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Predictive Factors of Outcome and Tumor Response to Systemic Chemotherapy in Patients with Metastatic Hepatocellular Carcinoma

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Objective: Systemic chemotherapy is an important treatment modality for metastatic hepatocellular carcinoma (HCC); however, the predictive factors of outcome and tumor response have not been fully investigated. The aim of this study was to identify factors that could be used to predict outcome and tumor response to systemic chemotherapy in patients with metastatic HCC.
Methods: We retrospectively examined 82 consecutive patients with metastatic HCC undergoing systemic chemotherapy to investigate factors associated with outcome and tumor response. The patients underwent 5-fluorouracil, mitoxantrone and cisplatin (FMP) therapy.
Results: The overall objective response rate was 22% (95% confidence interval, 14–32), and the median survival time and 1-year survival for all patients were 11.2 months and 43.5%, respectively. Multivariate analysis demonstrated that the absence of radiologically active intrahepatic disease ($P = 0.02$) and ascites ($P = 0.002$) was independent favorable prognostic factors. Although multivariate analysis revealed no significant predictive factors of tumor response, the response rates in patients without radiologically active intrahepatic disease (response rate, 46%) tended to be higher than those in patients with active intrahepatic disease (response rate, 17%) ($P = 0.05$).
Conclusion: Patients with metastatic HCC, who had sufficient hepatic function and no radiologically active intrahepatic disease, might be good candidates for systemic chemotherapy.

Key words: hepatocellular carcinoma – prognostic factor – tumor response – chemotherapy – metastasis

INTRODUCTION

The prognosis of patients with advanced hepatocellular carcinoma (HCC) remains poor, particularly for those with extrahepatic metastases (1). For patients with extrahepatic disease, systemic chemotherapy is one of the most important treatment modalities (2–6), but it has only limited value in clinical practice. Various clinical trials conducted after 1980s using different single agents reported overall response rates of 0–20%. Combination chemotherapy with cytotoxic agents yields higher response rates (2–7); however, a randomized controlled study comparing a promising combination therapy with a single agent failed to show any overall survival advantage (8). Recently, attention has focussed on molecularly targeted agents for the treatment of advanced HCC, because they have been reported to offer some degree of success for the treatment of challenging cancers like renal cell carcinoma (6,9,10). Among them, sorafenib, which is an oral multikinase inhibitor targeting Raf kinase and receptor tyrosine kinases, has been reported to confer an overall survival advantage of 12 weeks, with manageable toxicity, in comparison with placebo in a Phase III trial (11). These encouraging results suggest that sorafenib promise as a standard treatment for patients with advanced HCC. Recently, combination therapy using sorafenib with cytotoxic agents or other newly developed molecularly targeted agents have been tested for the activity against HCC (6,10).

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Analysis of predictive factors of outcome and tumor response can suggest the appropriate candidates for systemic chemotherapy. Although those in patients with advanced HCC, not limited to metastatic disease, have often been reported (12–18), these factors have not been investigated in patients with metastatic HCC receiving systemic chemotherapy. Since there may be some differences in predictive factors of outcome and tumor response between the patients with metastatic HCC alone and those with advanced HCC partially, including metastatic disease, the present study was conducted to evaluate a number of variables that may affect survival and tumor response in patients with metastatic HCC alone treated by systemic chemotherapy. To our knowledge, this is the first report concerning predictive factors of outcome and tumor response in patients with metastatic HCC alone receiving systemic chemotherapy.

PATIENTS AND METHODS

Patients

The study subjects were 82 consecutive patients with metastatic HCC for whom surgical resection was not indicated, and who underwent continuous infusion of 5-fluorouracil, mitoxantrone and cisplatin (FMP therapy) between September 1993 and January 2005 at the National Cancer Center Hospital, Tokyo, Japan (Table 1). The FMP therapy has been reported to show promising anti-tumor activity (response rate: 27%, median survival time: 11.6 months) with tolerable toxicity in a Phase II trial (19). HCC was diagnosed on the basis of histological examination or distinctive findings of computed tomography (CT) and/or angiography, along with the elevated levels of serum alpha-fetoprotein (AFP) or protein induced by vitamin K absence or antagonist-II (PIVKA II). Pretreatment evaluation included a complete medical history and a physical examination. The laboratory procedures included a complete differential blood count, biochemistry tests, viral markers including serum hepatitis B surface antigen and serum hepatitis C antibody, urinalysis and tumor markers including serum levels of AFP and PIVKA II. All patients underwent electrocardiography, chest radiography and CT/magnetic resonance imaging within 4 weeks before chemotherapy. Written informed consent was obtained from all patients before treatment.

Treatment Schedule

All patients received systemic chemotherapy using the FMP regimen as follows: 5-fluorouracil, mitoxantrone and cisplatin were given as a continuous intravenous infusion at a dose of 450 mg/m^2 on Days 1–5, 6 mg/m^2 on Day 1 and 80 mg/m^2 over a 2-h period on Day 1, respectively, with standard hydration. If there was no evidence of tumor progression or unacceptable toxicity with dose adjustments based on the toxic effects observed, the treatment was repeated every 4 weeks until a maximum of six courses were achieved. The patients who were refractory to this regimen

Table 1. Patient characteristics

	Number of patients (%)
Age (years)	
Median (range)	61 (34–74)
Gender	
Male	76 (93)
Female	6 (7)
Performance status	
0	72 (88)
1–2	10 (12)
History of blood transfusion	
Present	18 (22)
Alcohol abuse[a]	
Present	15 (18)
Smoking habit[b]	
Present	39 (48)
Hepatitis B surface antigen	
Positive	33 (40)
Hepatitis C virus antibody	
Positive	43 (52)
Prior treatment	
Hepatic resection	53 (65)
Local ablation	18 (22)
Transcatheter arterial chemoembolization	50 (61)
None	13 (16)
Organs affected by metastases	
Lung	55 (67)
Lymph nodes	38 (46)
Bone	11 (13)
Adrenal gland	5 (6)
Child-Pugh class	
A	65 (79)
B	17 (21)
Radiologically active intrahepatic disease	
Absent	13 (16)
Portal vein tumor thrombosis	
Present	14 (17)
Alpha-fetoprotein (ng/dl)	
Median (range)	261 (3–959 300)
PIVKA II (mAU/ml)	
Median (range)	860 (10–418 000)

PIVKA II, protein induced by vitamin K absence or antagonist-II. [a]Alcohol intake of $\geq$80 g/day $\times$ 5 years, [b]Smoking habit of $\geq$20 cigarettes/day for $\geq$10 years.

were allowed to undergo other anticancer treatments at their physician's discretion.

Factors Analyzed

Pretreatment clinical variables were evaluated for their relationship to the survival and tumor response by univariate and multivariate analyses. The pretreatment variables were chosen by considering possible effects on the prognosis and tumor response as indicated by previous investigations (2–6,12–18) or suggested from our own clinical experience. Each variable, which was classified as host- or tumor-related, was divided into two subgroups in accordance with clinically meaningful values as given in Table 2. No radiologically active intrahepatic disease was defined as complete tumor necrosis and no residual lesion in the entire liver as a result of prior local therapy on contrast-enhanced CT or magnetic resonance imaging before FMP therapy.

Overall survival was measured from the date of initial treatment to the date of death or last follow-up. The objective tumor response was assessed by CT or magnetic resonance imaging every 4 weeks after the start of FMP therapy. Response was evaluated according to the World Health Organization guidelines. The best overall response was recorded for each patient. Bone metastases were not regarded as measurable lesions.

Statistical Analyses

Survival curves were calculated by the Kaplan–Meier method, and the differences in survival were evaluated by log-rank test. The Cox proportional hazard model was used to determine the most significant variables related to survival. Differences in response rate were evaluated by the chi-squared test as univariate analyses. The logistic regression model was used to determine the most significant variables related to tumor response. In the multivariate analyses, all variables considered in univariate analysis were entered, and variable selection was not conducted. Statistical analyses were performed using SPSS 11.0J (SPSS Inc. Chicago, IL, USA). All P values presented in this report are of the two-tailed type. Differences at $P \leq 0.05$ were considered significant.

