGGCCTGGATCTTCTTTCTC-3′	5′-TTAGATCACCAGATCCTTGTTGATGTCC-3′
TRP2	5′-CCTTTGCGTTGCCCTACT-3′	5′-CTAGGCTTCCTCCGTGTATCTCTT-3′
mgp100	5′-CCCGTGCTTGTGCTGAGTGCTCTG-3′	5′-ATGCTCGACCTGGACACTGGAC-3′

*The sense strand primer used for detection of transgene-derived mRNA corresponded to the 5′ untranslated region included in the vector DNA (Fig. 1A).
ES-DC indicates embryonic stem cell-derived dendritic cells; GPC3, glypican-3; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; hgp100, human gp100; mgp100, mouse gp100; PCR, polymerase chain reaction; SPARC, secreted protein acidic and rich in cysteine; TRP2, tyrosinase-related protein-2.

assays using MCA, MCA-GPC3, B16-F10, EL-4, EL-4–SPARC, and peptide pulsed EL-4 as targets basically by the same method as described previously.[15] B16-F10 cells were pretreated with recombinant murine IFN-γ (1000 units/mL) before use as target cells as reported previously.[27]

Subcutaneous Tumor Model

The mice were immunized IP with ES-DC–SPARC or ES-DC–TRP2 twice on days −14 and −7, and B16-F10 cells were inoculated subcutaneously into the shaved back region on day 0. In some experiments, the mice were immunized with a mixture of ES-DC–GPC3, ES-DC–SPARC, and ES-DC–TRP2. The tumor sizes were determined biweekly in a blinded fashion and the survival rate was monitored. The tumor volume was calculated as follows: tumor volume (mm^3) = (length × width × height).

Measurement of Luciferase Activity

The cells or tissues were homogenized with 2 mL of lysis buffer (0.05% Triton X-100, 2 mM ethylenediaminetetraacetic acid, 0.1 M Tris, pH 7.8) and the homogenates were cleared by centrifugation at 10,000 × g for 5 minutes. Twenty-five microliter of the supernatant was mixed with 75 μL of dilution buffer (phosphate-buffered saline containing $CaCl_2$ 1.8 mM and $MgSO_4$ 0.82 mM) and 100 μL of luciferase assay buffer (Steadyliteplus, PerkinElmer, Norwalk, CT), and at 10 minutes after the mixing the light produced was measured for 1 second in a luminometer (Tristar LB941, Berthold Technologies, Bad Wildbad, Germany). The luciferase activity was converted to the number of tumor cells using a regression line ($y = 0.1134x^2 - 0.305x + 0.4213$; x = cell number, y = counts/s).

Experimental Peritoneal Dissemination

In the preventive experiments, the mice were immunized IP with ES-DC–SPARC, ES-DC–TRP2, ES-DC–hgp100, their mixture (ES-DC–STH), or nontransfectant ES-DC twice on days 0 and 7, and B16-BL6/Luc cells were inoculated IP into mice on day 14. On day 28, the mice were euthanized and the greater omentum and pancreas were excised together and then the total luciferase activities were measured. In 1 experiment, the luciferase activities of the liver, kidney, spleen, and peritoneum were also measured. In the therapeutic experiments, B16-BL6/Luc cells were inoculated IP into mice on day 0. On days 3 and 10, ES-DC were transferred IP into mice. On day 17, mice were euthanized and luciferase activities of the greater omentum and pancreas were measured. In some experiments, ES-DC were cultured in the presence of α-GalCer (100 ng/mL) or vehicle (0.00025% Polysorbate-20) for 20 hours, and washed twice before injection. In 1 experiment, free α-GalCer (1 μg/mouse/transfer) either with or without ES-DC was transferred.

Analysis of the Activation of NKT Cells

The activation of NKT cells in vitro was analyzed as previously described.[28] In the analysis of the activation of NKT cells in vivo, on day 0, ES-DC loaded with either α-GalCer or vehicle were transferred IP into mice. On days 1, 7, 17, or 27, the mice were euthanized and the cytotoxic activities of the whole spleen cells against Yac-1 cells were analyzed.

In Vivo Depletion Experiments

The mice were transferred IP twice with either α-GalCer or vehicle loaded ES-DC–STH on days 0 and 7, and B16-BL6/Luc cells were inoculated IP into mice on day 14. To deplete the specific types of cells, the mice were given a total of 4 IP transfers (days −2, 5, 12, and 19) of monoclonal antibodies (mAbs), ascites (0.1 mL/mouse/transfer) from hybridoma-bearing nude mice, or polyclonal rabbit antiasialo GM1 antibody (Wako, Tokyo, Japan; 20 μL/mouse/transfer). The mAbs used were rat antimouse CD4 mAb (clone GK1.5) and rat antimouse CD8 mAb (clone 2.43). Normal rat IgG (Sigma-Aldrich, St Louis, MO; 200 μg/mouse/transfer) was used as a control. The depletion of specific cell subsets by treatment with antibodies was confirmed by a flow cytometric analysis of spleen cells, which showed a > 90% specific depletion.