RESULTS

Patient Characteristics

The characteristics of all 82 patients are given in Table 1. The diagnosis of HCC was made on the basis of either histological examination (71 patients, 87%) or distinctive findings of CT and/or angiography with elevated serum levels of AFP or PIVKA II (11 patients, 13%). Prior treatments included hepatic resection in 53 patients (65%), local ablative therapy in 18 (22%), transcatheter arterial chemoembolization in 50 (61%) and no treatment in 13 (16%). Thirteen patients (16%) had been judged as having no active intrahepatic disease radiologically by two radiologists, and their prior treatments for the primary tumor had been hepatic resection in 12 patients, radiofrequency ablation therapy in one, percutaneous ethanol injection in one and transcatheter arterial chemoembolization in two. The median period between the latest prior treatment and the start of FMP therapy was 4.1 (range: 1.1–51.7) months. The median number of courses of FMP therapy was 2 (range: 1–6).

Survival and Tumor Response

The median survival time, 1-year survival proportion and median progression-free survival in all 82 patients were 11.2 months, 43.5%, 3.2 months, respectively (Fig. 1). At the time of analysis, 70 patients had died, and the causes of death were tumor progression and/or hepatic decompensation (65 patients), rupture of esophageal varices (one patient), cerebral bleeding from brain metastasis (three patients) and treatment-related death (one patient).

Eighty-one patients were evaluable for response; the remaining one patient could not be evaluated because of treatment-related death on Day 22 of the first course of FMP therapy. Although no patient achieved a complete response, 18 patients achieved a partial response, giving an overall response rate of 22% (95% confidence interval, 14–32). Forty-two patients (51%) showed no change and the remaining 21 patients (26%) had progressive disease. The median survivals of the patients with partial response, no change and progressive disease were 22.3, 11.9 and 5.5 months, respectively ($P < 0.01$). After this chemotherapy, two partial responders underwent surgical resection for residual HCC lesions in the lung and liver, respectively. These resections were successful and both patients achieved complete clinical remission after surgery, and one of both has survived with no recurrence over 7.5 years although the remaining had died of hepatic failure 3 months after resection.

Prognostic Factors

Median survival times, hazard ratios and P values of survival time for univariate analysis are given in Table 2. Among host-related factors, absence of ascites and an alkaline phosphatase level of $\leq$333 U/l were significantly associated with longer survival times. Among tumor-related factors, absence of lymph node metastasis and active intrahepatic disease was significantly associated with longer survival times. The results of multivariate analysis are given in Table 3. Absence of active intrahepatic disease and ascites was shown by multivariate analysis to be significantly favorable prognostic factors. The overall survival of patients without active intrahepatic disease (median: 22.3 months) was significantly better than that of patients with active intrahepatic disease (median: 10.6 months) (Fig. 2). The overall survival of

Table 2. Predictive factors of outcome and tumor response to FMP therapy for metastatic HCC

	Number of patients	Median survival survival (months)	Hazard ratio (95% CI)	*P* value*	Response (%)	*P* value
Host-related variables						
Age (years)						
>65	21	9.1			14.2	
≤65	61	12.1	0.69 (0.40–1.19)	0.18	24.5	0.50
Gender						
Male	76	11.7			21.0	
Female	6	7.3	2.15 (0.91–5.09)	0.07	33.3	0.85
Performance status						
0	72	11.2			22.7	
1–2	10	6.9	0.93 (0.46–1.89)	0.84	20.0	0.99
Alcohol abuse[a]						
Present	15	12.1			20.0	
Absent	67	10.5	1.06 (0.59–1.90)	0.86	22.4	0.99
Smoking habit[b]						
Present	39	12.1			17.9	
Absent	43	9.8	1.30 (0.81–2.10)	0.27	25.6	0.57
Blood transfusion						
Present	18	14.8			27.8	
Absent	64	10.0	1.66 (0.92–3.00)	0.09	20.3	0.72
Hepatitis B surface antigen						
Negative	49	11.8			20.4	
Positive	33	9.8	1.17 (0.72–1.91)	0.52	24.2	0.89
Hepatitis C virus antibody						
Negative	39	10.5			25.6	
Positive	43	12.1	0.84 (0.52–1.35)	0.47	18.6	0.62
Ascites						
Present	4	2.0			25.0	
Absent	78	11.7	0.17 (0.06–0.48)	0.0002	21.8	0.99
White blood cells $\times 10^3$ (/mm^3)						
≥4.0	67	11.5			20.9	
<4.0	15	9.1	1.21 (0.64–2.26)	0.56	26.7	0.89
Hemoglobin (g/dl)						
≥11	77	11.2			20.8	
<11	5	9.3	1.07 (0.39–2.96)	0.90	40.0	0.65
Platelets $\times 10^4$ (/mm^3)						
≥10	69	11.5			24.6	
<10	13	9.9	1.17 (0.63–2.20)	0.62	7.7	0.32
Total bilirubin (mg/dl)						
>1.0	18	10.0			11.1	
≤1.0	64	11.5	0.73 (0.42–1.28)	0.27	25.0	0.35
Albumin (g/dl)						
>3.5	52	10.6			23.1	
≤3.5	30	11.7	0.99 (0.60–1.62)	0.96	20.0	0.96

Continued

Table 2. *Continued*

	Number of patients	Median survival survival (months)	Hazard ratio (95% CI)	*P* value*	Response (%)	*P* value
Aspartate aminotransferase (U/l)						
>82	18	8.1			11.1	
≤82	64	11.7	0.76 (0.44–1.34)	0.35	25.0	0.35
Alanine aminotransferase (U/l)						
>70	16	11.5			0.0	
≤70	66	10.6	1.19 (0.65–2.19)	0.57	27.3	0.13
Alkaline phosphatase (U/l)						
>333	32	9.4			12.5	
≤333	50	10.1	0.59 (0.36–0.97)	0.03	28.0	0.17
Prothrombin time (%)						
>80	31	11.7			22.5	
<80	44	10.0	0.81 (0.49–1.35)	0.42	20.5	0.99
Prior treatments						
Absent	13	11.7			33.3	
Present	69	11.2	0.78 (0.41–1.48)	0.44	20.0	0.51
Tumor-related variables						
Metastatic site						
Lung						
Absent	27	9.9			18.5	
Present	55	11.4	0.78 (0.47–1.30)	0.34	23.6	0.81
Lymph node						
Absent	44	12.1			25.0	
Present	38	9.8	1.71 (1.05–2.77)	0.03	18.4	0.65
Bone						
Absent	71	11.1			20.2	
Present	11	14.7	0.77 (0.38–1.55)	0.46	36.4	0.40
Adrenal gland						
Absent	77	10.6			23.4	
Present	5	11.8	1.60 (0.64–4.04)	0.31	0.0	0.51
Radiologically active intrahepatic disease						
Absent	13	22.3			46.1	
Present	69	10.6	2.43 (1.17–5.03)	0.01	17.4	0.05
Portal vein tumor thrombosis						
Absent	68	11.7			22.1	
Present	14	10.0	1.49 (0.80–2.80)	0.21	21.4	0.99
Alpha-fetoprotein (ng/dl)						
>1000	32	8.1			18.8	
<1000	50	12.1	0.76 (0.47–1.24)	0.27	24.0	0.77
PIVKA II (mAU/ml)						
>1000	38	11.7			26.3	
<1000	40	11.2	1.24 (0.76–2.02)	0.40	17.5	0.94

HCC, hepatocellular carcinoma; FMP therapy, combination therapy of 5-fluorouracil, mitoxantrone and cisplatin; CI, confidence interval, −; reference category. *Log-rank test. [a]Alcohol intake of ≥80 g/day × 5 years, [b]Smoking habit of ≥20 cigarettes/day for ≥10 years.

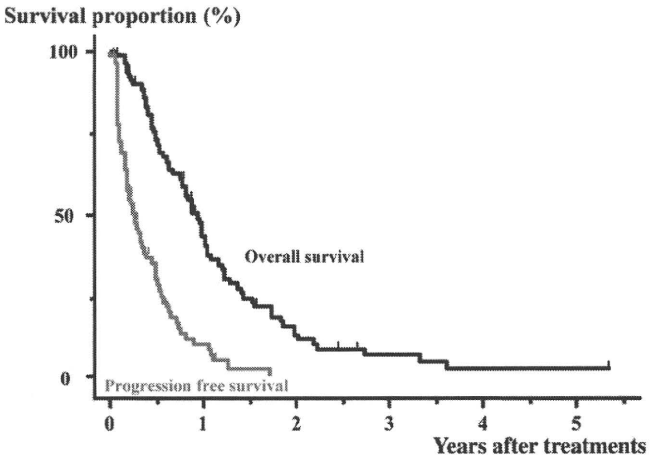

Figure 1. Overall survival and progression free survival curves of 82 patients who received 5-fluorouracil, mitoxantrone and cisplatin (FMP) therapy for metastatic hepatocellular carcinoma (HCC).

Table 3. Significant prognostic factors determined by multivariate analysis with the Cox proportional hazard model

Variable	Hazard ratio (95% CI)	*P* value
Radiologically active intrahepatic disease (−)	0.42 (0.21–0.89)	0.02
Ascites (−)	0.195 (0.07–0.54)	0.002

patients without ascites (median: 11.7 months) was significantly better than that of patients with ascites (median: 2.0 months) (Fig. 3).

Predictive Factors of Tumor Response

The response rates of the two subgroups for each variable are given in Table 2. Univariate and multivariate analyses revealed no significant predictive factors of tumor response. However, the response rates in patients without active intrahepatic disease in the liver (response rate, 46%) tended to be higher than those in patients with active intrahepatic disease (response rate, 17%) ($P = 0.05$).

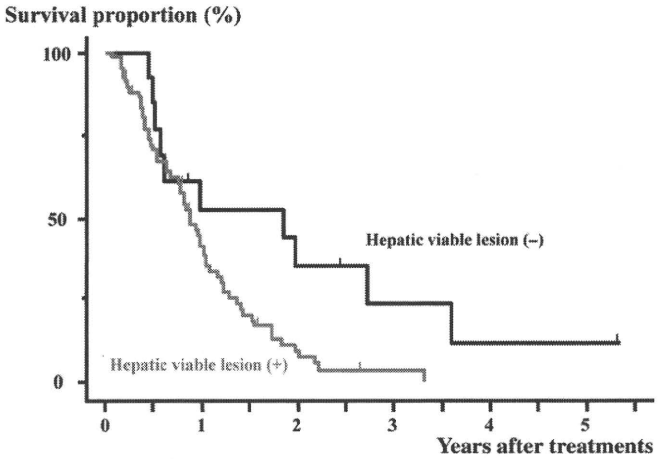

Figure 2. Comparison of overall survival in patients with metastatic HCC receiving FMP therapy with and without radiologically active intrahepatic disease.

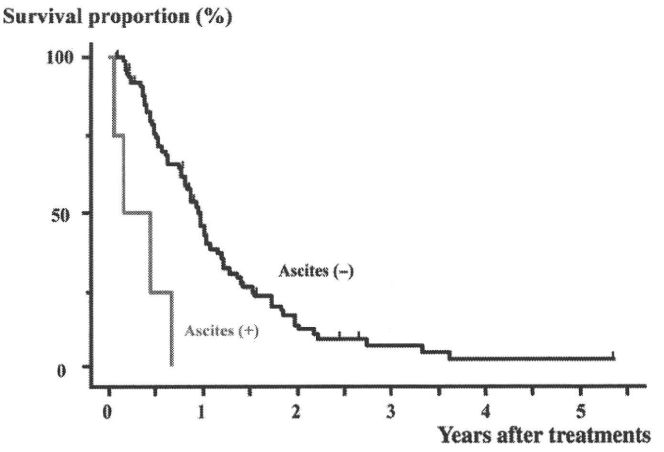

Figure 3. Comparison of overall survival in patients with metastatic HCC with and without ascites receiving FMP therapy.

DISCUSSION

Systemic chemotherapy for metastatic HCC is an important treatment modality (2–6), especially in view of the verified survival benefit of sorafenib (10,11). In this Phase III trial comparing sorafenib with placebo for advanced HCC (11), the rate of response to sorafenib was low (complete response: 0%, partial response: 2.3%), but the time-to-progression and overall survival were significantly longer for sorafenib than for placebo (median time-to-progression: 24.0 versus 12.3 weeks, hazard ratio 0.69, $P = 0.000007$, overall median survival: 46.3 versus 34.4 weeks, hazard ratio 0.58, $P = 0.00058$). Sorafenib is the first agent that has been proven to confer a survival benefit, and to show promise as a standard treatment, for patients with advanced HCC (10). To improve the treatment efficacy, development of further regimens of systemic chemotherapy, such as combination therapy comprising sorafenib and cytotoxic agents or other molecularly targeted agents, remains challenging.

In this study, to clarify the appropriate candidates for systemic chemotherapy, analysis of predictive outcomes and tumor response was conducted in patients with metastatic HCC receiving systemic chemotherapy. The study subjects were patients with metastatic HCC receiving FMP therapy, which has been shown in a Phase II trial to have promising anti-tumor activity (response rate: 27%, median survival time: 11.6 months) with tolerable toxicity for metastatic HCC (19). This regimen consists of three kinds of cytotoxic agents, not molecularly targeted agents like sorafenib. There were some differences between conventional cytotoxic chemotherapy and molecularly targeted agents. First, the tumor responses to conventional cytotoxic chemotherapy were greater than for molecularly targeted agents. Secondly, the

toxicities of conventional cytotoxic chemotherapy, especially hematological and hepatic toxicities, were more severe and difficult to manage than those of molecularly targeted agents. Thirdly, some conventional cytotoxic regimens need to be administered on an inpatient basis with standard hydration. However, many molecularly targeted agents are orally active and can be taken by patients on an outpatient basis. Therefore, there may be some differences in predictive outcome and tumor response between combination therapy comprising cytotoxic agents alone and therapy that includes molecularly targeted agents. Recently, trials of combinations of cytotoxic and molecularly targeted agents have been reported increasingly (10), and it would be worthwhile to analyze the predictive outcomes and tumor response in these subjects.

Multivariate analysis of prognostic factors in this study showed that absence of ascites and radiologically active intrahepatic disease was independent favorable factors. Presence of ascites is one of the most important factors to consider when evaluating hepatic reserve, being included in the Okuda staging system (20) and Child-Pugh classification (21) and has been shown to be a prognostic factor in previous studies of patients with advanced HCC (3,12,18). Although patients with massive or moderate ascites were not included in the present study, the outcome for patients with even a small amount of ascites was extremely poor, with a median survival of only 2.0 months. In such patients with impaired hepatic reserve, the toxicity of chemotherapy might outweigh its benefits.

There were some possible reasons why patients without active intrahepatic disease showed better survival than those with active intrahepatic disease. First, those without active intrahepatic disease might have a lower risk of hepatic failure due to progression of the intrahepatic tumor compared with those with active intrahepatic disease. Second, they might have a smaller tumor burden than those with active intrahepatic disease. A smaller tumor burden has been reported to be a favorable, independent prognostic indicator for advanced HCC (3,12,18). Finally, they might obtain a better tumor shrinkage effect of FMP therapy compared with patients with active intrahepatic disease, and this in turn might result in longer survival. Therefore, we also analyzed the predictive factors of tumor response to identify patients who might obtain a tumor response to FMP therapy. In patients receiving conventional cytotoxic agents/regimens like FMP therapy, it is important to predict the tumor response, because patients who achieve tumor shrinkage may show prolonged survival and improvement of clinical symptoms, such as tumor-related pain, and their general condition.

Among the variables investigated, the response rates in patients without active intrahepatic disease tended to be higher than those in patients with active intrahepatic disease, although the differences did not reach significance in univariate and multivariate analyses. The response rates in the patients with active intrahepatic disease were also analyzed, but no specific findings were obtained (data not shown). The precise reasons why patients without active intrahepatic disease obtained a better tumor response than those with such lesions remain unknown. There were no differences in patient characteristics, such as performance status, hepatic function, tumor burden outside the liver and tumor markers, between patients with and without active intrahepatic disease. The limited tumor heterogeneity in this population might have been a factor: heterogeneity of HCC has been reported to be closely related to chemoresistance (2–6,10,22). Yang et al. (18) have also reported a better response in patients with distant metastases than in those without, the difference perhaps suggesting heterogeneity of intrahepatic HCC. The limited heterogeneity in patients without active intrahepatic disease might result in a better response to FMP therapy. From these analyses of predictive factors of outcome and tumor response in patients with metastatic HCC receiving systemic chemotherapy, the best candidates were considered to be patients without ascites and active intrahepatic disease, although it may be considered that the results merely reflect the patients' conditions classified according to outcome. In such patients, FMP therapy resulted in favorable survival (median: 22.3 months) and tumor response (46%), although such patients comprised a very small population (only 16% in this study). These factors need to be considered in future clinical trials, including randomized trials, for patients with advanced HCC.