Spontaneous Metastasis Experiments

In the preventive experiments, α-GalCer–loaded ES-DC or ES-DC–STH were transferred IP into mice twice on days 0 and 7. On day 14, the footpad of mice was inoculated with B16-BL6/Luc cells. On day 35, mice were euthanized, the lungs were excised, and total luciferase activities were measured. In the therapeutic experiments, B16-BL6/Luc cells were inoculated into the footpad on day 0. On days 3 and 10, ES-DC were transferred IP into mice. On day 21, mice were euthanized and luciferase activities of the inguinal lymph nodes were measured.

Statistical Analysis

Two-tailed Student *t* test was used to determine the statistical significance of differences in the tumor growth

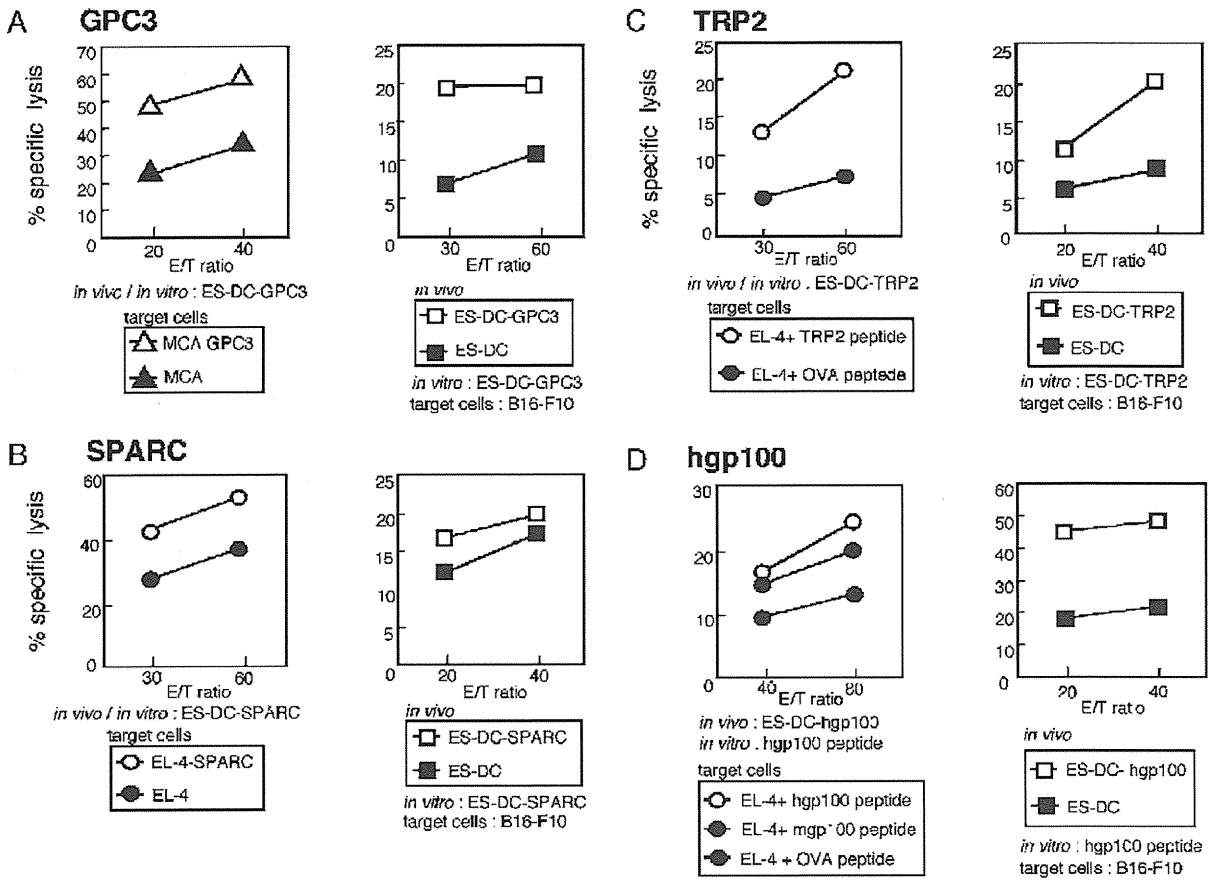

FIGURE 2. The priming of antigen-specific CTL in vivo with ES-DC expressing tumor-associated antigen. The mice were immunized intraperitoneally twice with each 1×10^5 ES-DC (as indicated in figure) on days 0 and 7. On day 14, spleen cells were isolated and cultured with 1×10^5 ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, or 0.1 μM human gp100 peptide per well in the presence of rhIL-2 (100 units/mL) for 5 days. Four-hour ^{51}Cr release assays were carried out using the obtained resultant cells to evaluate the capacity to kill MCA and MCA-GPC3 (A; left), EL-4 and EL-4–SPARC (B; left), EL-4 pulsed with the epitope peptide of TRP2 (C; left) and human or mouse gp100 (D; left), or B16-F10 (A–D; right). The results are expressed as percentage specific lysis from triplicate assays. The data are each representative of 2 independent experiments with similar results. CTL indicates cytotoxic T lymphocytes; ES-DC, embryonic stem cell-derived dendritic cells; GPC3, glypican-3; rhIL, recombinant human interleukin; SPARC, secreted protein acidic and rich in cysteine; TRP2, tyrosinase-related protein-2.

between the treatment groups. The Kaplan-Meier analysis with the Breslow-Gehan-Wilcoxon test was used to determine that of survival. The Mann-Whitney *U* test was used to examine the differences of luciferase activities. $P < 0.05$ was considered to be significant. Statistical analyses were performed by using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

RESULTS

Generation of ES-DC expressing Melanoma Antigens

B6 ES cells were transfected with the *GPC3*, *SPARC*, *TRP2*, and *hgp100* expression vectors; pCAGGS-GPC3-IRES–puro-R, pCAGGS-SPARC-FLAG-HA-IRES–puro-R, pCAGGS-TRP2-HA-IRES–puro-R, pCAGGS-hgp100-HA-IRES–puro-R (Fig. 1A), and several transfectant clones were isolated. The transfectant ES cell clones were subjected to differentiation to ES-DC, and the transfectant clone expressing the highest level of each TAA was selected on the basis of the RT-PCR, fluorescence-activated cell sorting analysis, and ELISA (Fig. 1B–D).

Priming of TAA-specific CTL With Genetically Modified ES-DC

The capacity of ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 to prime each TAA-specific CTL was analyzed. Other investigators reported that immunization of mice with hgp100 elicited a mouse gp100-specific $CD8^+$ T-cell response more efficiently than that with mouse gp100.[29] Therefore, ES-DC expressing hgp100 were generated in the present study. The mice were immunized with ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, ES-DC–hgp100, or nontransfectant ES-DC, respectively, on days 0 and 7. On day 14, the spleen cells were isolated and cultured with ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, or hgp100 peptide for 5 days. Next, the cells were recovered and their TAA-specific killing activities were analyzed. For the analysis of GPC3-specific and SPARC-specific CTL, MCA-GPC3 and EL-4–SPARC were used as targets. For TRP2-specific and gp100-specific CTL, EL-4 cells pulsed with previously