In conclusion, patients with metastatic HCC who have sufficient hepatic function and no active intrahepatic disease might be good candidates for systemic chemotherapy. This analysis may be helpful for predicting life expectancy and tumor response, determining treatment strategies and designing future clinical trials, including randomized trials for patients with advanced HCC.

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Conflict of interest statement

None declared.

References

1. Llovet JM, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M, et al. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. *Hepatology* 1999;29:62–7.
2. Forner A, Hessheimer AJ, Isabel Real M, Bruix J. Treatment of hepatocellular carcinoma. *Crit Rev Oncol Hematol* 2006;60:89–98.
3. Okada S. Chemotherapy in hepatocellular carcinoma. *Hepatogastroenterology* 1998;45(Suppl 3):1259–63.

4. Nowak AK, Chow PK, Findlay M. Systemic therapy for advanced hepatocellular carcinoma: a review. *Eur J Cancer* 2004;40:1474–84.
5. Aguayo A, Patt YZ. Nonsurgical treatment of hepatocellular carcinoma. *Semin Oncol* 2001;28:503–13.
6. Thomas MB, Zhu AX. Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 2005;23:2892–9.
7. Leung TW, Patt YZ, Lau WY, Ho SK, Yu SC, Chan AT, et al. Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma. *Clin Cancer Res* 1999;5:1676–81.
8. Yeo W, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, et al. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *J Natl Cancer Inst* 2005;97:1532–8.
9. Abou-Alfa GK, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, et al. Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2006;24:4293–300.
10. Zhu AX. Development of sorafenib and other molecularly targeted agents in hepatocellular carcinoma. *Cancer* 2008;112:250–9.
11. Llovet J, Ricci S, Mazzaferro V, Hilgard P, Raoul J, Zeuzem S, et al. Sorafenib improves survival in advanced hepatocellular carcinoma (HCC): results of a Phase III randomized placebo-controlled trial (SHARP trial). *J Clin Oncol* 2007;25:18S LBA1.
12. Okada S, Okazaki N, Nose H, Yoshimori M, Aoki K. Prognostic factors in patients with hepatocellular carcinoma receiving systemic chemotherapy. *Hepatology* 1992;16:112–7.
13. Patt YZ, Yoffe B, Charnsangavej C, Pazdur R, Fischer H, Cleary K, et al. Low serum alpha-fetoprotein level in patients with hepatocellular carcinoma as a predictor of response to 5-FU and interferon-alpha-2b. *Cancer* 1993;72:2574–82.
14. Stuart KE, Anand AJ, Jenkins RL. Hepatocellular carcinoma in the United States. Prognostic features, treatment outcome, and survival. *Cancer* 1996;77:2217–22.
15. Okusaka T, Okada S, Ishii H, Nose H, Nagahama H, Nakasuka H, et al. Prognosis of hepatocellular carcinoma patients with extrahepatic metastases. *Hepatogastroenterology* 1997;44:251–7.
16. Nagahama H, Okada S, Okusaka T, Ishii H, Ikeda M, Nakasuka H, et al. Predictive factors for tumor response to systemic chemotherapy in patients with hepatocellular carcinoma. *Jpn J Clin Oncol* 1997;27: 321–4.
17. Leung TW, Tang AM, Zee B, Yu SC, Lai PB, Lau WY, et al. Factors predicting response and survival in 149 patients with unresectable hepatocellular carcinoma treated by combination cisplatin, interferon-alpha, doxorubicin and 5-fluorouracil chemotherapy. *Cancer* 2002;94:421–7.
18. Yang TS, Chang HK, Chen JS, Lin YC, Liau CT, Chang WC. Chemotherapy using 5-fluorouracil, mitoxantrone, and cisplatin for patients with advanced hepatocellular carcinoma: an analysis of 63 cases. *J Gastroenterol* 2004;39:362–9.
19. Ikeda M, Okusaka T, Ueno H, Takezako Y, Morizane C. A phase II trial of continuous infusion of 5-fluorouracil, mitoxantrone, and cisplatin for metastatic hepatocellular carcinoma. *Cancer* 2005;103:756–62.
20. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. *Cancer* 1985;56: 918–28.
21. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973;60:646–9.
22. Dexter DL, Leith JT. Tumor heterogeneity and drug resistance. *J Clin Oncol* 1986;4:244–57.

Activation of Antigen-Specific Cytotoxic T Lymphocytes by β_2-Microglobulin or TAP1 Gene Disruption and the Introduction of Recipient-Matched MHC Class I Gene in Allogeneic Embryonic Stem Cell-Derived Dendritic Cells[1]

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A method for the genetic modification of dendritic cells (DC) was previously established based on the in vitro differentiation of embryonic stem (ES) cells to DC (ES-DC). The unavailability of human ES cells genetically identical to the patients will be a problem in the future clinical application of this technology. This study attempted to establish a strategy to overcome this issue. The TAP1 or β_2-microglobulin (β_2m) gene was disrupted in 129 (H-2^b)-derived ES cells and then expression vectors for the H-2K^d or β_2m-linked form of K^d (β2m-K^d) were introduced, thus resulting in two types of genetically engineered ES-DC, TAP1$^{-/-}$/K^d ES-DC and β_2m$^{-/-}$/β_2m-K^d ES-DC. As intended, both of the transfectant ES-DC expressed K^d but not the intrinsic H-2^b haplotype-derived MHC class I. β_2m$^{-/-}$/β_2m-K^d and TAP1$^{-/-}$/K^d ES-DC were not recognized by pre-activated H-2^b-reactive CTL and did not prime H-2^b reactive CTL in vitro or in vivo. β_2m$^{-/-}$/β_2m-K^d ES-DC and TAP1$^{-/-}$/K^d ES-DC had a survival advantage in comparison to β_2m$^{+/-}$/β_2m-K^d ES-DC and TAP1$^{+/+}$/K^d ES-DC, when transferred into BALB/c mice. K^d-restricted RSV-M2-derived peptide-loaded ES-DC could prime the epitope-specific CTL upon injection into the BALB/c mice, irrespective of the cell surface expression of intrinsic H-2^b haplotype-encoded MHC class I. β_2m$^{-/-}$/β_2m-K^d ES-DC were significantly more efficient in eliciting immunity against RSV M2 protein-expressing tumor cells than β_2m$^{+/-}$/β_2m-K^d ES-DC. The modification of the β_2m or TAP gene may therefore be an effective strategy to resolve the problem of HLA class I allele mismatch between human ES or induced pluripotent stem cells and the recipients to be treated. ***The Journal of Immunology,*** **2008, 181: 6635–6643.**

An efficient means for the activation of the CTL reactive to tumor Ags is crucial for T cell-mediated antitumor immunotherapy (1). Dendritic cells (DC)[3] are potent T cell stimulators and cellular vaccination using Ag-loaded DC has proven to be an efficient means for priming CTL specific to Ags (2, 3). This laboratory and others have established methods to generate DC from mouse and human embryonic stem (ES) cells (4–8). The capacity of ES cell-derived DC (ES-DC or esDC) to simulate alloreactive T cells and to prime Ag-specific CTL is comparable to that of conventional bone marrow-derived DC (BM-DC). Genetically modified ES-DC can be readily generated by introducing expression vectors into ES cells and the subsequent induction of their differentiation into ES-DC (9). The transfection of ES cells can be done by electroporation with plasmid vectors and the use of virus-based vectors is not necessary. Once an ES cell clone with proper genetic modification is established, it then serves as an infinite source for genetically modified DC. Mouse models have demonstrated that vaccination with genetically engineered ES-DC expressing tumor Ags (10) and T cell-attracting chemokines (11) is very effective for the induction of antitumor immunity.

In the future, the clinical application of ES-DC technology will require a solution to the problem of histoincompatibility between patients to be treated and the ES-DC. Specifically, the HLA allele mismatch may cause a rapid immune response and rejection of the inoculated cells (12), although a discrepancy in the minor histocompatibility Ag can also be a cause of alloreaction (13). The present study addressed this problem by using the strategy of modification of the genes that control the cell surface expression of MHC class I, i.e., β_2-microglobulin (β_2m) and TAP. TAP1$^{-/-}$ and β_2m$^{-/-}$ ES cell clones were generated from the ES cell lines derived from the 129 mouse (H-2^b) embryo. The expression vectors for H-2K^d and β_2m-linked form of H-2K^d (β2m-K^d) were then introduced into TAP1$^{-/-}$ and β_2m$^{-/-}$ ES cell clones, respectively. Subsequently, these genetically modified ES cells were subjected to a differentiation culture to generate ES-DC. The MHC class I molecules encoded by the genes in the H-2^b haplotype were either absent or at very low levels on the cell surface of these genetically modified ES-DC. The effect of the alteration of cell

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[3] Abbreviations used in this paper: DC, dendritic cell; ES cell, embryonic stem cell; ES-DC, embryonic stem cell-derived DC; BM-DC, bone marrow-derived DC; β_2-microglobulin, β_2m; HA, hemagglutinin; RSV, respiratory syncytical virus; PEF, primary mouse embryonic fibroblast; CMTR, chloromethyl-benzoyl-amino-tetramethyl-rhodamine; CMFDA, chloromethylfluorescein diacetate; iPS cell, induced pluripotent stem cell; Luc, luciferase; IRES, Internal ribosomal entry site.