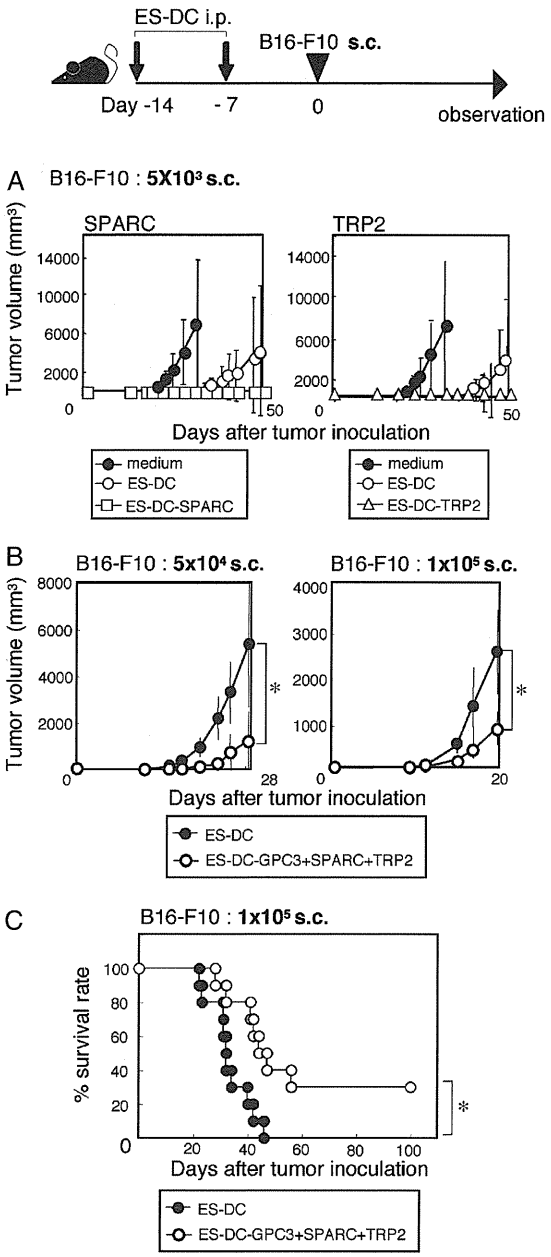

FIGURE 3. Protection against a subcutaneously inoculated tumor. A, About 1×10^5 ES-DC–SPARC or ES-DC–TRP2 were transferred IP into mice twice on days -14 and -7, and 5×10^3 B16-F10 cells were inoculated subcutaneously into the shaved back region on day 0. B, About 3×10^5 of nontransfectant ES-DC (control) or a mixture of ES-DC–GPC3, ES-DC–SPARC, and ES-DC–TRP2 that consisted of 1×10^5 of each ES-DC was transferred IP, and 5×10^4 or 1×10^5 B16-F10 cells were inoculated in the same schedule as above. The tumor sizes were determined biweekly in a blinded fashion and the survival rate was monitored (C). The tumor volume was calculated as follows: tumor volume (mm^3) = (length × width × height). The data are the mean ± SD (A–C, n = 5; D, n = 10; *P<0.05). The data are each representative of 2 independent experiments with similar results. ES-DC indicates embryonic stem cell-derived dendritic cells; GPC3, glypican-3; IP, intraperitoneal; SPARC, secreted protein acidic and rich in cysteine; TRP2, tyrosinase-related protein-2.

reported dominant epitopes of TRP2 and gp100 we used. As shown in Fig. 2A–D; left, the effector cells primed with ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, or ES-DC–hgp100 exhibited significantly higher killing activities against the target cells that specifically express each TAA. Furthermore, the effector cells activated with each TAA-expressing ES-DC in vivo showed significantly higher killing activities against the B16-F10 that naturally express each TAA (Fig. 2A–D; right). In contrast, spleen cells isolated from mice transferred with nontransfectant ES-DC and cocultured in vitro with each TAA exhibited a basal level killing activity. These results suggest that ES-DC–GPC3, ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 have the capacity to prime TAA-specific CTL in vivo.

Protection Against Subcutaneously Inoculated Tumor

ES-DC–GPC3 can induce an antigen-specific protective effect against 5×10^3 subcutaneously inoculated B16-F10 cells naturally expressing GPC3.[15] However, the anticancer effect was insufficient when the mice were challenged with a larger number (1×10^4) of melanoma cells (unpublished observation). The present study examined whether ES-DC–SPARC or ES-DC–TRP2 could protect the recipient mice from a subcutaneous inoculation of 5×10^3 B16-F10 cells. Figure 3A shows that immunization with ES-DC–SPARC or ES-DC–TRP2 provided complete protection against 5×10^3 B16-F10 inoculations. We observed a certain antitumor effect of nontransfectant ES-DC (Fig. 3A). This phenomenon may be owing to the fact that even nontransfectant ES-DC produced cytokines with an antitumor effect, such as IL-12 and tumor necrosis factor-α. In addition, nontransfectant ES-DC may have presented tumor cell-derived TAA and activated specific CTL, as intrinsic natural DC did. However, the TAA-specific effect elicited by these ES-DC was not significant when the mice were inoculated with 5×10^4 B16-F10 cells (unpublished observation). Collectively, ES-DC expressing single TAA could protect the mice from subcutaneous challenge with a relatively small number of tumor cells, however, the effect was insufficient to protect the mice from a challenge with a larger number of tumor cells.

The simultaneous in vivo transfer of ES-DC–GPC3, ES-DC–SPARC, and ES-DC–TRP2 might protect the recipient mice from a challenge with a relatively large number of B16-F10 cells. Figure 3B demonstrates the transfer of a combination of these TAA-transfectant ES-DC to elicit a significant protection against inoculation with 5×10^4 and 1×10^5 B16-F10 cells, thus resulting in a significant prolongation of the survival time of the treated mice (Fig. 3C). Therefore, immunotherapy with multiple TAA is more effective than that with a single TAA.

Prevention of Peritoneal Dissemination of Melanoma by Preimmunization With Multiple TAA-targeted ES-DC

Experiments on preventing the growth of tumors inoculated subcutaneously are often performed and they are convenient models to observe the effects of cancer immunotherapy, but they do not accurately reflect clinical situations. In a preclinical study of cancer immunotherapy, it is important to examine in the metastatic models that are resistant to cancer immunotherapy. Therefore, the effects

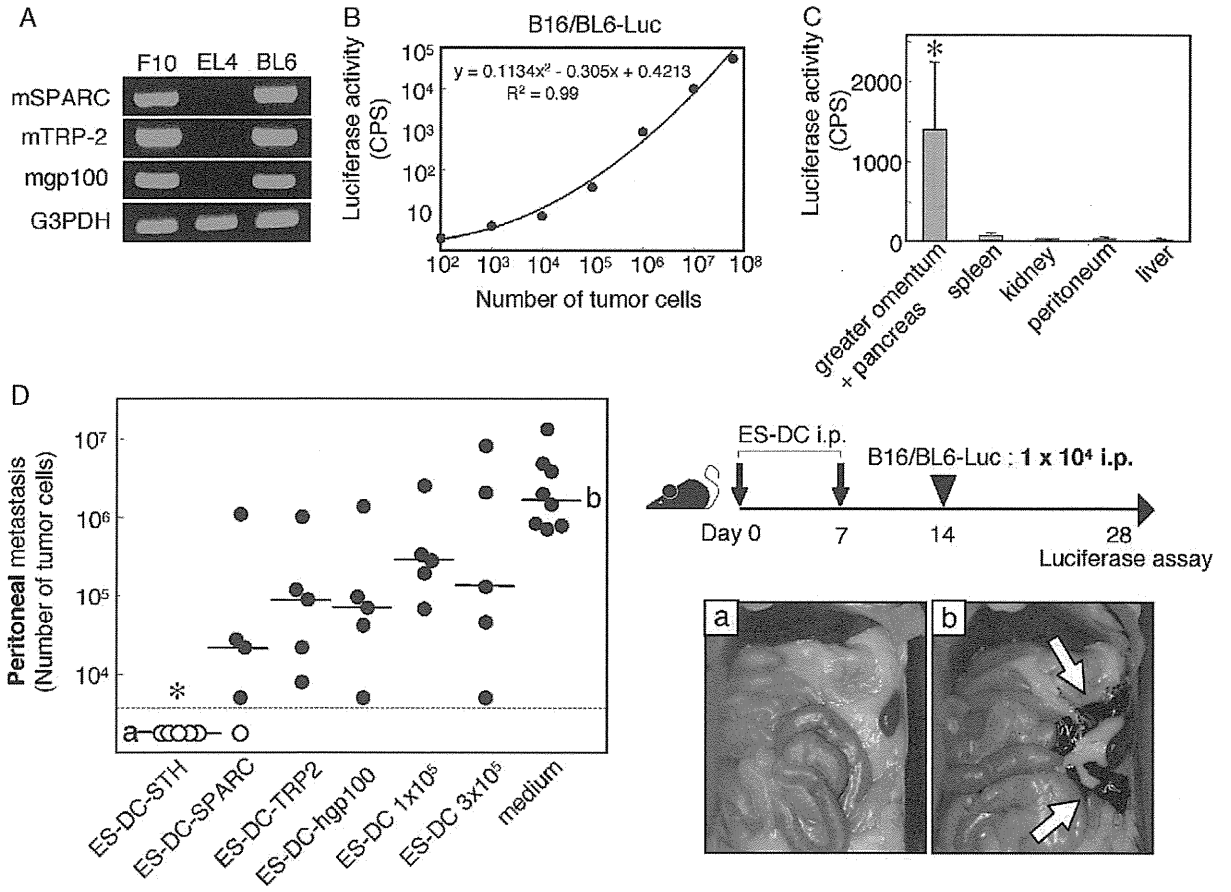

FIGURE 4. The prevention of peritoneal dissemination of melanoma by multiple tumor-associated antigen-targeted immunization with ES-DC. A, The expressions of SPARC, TRP2, and mouse gp100 mRNA in B16-BL6 were detected by RT-PCR. B, Luciferase activities in the homogenates of B16-BL6/Luc cells at the indicated numbers are shown. C, Luciferase activity of the homogenate of organs isolated on 17 days after IP inoculation of 1×10^4 B16-BL6/Luc cells. The data are shown as the counts per second (CPS). D, ES-DC–SPARC (1×10^5 cells), ES-DC–TRP2 (1×10^5 cells), ES-DC–hgp100 (1×10^5 cells), nontransfectant ES-DC (1×10^5 cells), nontransfectant ES-DC (3×10^5 cells), or a equal mixture of ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 (ES-DC–STH; 3×10^5 cells in total) were inoculated IP into mice twice on days 0 and 7, and 1×10^4 B16-BL6/Luc cells were inoculated IP into mice on day 14. Mice were euthanized on day 28. The greater omentum and pancreas were excised together and the total luciferase activity was measured. The luciferase activity was converted to the number of tumor cells using a regression line shown in B. Typical examples of peritoneal dissemination of B16-BL6/Luc cells are shown in mice inoculated with (left; A) ES-DC–STH or (right; B) medium (C, n = 5; D, n = 5 to 8; *P<0.05). Dotted line indicates the detection limit. The data are each representative of 2 independent experiments with similar results. ES-DC indicates embryonic stem cell-derived dendritic cells; IP indicates intraperitoneal; RT-PCR, reverse transcription polymerase chain reaction; SPARC, secreted protein acidic and rich in cysteine; TRP2, tyrosinase-related protein-2.

of ES-DC were evaluated in the peritoneal dissemination model.[18,28] Previously, the survival rate was the only end point in the peritoneal dissemination model. To evaluate tumor metastasis, labeling of cells by introducing reporter gene is a promising technique because the metastatic cells can be quantified. A firefly luciferase gene was introduced into a mouse melanoma B16-BL6 cell line that is a highly metastatic subline of B16, as previously described[30] and established a transfectant clone B16-BL6/Luc. The expressions of *SPARC*, *TRP2*, and mouse *gp100* mRNA were detected by RT-PCR analysis in B16-BL6 (Fig. 4A) and *gpc3* mRNA was not detected (unpublished observation). The luciferase activities in the homogenates of B16-BL6/Luc cells indicated that the luciferase activity was correlated with the number of B16-BL6/Luc cells at least in the range from 7 to 60,000 counts/s (Fig. 4B). The luciferase activity of the homogenate of each organ was confirmed on day 17 after IP inoculation of 1×10^4 B16-BL6/Luc cells (Fig. 4C). As others have reported, the greater omentum and pancreas were the most common site of metastasis in the early stage of peritoneal dissemination.[31] Because the luciferase activities of the greater omentum and pancreas were significantly higher than other organs such as the spleen, liver, kidney, and peritoneum, the luciferase activities of greater omentum and pancreas were measured to evaluate the metastasis of abdominal organs in the subsequent experiments.