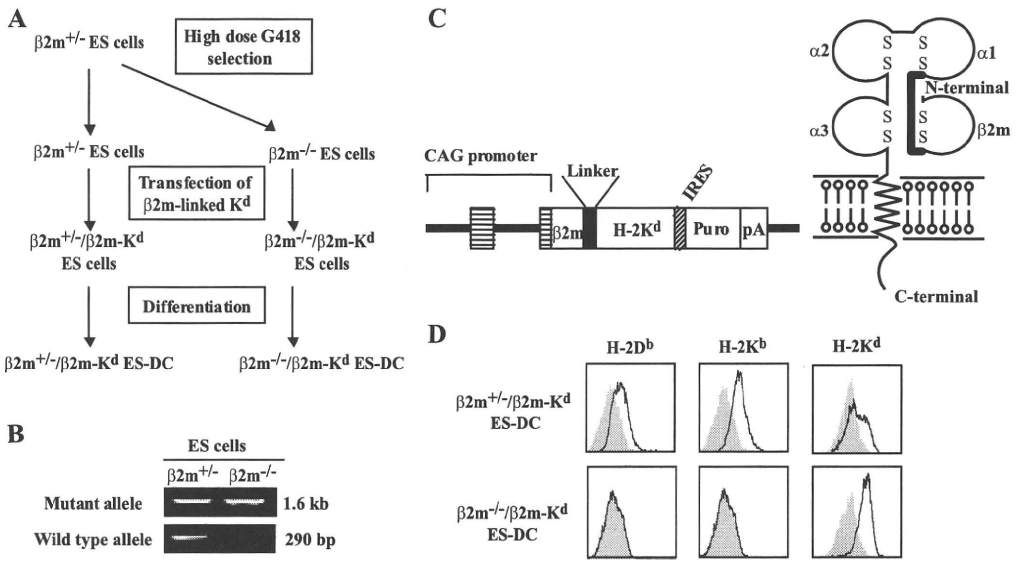

FIGURE 1. Generation of ES-DC deficient in the intrinsic β_2m gene and expressing recipient-matched MHC class I linked to β_2m. *A*, Overview of the method for generation of ES-DC-expressing β_2m-linked K^d, $\beta_2m^{+/-}/\beta_2$m-K^d ES-DC and $\beta_2m^{-/-}/\beta_2$m-K^d ES-DC. $\beta_2m^{-/-}$ ES cells were generated from $\beta_2m^{+/-}$ ES cells by high dose G418 selection. $\beta_2m^{+/-}$ and $\beta_2m^{-/-}$ ES cells were introduced with cDNA for the β2m-linked form of K^d and subsequently differentiated to generate $\beta2m^{+/-}/\beta$2m-K^d ES-DC and $\beta2m^{-/-}/\beta$2m-K^d ES-DC. *B*, The absence of wild-type β_2m gene in the $\beta_2m^{-/-}$ ES cell clone was confirmed by genomic PCR. *C*, Structure of the expression vector for β_2m-linked H-2K^d (*left*) and a schematic representation of the encoded molecule (*right*). The β_2m was fused to H-2K^d via a flexible 15 amino acid-long linker ($[Gly_4\text{-}Ser]_3$). The vector is driven by CAG promoter (pCAG) and cDNA for β_2m-linked H-2K^d are followed by the IRES-puromycin-resistance gene (Puro^R)-polyadenylation signal sequence (pA). *D*, Analysis of the cell surface expression of MHC class I on ES-DC by flow cytometry. The staining patterns with specific Abs (thick lines) and isotype-matched controls (gray) are shown.

surface expression of MHC class I on activation of alloreactive (H-2^b haplotype-encoded MHC class I-reactive) CTL was analyzed in both in vitro and in vivo experiments. After loading ES-DC with the antigenic peptide having a capacity to bind H-2K^d molecule, these ES-DC were transferred into BALB/c mice (H-2^d haplotype) to determine whether the peptide-specific, H-2K^d-restricted CTL could be primed in the recipient mice and whether Ag-specific antitumor immunity could be induced.

Materials and Methods

Mice

Six- to eight-week-old female BALB/c and 129/Sv (129) mice were purchased from Japan SLC and Clea Japan, respectively. The mice were housed at the Center for Animal Resources and Development (CARD, Kumamoto University) under specific pathogen-free conditions. All studies were performed under the approval of the animal experiment committee of Kumamoto University.

Peptides and cell lines

The respiratory syncytial virus (RSV) $M2_{82-90}$ epitope (SYIGSINNI) restricted to H-2K^d has been described previously (14). H-2K^d-restricted HIV gag protein-derived $p24_{199-207}$ epitope (AMQMLKETI) was used as an irrelevant control peptide (15). The peptides were commercially synthesized and supplied at >98% purity (Anygen). Murine mastcytoma P815 cells were used as target cells for a ^{51}Cr release assay. A RSV-M2-transduced colon26/luciferase (Luc) cell line (colon26/M2-Luc) was established by the transfection of murine adenocarcinoma colon26 cells with an influenza virus hemagglutinin (HA)-tagged RSV M2 expression vector (pCAG-M2- internal ribosomal entry site (IRES)-neo^R) and a firefly luciferase expression vector (pCAG-luc-IRES-puro^R) by electroporation. After the transfection, G418 and puromycin were added to the culture medium for selection and single clones were obtained by limiting dilution. The expression of firefly luciferase was verified by measuring the luciferase activity in the cell lysates as described below. The expression of the HA-RSV-M2 protein in the selected transfectant clones was confirmed by a flow cytometric analysis following intracellular anti-HA staining and also using ELISA detecting IFN-γ-production by $M2_{82-90}$ specific K^d-restricted CTL cocultured with the transfectants.

Generation of TAP1- or β_2m-deficient ES cells and differentiation of DC from ES cells

ES cells were cultured on primary mouse embryonic fibroblast (PEF) feeder layers in complete ES cell medium, DMEM containing 20% Knock-Out Serum Replacement (Invitrogen Life Technologies), 2-ME (50 μM), and mouse leukemia inhibitory factor (1000 U/ml). A $\beta_2m^{+/-}$ ES cell clone established from D3 cell line derived from a 129 mouse embryo (H-2^b) was a generous gift from Dr. R. Jaenisch (Massachusetts Institute of Technology, Cambridge, MA) (16). To generate $\beta_2m^{-/-}$ ES cells, $\beta_2m^{+/-}$ ES cells (5×10^5 cells/90-mm culture dish) were cultured on feeder layers of neomycin-resistant PEF derived from GTPBP1$^{-/-}$ mouse embryos (17) in ES cell medium containing high dose G418 (1.5–2.0 mg/ml) for 10 days (18). After a further culture for 7 days without G418, the surviving ES cell colonies were picked up from the dishes, transferred to 24-well culture plates, and then expanded. For each isolated ES cell clones, a part of the expanded cells were cultured in gelatin-coated 6-well plates without PEF feeders. Genomic DNA extracted from the feeder-free ES cells was used for genotyping of the β_2m locus by genomic PCR and $\beta_2m^{-/-}$ ES clones were selected. TAP1$^{+/-}$ ES cells were generated from E14 ES cells derived from 129 mouse embryo (H-2^b). E14 cells were transfected with 30 μg of linearized targeting vector by electroporation (19). G418 and ganciclovir were added to the culture medium 24 h after the transfection and the surviving colonies were isolated during days 7–10 of selection. The isolated clones were analyzed by PCR and Southern blotting to identify cell clones with homologous recombination. Subsequently, one of the TAP1$^{+/-}$ clones was subjected to selection with high dose of G418 as in the case of $\beta2m^{-/-}$ ES cells. The clones were expanded and analyzed by Southern blotting to select TAP1$^{-/-}$ clones. Expression vectors for H-2K^d and β_2m-linked form of H-2K^d (β2m-K^d) were introduced into TAP1$^{-/-}$ and $\beta_2m^{-/-}$ ES cell clones, respectively. The induction of differentiation of ES cells into ES-DC was done as described previously (20). On day 17–19 of cultures, the floating or loosely adherent cells were recovered from culture dishes by pipeting and then were used for the experiments.

PCR

The initial screening of ES cells was done by PCR using the following primers: the wild type TAP1 allele (5′-ATGGGACACATGCACGGC-3′ and 5′-CCACAGTAGCAGGCTCAG-3′), the mutant TAP1 allele (5′-TG TAGCTTTGGCTCTTCTGGAA-3′ and 5′-GGGCCAGCTCATTCCTC CACTC-3′), β_2m (5′-CCTCAGAAACCCCTCAAATTCAAG-3′ and

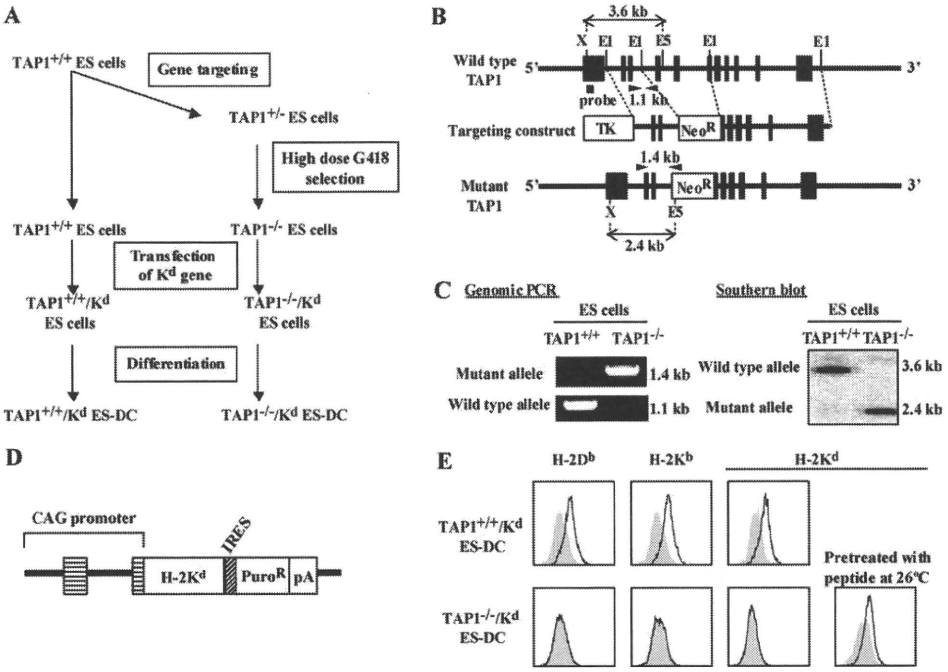

FIGURE 2. Generation of TAP1-deficient, recipient-matched MHC class I gene-introduced ES-DC. *A*, Overview of the method for generation of K^d expressing, TAP1-deficient ES-DC. $TAP1^{+/+}$ and $TAP1^{-/-}$ ES cells were transfected with cDNA for K^d and subsequently induced to differentiate into ES-DC. *B*, Structure of the mouse TAP1 genomic locus, the targeting construct and the mutant allele. Closed boxes indicate exons. The targeting construct contains a neomycin resistant gene (Neo^R) and herpesvirus thymidine kinase gene (TK). The sets of PCR primers for the wild-type allele and mutant allele are shown as arrowheads. The position of the probe used for the Southern blot analysis is indicated as a small black box. The sizes of bands generated from the wild-type and mutant allele by digestion with *Xho*I (X) and EcoR V (E5) are indicated. The EcoR I (E1) restriction site is also shown. *C*, A genotype analysis of ES cells. Genomic DNA from ES cells was analyzed by genomic PCR and Southern blotting. *D*, Structure of the H-$2K^d$ expression construct. The vector is driven by a CAG promoter (pCAG) and cDNA for H-$2K^d$ are followed by the IRES-puromycin-resistance gene ($Puro^R$)-polyadenylation signal sequence (pA). *E*, Surface phenotypes of genetically modified ES-DC. The surface expression of indicated MHC class I molecules on ES-DC were analyzed using flow cytometry. The expression of cell surface H-$2K^d$ by $TAP1^{-/-}/K_d$ ES-DC were detected after cells were incubated with 10 μM H-$2K^d$-binding peptide (RSV $M2_{82-90}$) at 26°C for 12 h, subsequently incubated at 37°C for 4 h and stained with anti-H-$2K^d$ Ab. The staining patterns with specific Abs (thick lines) and isotype-matched controls (gray) are shown.

5′-GCTTACCCCAGTAGACGGTCTTGG-3′). The set of primers for β_2m was designed to amplify both the wild type and targeted loci.

Southern blot analysis

To analyze the genotype of ES cells, genomic DNA isolated from ES cells was digested with *Xho*I and EcoR V. The DNA fragments were separated by electrophoresis in 0.8% agarose gels. Subsequently, the DNA was transferred onto nylon membranes. Probes for the Southern blot analysis were obtained by PCR with sets of primers for TAP1 locus (5′-GACCA GACTCTGGACAGCTCAC-3′ and 5′-AAGGCAAGAGAGAATCAA GAG-3′) from the genomic DNA of ES cells. Labeling of probe DNA with ^{32}P-dCTP was done by using a Megaprime DNA Labeling Kit (GE Healthcare) and the standard Southern blot procedure was conducted.

Abs and flow cytometric analysis

FITC-conjugated anti-H-$2K^d$ (BD Pharmingen), H-$2K^b$ (Caltag Laboratories), and H-$2D^b$ (Caltag Laboratories) Abs were purchased from the indicated sources. A flow cytometric analysis was done on a FACScan flow cytometer (BD Biosciences) and the data analysis was performed using the CellQuest software program (BD Biosciences).

Preparation of BM-DC

Bone marrow cells prepared from BALB/c or 129 mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 500 U/ml GM-CSF, and 50 μM 2-ME. On day 7 of the culture, the cells were recovered and used as BM-DC for the experiments.

Analysis of stimulation of BALB/c -derived H-2^b-reactive $CD8^+$ T cell lines by DC

To generate BALB/c (H-2^d)-derived H-2^b-reactive $CD8^+$ T cell lines, 5×10^6 BALB/c spleen cells were cultured with 2×10^6 irradiated 129 (H-2^b) spleen cells in 2 ml of RPMI 1640 medium supplemented with 10% FCS, 100 U/ml IL-2 and 50 μM 2-ME in a well of 24-well plates for 5 days (21) and after that $CD8^+$ T cells were isolated by using anti-CD8 magnetic beads (Miltenyi Biotec). The H-2^b-reactive $CD8^+$ T cells (1×10^3) were cultured with the indicated stimulator DC (5×10^3) for 16 h and the activation of T cells was detected by IFN-γ-production by using ELISPOT (BD Biosciences). For the analysis of the priming of alloreactive (H-2^b-reactive) $CD8^+$ T cells by ES-DC, BALB/c spleen cells (5×10^6) were cultured with irradiated ES-DC (2×10^6) for 5 days and then $CD8^+$ T cells were isolated as described above. The magnitude of priming of H-2^b-reactive $CD8^+$ T cells was analyzed by IFN-γ-production detected by ELISPOT upon coculture with 129 mice-derived BM-DC as stimulators.

Analysis of frequency of alloreactive CTL in ES-DC-injected mice

Spleen cells were isolated from naive BALB/c mice or those that received multiple ES-DC injections and $CD8^+$ T cells were isolated by using anti-CD8 magnetic beads (Miltenyi Biotech). To analyze the frequency of auto (H-2^d)- or allo (H-2^b)-reactive $CD8^+$ T cells, the cells (5×10^3) were cocultured with BALB/c or 129 BM-DC (5×10^3) for 16 h and IFN-γ producing cells were detected using the ELISPOT assay.