The preventive effects of ES-DC were evaluated in the peritoneal tumor dissemination model (Fig. 4D). Immunization with ES-DC–SPARC, ES-DC–TRP2, or ES-DC–hgp100 did not show a TAA-specific, antitumor effect to inhibit the dissemination when 1×10^4 B16-BL6/Luc cells were inoculated. However, simultaneous in vivo transfer of ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 completely prevented the tumor dissemination.

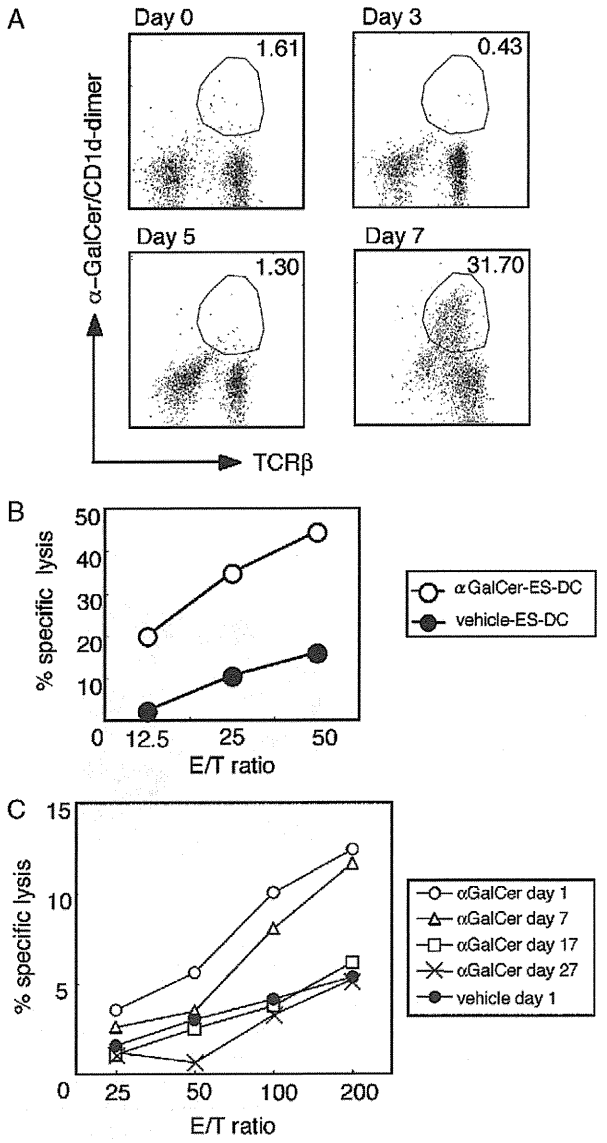

FIGURE 5. The activation of NKT cells by α-GalCer–loaded ES-DC. A, ES-DC were cultured in the presence of α-GalCer or vehicle alone for 20 hours, washed twice, and cocultured with splenic T cells of syngeneic mice (5×10^4 DC+2.5×10^6T cells/well in 24-well culture plate). On day 0, 3, 5, or 7, the cells were analyzed by flow cytometric analysis. The percentage of NKT cells, defined as TCRβ$^+$ and α-GalCer/CD1d dimer reactive cells, is indicated. B, On day 5, the effecter cells recovered from the culture were analyzed for their cytotoxic activities by a 4-hour ^{51}Cr-release assay using Yac-1 cells as target cells (1×10^4 cells/well) at the indicated effecter:target ratio. C, In vivo activation of NKT cells by α-GalCer–loaded ES-DC. On day 0, ES-DC loaded with either α-GalCer or vehicle alone were transferred intraperitoneally into mice (1×10^6 cells/mouse). On day 1, 7, 17, or 27, the mice were euthanized and the cytotoxic activities of whole spleen cells against Yac-1 cells were analyzed, as described above. The results are expressed as percentage specific lysis from triplicate assays. ES-DC indicates embryonic stem cell-derived dendritic cells; NKT, natural killer T; TCR, T-cell receptor; α-GalCer, α-galactosylceramide.

Therefore, the potency of immunotherapy with multiple TAA was confirmed also in the peritoneal dissemination model.