Detection of in vivo administered ES-DC in the draining lymph nodes

The in vivo elimination of ES-DC was assessed according to the reported procedures with some modification (22). In brief, ES-DC were labeled with the 10 μM chloromethyl-benzoyl-amino-tetramethyl-rhodamine (CMTMR; Molecular Probes) and BALB/c BM-DC were labeled with 10 μM chloromethylfluorescein diacetate (CMFDA; Molecular Probes) according to the manufacturer's instructions. The mice were injected s.c. in the forelimb with 2×10^6

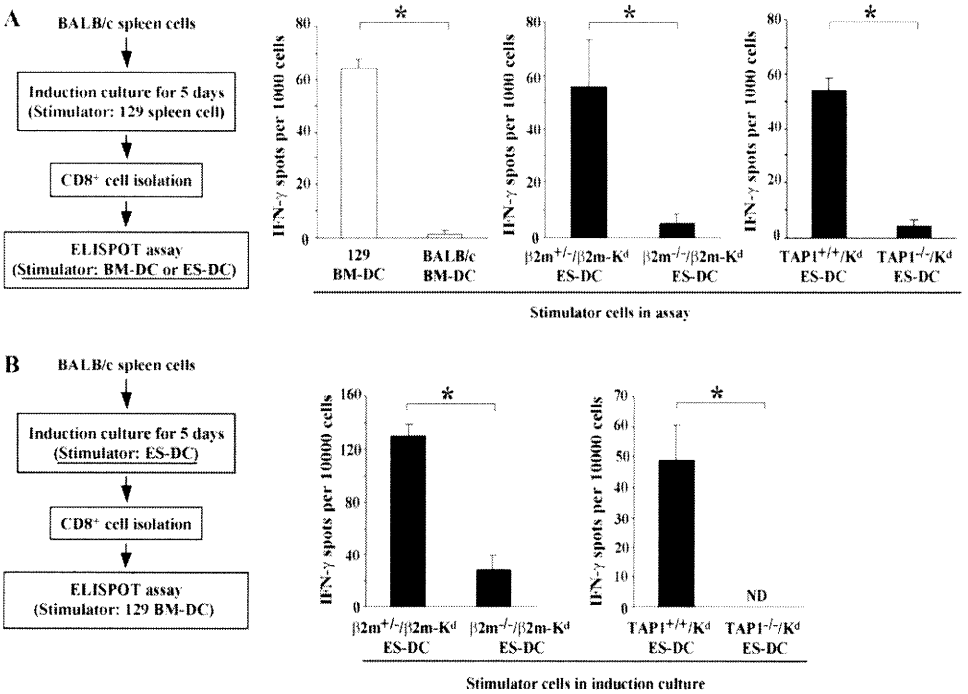

FIGURE 3. Reduced in vitro stimulation of alloreactive CD8$^+$ T cells by β_2m or TAP1 gene-deficient ES-DC. *A*, The response of H-2^b-reactive CD8$^+$ T cells to ES-DC was reduced by modification of the β_2m (*middle panel*) or TAP1 (*right panel*) gene. To generate alloreactive (anti-H-2^b) CTL, 5×10^6 BALB/c (H-2^d) spleen cells were cultured with 2×10^6 irradiated 129 (H-2^b) spleen cells for 5 days and after that the CD8$^+$ cells were purified from the culture. The alloreactive CD8$^+$ T cells (1×10^3) were cultured with the ES-DC (5×10^3) of the indicated genotype for 16 h. The 129 or BALB/c BM-DC were used as additional controls (*left panel*). The number of IFN-γ producing cells was measured using an ELISPOT assay. *B*, Allogeneic MHC class I-deficient ES-DC showed reduced potency to prime alloreactive CD8$^+$ T cells in vitro. Splenocytes (5×10^6) derived from BALB/c mice were cultured with irradiated ES-DC (2×10^6) of the indicated genotype for 5 days and after that CD8$^+$ T cells were purified from the culture. The IFN-γ producing response of the CD8$^+$ T cells was measured with an ELISPOT with 129 BM-DC as stimulators. The results of the experiments with β_2m deficient ES-DC (*left panel*) and TAP1-deficient ES-DC (*right panel*) are shown. Data are representative of at least two experiments with similar results. The data are the mean ± SD of triplicate assays. The asterisks indicate significant ($p < 0.05$, Student's *t* test) differences between the two groups. ND, not detectable.

cells containing equal numbers of CMTMR-labeled ES-DC and CMFDA-labeled BM-DC. After 48 h, the draining axillary and brachial lymph nodes were removed, digested with collagenase type II and DNase I and analyzed for the presence of fluorescent cells by flow cytometry. The number of ES-DC was normalized to control syngeneic BALB/c BM-DC.

Priming of RSV-M2 specific CD8$^+$ T cells by ES-DC

ES-DC were incubated with RSV-M2$_{82-90}$ peptide (10 μM) for 3 h and then washed three times with FCS-free DMEM. Ag-loaded ES-DC were injected i.p. (1×10^5 cells/injection/mouse) into the mice twice, with a 7-day interval. In some experiments, non-Ag-loaded ES-DC were injected five or ten times with 7-day intervals before the injection of Ag-loaded ES-DC. Seven days after the last injection of ES-DC, the mice were sacrificed and the spleen cells were isolated. After hemolysis, the spleen cells were cultured in the presence of M2$_{82-90}$ peptide (1 μM). Six days later, the cells were recovered and cytotoxic activity against M2$_{82-90}$ peptide pulsed-P815 target cells were measured using the standard ^{51}Cr- release assay.

Tumor challenge experiments

The mice were immunized with ES-DC and, 7 days after the immunization, colon26/M2-Luc (1×10^6/mouse) cells were injected into the mice i.p. Ten days later, the mice were sacrificed and the luciferase activity of the lysates of the abdominal organs was measured to quantify tumor growth. The tissue specimens were homogenized in 3 ml of lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris (pH 7.8)) and the homogenates were cleared by centrifugation at 10,000 × *g* for 5 min. Fifty μl of the supernatant was mixed with 50 μl of dilution buffer (PBS containing 2.4 mM $CaCl_2$ and 0.82 mM $MgSO_4$) and 100 μl of luciferase assay buffer (Steadyliteplus, PerkinElmer) and at 5 min after the mixing the light produced was measured for 1 second in a luminometer (Tristar LB941, Berthold Technologies).

Statistical analysis

Student's *t* test was used for the statistical analysis of data except for the data regarding the tumor invasion experiments. Because some of data in the tumor invasion experiments did not follow a normal distribution, the data were analyzed using the Mann-Whitney *U* test, a nonparametric test. A value of $p < 0.05$ was considered to be significant.

Results

Generation of ES-DC expressing recipient-matched MHC class I but not intrinsic MHC class I

The present study evaluated a strategy to prime Ag-specific CTL by transfer of genetically engineered 129 (H-2^b)-derived ES-DC into BALB/c (H-2^d) recipient mice, thus avoiding the recognition of ES-DC by allo (H-2^b)-reactive CD8$^+$ T cells. To modify the cell surface expression of MHC class I, two strategies were tested in parallel: 1) disruption of the β_2m gene in ES cells and introduction of β_2m-linked form of recipient-matched MHC class I (β_2m-K^d) (Fig. 1*A*), 2) disruption of TAP1 gene, introduction of recipient-matched MHC class I (K^d) and loading of K^d-binding epitopes to the ES-DC (Fig. 2*A*).

$\beta_2m^{-/-}$ ES cells were generated by the selection of the previously established $\beta_2m^{+/-}$ ES cells (16) with high dose (1.5–2 mg/ml) of G418. Two of the 39 clones were found to have a $\beta2m^{-/-}$ genotype by genomic PCR (Fig. 1*B*). Subsequently, an expression vector for β_2m-K^d (Fig. 1*C*) was introduced into both $\beta_2m^{+/-}$ and $\beta_2m^{-/-}$ ES cells to generate $\beta_2m^{+/-}$/β_2m-K^d and $\beta_2m^{-/-}$/β_2m-K^d ES cells, respectively. The genetically modified ES cell clones were subjected to differentiation culture to generate ES-DC. Theoretically, β_2m-K^d is the only MHC class I molecule expressed on the cell surface of $\beta_2m^{-/-}$/β_2m-K^d ES-DC. In the flow cytometric analysis, the cell surface expression of MHC class I molecules of H-2^b haplotype, H-2D^b and K^b, was detected in $\beta_2m^{+/-}$/β_2m-K^d ES-DC but not in $\beta_2m^{-/-}$/