Activation of NKT Cells by α-GalCer–loaded ES-DC

Others have reported that α-GalCer loaded on mature DC induced more prolonged IFN-γ production by NKT cells and better protection against B16 melanoma than free α-GalCer.[20] The capacity of ES-DC was first confirmed to activate NKT cells in vitro. As shown in Figure 5A, NKT cells detected by α-GalCer/CD1d dimer represented 1.61% of splenic T cells before stimulation with α-GalCer–loaded ES-DC (day 0). After stimulation, TCRβ$^+$ dimer$^+$ cells almost disappeared on day 3, probably reflecting activation-induced down-regulation of the surface TCR.[32,33] This population began to reappear on day 5 and was dramatically increased (31.7%) on day 7, consistent with previous reports that activated NKT cells remain quiescent for a while, but eventually proliferate. The cultured cells recovered on day 5 exhibited strong cytotoxic activities against Yac-1 cells (Fig. 5B). These results indicate that α-GalCer–loaded ES-DC induced significant activation and of NKT cells in vitro. Next, the duration of activation of NKT cells induced by ES-DC was analyzed in vivo. On day 0, ES-DC loaded with either α-GalCer or vehicle were injected IP into mice. On days 1, 7, 17, or 27, the mice were euthanized and the cytotoxic activity of whole spleen cells against Yac-1 cells was analyzed. Figure 5C showed the Yac-1 cell-killing activities of spleen cells reflecting activation of NKT cells. The killing activities sustained for 1 week, and after 2 weeks the effect decreased to the background level.

Potent Anticancer Effects of Multiple TAA-targeted ES-DC Loaded With α-GalCer

We evaluated the effects of multiple TAA-targeted and α-GalCer–loaded ES-DC in several metastatic models. As shown in Figure 6A, simultaneous in vivo transfer of vehicle-loaded ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 did not show a significant inhibition of the dissemination of 5×10^4 B16-BL6/Luc cells, when 5 times more cells were injected IP in comparison with the experiments shown in Figure 4D. Although α-GalCer–loaded ES-DC without TAA induced a significant but a partial protection, a mixture of 3 kinds of TAA-transfectant ES-DC loaded with α-GalCer completely protected the mice from tumor dissemination. As shown in Figure 6B, α-GalCer–loaded ES-DC expressing TAA induced a significantly more potent protection than either 1 μg of free α-GalCer alone (the commonly used dose) or that injected simultaneously with ES-DC expressing TAA. These results clearly indicate the advantage of loading α-GalCer to DC to improve the anticancer effects. To analyze the effector cell populations induced by α-GalCer–loaded ES-DC with TAA, in vivo depletion of CD4$^+$T, CD8$^+$T, or natural killer (NK) cells with specific antibodies was performed as shown in Figure 6C. The preventive effects of the immunization with α-GalCer–loaded TAA-expressing DC (compared with vehicle-loaded TAA-expressing DC) were almost totally abrogated when CD4$^+$T cells, CD8$^+$T cells, or NK cells were depleted. These results suggest that all of 3 effector cell subsets were essential to achieve the protective effect.

Spontaneous pulmonary metastasis was induced by inoculation of B16-BL6/Luc cells into the footpad as previously described.[34] The mixture of α-GalCer–loaded ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 or α-GalCer–loaded ES-DC without TAA were transferred

IP into mice twice on days 0 and 7. On day 14, 2×10^6 B16-BL6/Luc cells were inoculated into the footpad of mice. On day 35, the mice were euthanized, the lungs were excised, and luciferase activities were measured. As shown in Figure 7A, in vivo transfer of a mixture of 3 kinds of TAA-transfectant ES-DC loaded with α-GalCer induced significant protection compared with α-GalCer–loaded ES-DC without TAA also in the spontaneous pulmonary metastasis model. In this model, we have no evidence indicating that the loading of α-GalCer to TAA-expressing ES-DC provided any benefit in regard to inhibiting the local tumor growth in the primary lesion. This result may be owing to the tissue distribution of NKT cells. NKT cells are known to mainly localize in the liver, lung, spleen, bone marrow, and peritoneal cavity.

Finally, the effects of the in vivo transfer of a mixture of 3 kinds of TAA-transfectant ES-DC loaded with α-GalCer in the therapeutic setting on lymph node metastasis and peritoneal dissemination were evaluated. On day 0, 2×10^6 B16-BL6/Luc cells were inoculated into the footpad or 1×10^4 tumor cells were inoculated IP into mice. On days 3 and 10, ES-DC were transferred IP, and on day 21 or 17, respectively, the mice were euthanized and luciferase activities were measured. Multiple TAA-targeted ES-DC loaded with α-GalCer induced significant therapeutic effects compared with α-GalCer–loaded ES-DC without TAA in the model of spontaneous metastasis to inguinal lymph node (Fig. 7B). As shown in Figure 7C, although the in vivo transfer of α-GalCer–loaded ES-DC without TAA or vehicle-loaded ES-DC with TAA showed insufficient effects, a mixture of 3 kinds of TAA-transfectant ES-DC loaded with α-GalCer induced a significant therapeutic effect in the peritoneal dissemination model.

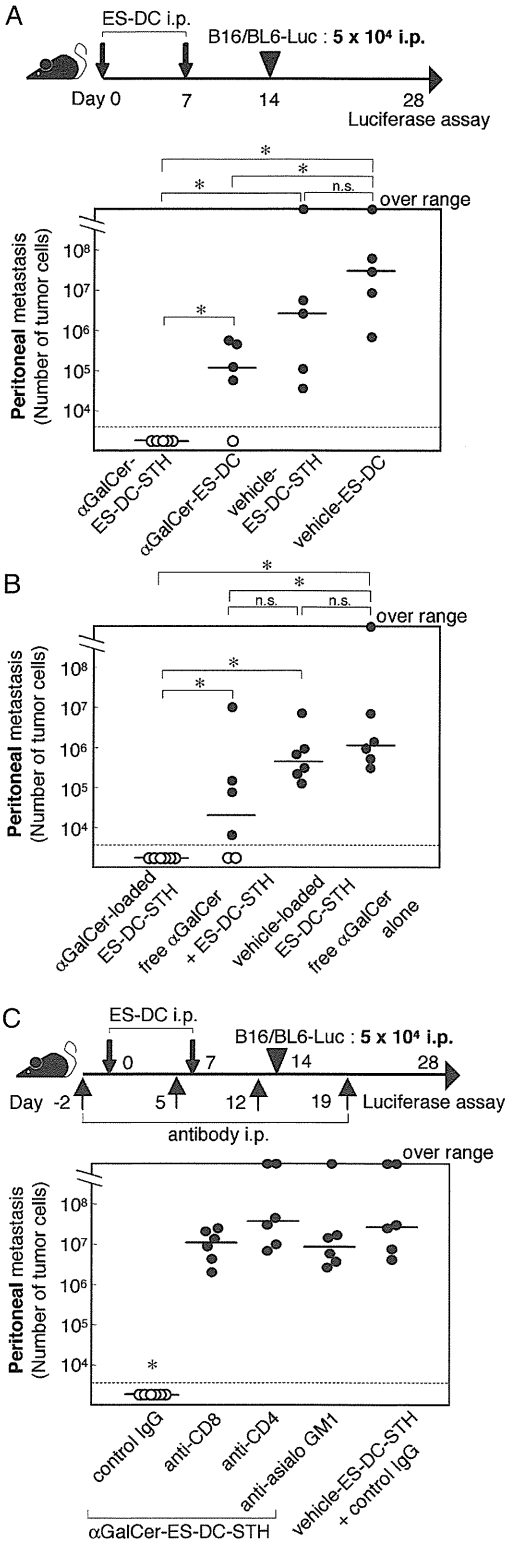

FIGURE 6. Preventive anticancer effects of multiple tumor-associated antigen-targeted vaccinations with α-GalCer–loaded ES-DC. A, The mixture of ES-DC–SPARC, ES-DC–TRP2, and ES-DC–hgp100 (3×10^5) loaded with either α-GalCer (αGalCer–ES-DC–STH) or vehicle (vehicle–ES-DC–STH) or nontransfectant ES-DC loaded with either α-GalCer (α-GalCer–ES-DC) or vehicle (vehicle–ES-DC) were transferred IP into mice twice on days 0 and 7, and 5×10^4 B16-BL6/Luc cells were inoculated IP into mice on day 14. On day 28, the mice were euthanized and the greater omentum and pancreas were excised together and the total luciferase activities were measured. B, 3×10^5 α-GalCer–loaded ES-DC–STH, free α-GalCer (1 μg/mouse/transfer) combined with ES-DC–STH, vehicle-loaded ES-DC–STH, and free α-GalCer alone (1 μg/mouse/transfer) were transferred IP into mice twice on days 0 and 7. About 5×10^4 B16-BL6/Luc cells were inoculated and luciferase activities were measured using the same protocol as A. C, $CD4^+$ T, $CD8^+$ T, or NK cells were depleted in vivo by the IP transfer of anti-CD4 mAb, anti-CD8 mAb, or polyclonal rabbit anti-asialo GM1 Ab. During this procedure, the mice were immunized with α-GalCer–loaded or vehicle-loaded ES-DC–STH and challenged IP with B16-BL6/Luc cells in the same protocol as A. Dotted line indicates the detection limit (A, n = 5; B and C, n = 6; *P<0.05). The data are each representative of 2 independent experiments with similar results. ES-DC indicates embryonic stem cell-derived dendritic cells; hgp100, human gp100; IP, intraperitoneal; mAb, monoclonal antibody; NK, natural killer; SPARC, secreted protein acidic and rich in cysteine; TRP2, tyrosinase-related protein-2; α-GalCer, α-galactosylceramide.

DISCUSSION

The anticancer effects of multiple TAA-targeted immunotherapies against mouse melanoma were evaluated by using α-GalCer–loaded and genetically engineered ES-DC. Four TAA that were naturally overexpressed in melanoma were selected, GPC3, SPARC, TRP2, and gp100. SPARC is expressed in various types of cancer tissues[35,36] and implicated in evasion of cancers from