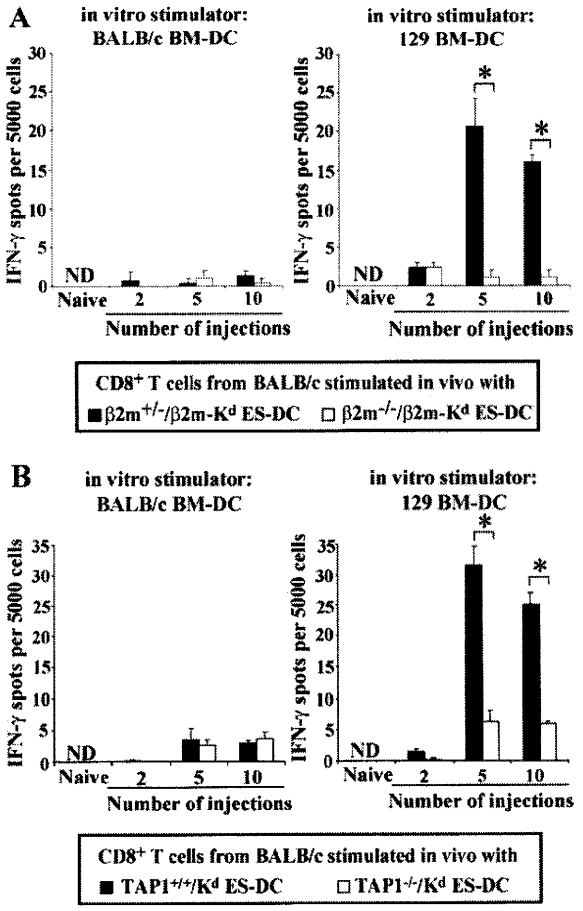

FIGURE 4. Reduced in vivo stimulation of alloreactive $CD8^+$ T cells by β_2m or TAP1 gene-modified ES-DC. Splenic $CD8^+$ T cells were isolated from naive BALB/c mice or those received multiple injections of β_2m deficient (*A*) or TAP1 deficient (*B*) ES-DC (1×10^5/injection). Isolated $CD8^+$ T cells (5×10^3) were cultured with BALB/c BM-DC (*left panels*) or 129 BM-DC (*right panels*) (5×10^3) for 16 h. The numbers of IFN-γ producing cells were measured using an ELISPOT assay. Data are representative of at least two experiments with similar results. The data are the mean ± SD of triplicate assays. Asterisks indicate significant ($p < 0.05$, Student's *t* test) differences. ND, not detectable.

β_2m-K^d ES-DC. Expression of K^d was detected in the both types of ES-DC, as expected (Fig. 1*D*).

To mutate the TAP1 gene in ES cells, the targeting vector (Fig. 2*B*) was introduced into E14 ES cells to make several $TAP1^{+/-}$ ES cell clones. Subsequently, one of the $TAP1^{+/-}$ clones was subjected to selection with high dose of G418 as in the case of $\beta_2m^{-/-}$ ES cells and $TAP1^{-/-}$ ES cell clones were isolated. Eight of the 88 surviving clones were found to be of $TAP^{-/-}$ genotype by genomic PCR (Fig. 2*C*, *left*) and Southern blotting (Fig. 2*C*, *right*). Next, an expression vector for K^d (Fig. 2*D*) was introduced into both $TAP1^{+/+}$ and $TAP1^{-/-}$ ES cells to generate $TAP1^{+/+}$/K^d and $TAP1^{-/-}$/K^d ES cells, respectively. Intrinsic MHC class I molecules, D^b and K^b, as well as transgene-derived K^d, were not detected on the cell surface of $TAP1^{-/-}$/K^d ES-DC (Fig. 2*E*). A low level of cell surface expression of K^d on $TAP1^{-/-}$/K^d ES-DC was observed after incubation of the ES-DC with K^d-binding peptide ($RSV\text{-}M2_{82-90}$) at 26°C for 12 h followed by the incubation at 37°C for 4 h (Fig. 2*E*).

Collectively, ES-DC expressing only transgene-derived K^d but not intrinsic ($H\text{-}2^b$ haplotype-derived) MHC class I molecules on the cell surface were generated by the two methods of genetic modifications of ES cells.

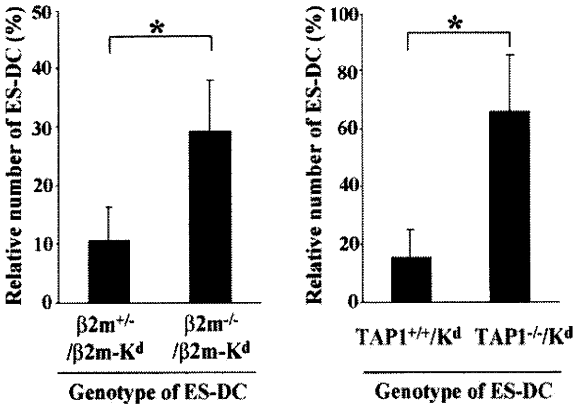

FIGURE 5. The survival advantage of β_2m or TAP1 gene-deficient ES-DC in allogeneic recipient mice. BALB/c mice were injected s.c. into the forelimb with CMFDA-labeled BALB/c BM-DC (1×10^6) and CMTMR-labeled ES-DC (1×10^6). After 48 h, the mice were killed and the number of labeled BM-DC and ES-DC in the axillary and brachial lymph nodes was determined by flow cytometry. The number of detected ES-DC was normalized to control syngeneic BALB/c BM-DC [($CMTMR^+$ ES-DC/$CMFDA^+$ BM-DC) × 100]. The results of experiments with β_2m-deficient ES-DC (*left*) and TAP1-deficient ES-DC (*right*) are shown. Data are representative of two experiments with similar results. The data are the mean ± SD (5–6 mice per each group). Asterisks indicate significant ($p < 0.05$, Student's *t* test) differences.

Avoidance of recognition by alloreactive $CD8^+$ T cells by genetic modification of ES-DC

$H\text{-}2^b$ MHC class I-reactive $CD8^+$ T cells were prepared by a 5-day-culture of BALB/c ($H\text{-}2^d$) spleen cells with irradiated 129 ($H\text{-}2^b$) spleen cells. They produced IFN-γ in response to 129 mouse-derived BM-DC but not against BALB/c BM-DC, confirming that they responded specifically to $H\text{-}2^b$ MHC class I (Fig. 3*A*, *left panel*). The four types of ES-DC derived from $H\text{-}2^b$ ES cells with genetic modification described above were cocultured with the $H\text{-}2^b$-reactive $CD8^+$ T cell line and the activation of the T cells was analyzed. Fig. 3*A* shows that $\beta_2m^{+/-}/\beta_2m\text{-}K^d$ ES-DC (*middle panel*) and $TAP1^{+/+}/K^d$ ES-DC (*right panel*), expressing MHC class I of $H\text{-}2^b$ haplotype along with K^d, were recognized by the $H\text{-}2^b$-reactive $CD8^+$ T cells, thus resulting in IFN-γ production at the magnitude similar to that observed in the case of 129 BM-DC. In contrast, $H\text{-}2^b$-reactive $CD8^+$ T cells showed practically no response to $\beta2m^{-/-}/\beta2m\text{-}K^d$ ES-DC or $TAP1^{-/-}/K^d$ ES-DC. These results indicate that ES-DC that were not recognized by alloreactive $CD8^+$ T cells could be generated by the modification of β_2m or TAP1 gene to inhibit surface expression of intrinsic MHC class I molecules.

Reduced priming of $H\text{-}2^b$-reactive $CD8^+$ T cells in vitro by genetically modified ES-DC

Next, the in vitro priming of alloreactive $CD8^+$ T cells by ES-DC was examined. Spleen cells from BALB/c mice were cocultured with either of the four types of genetically engineered ES-DC for 5 days. After that, $CD8^+$ T cells were isolated from the culture and their reactivity to 129-derived BM-DC (Fig. 3*B*) was measured to assess the magnitude of priming in vitro of $H\text{-}2^b$-reactive $CD8^+$ T cells by the ES-DC. $CD8^+$ T cells cultured with $\beta_2m^{+/-}/\beta_2m\text{-}K^d$ or $TAP1^{+/+}/K^d$ ES-DC in the induction phase responded to 129-derived BM-DC, indicating that both $\beta_2m^{+/-}/\beta_2m\text{-}K^d$ and $TAP1^{+/+}/K^d$ ES-DC primed $H\text{-}2^b$-reactive $CD8^+$ T cells. In contrast, $CD8^+$ T cells cultured with $\beta_2m^{-/-}/\beta2m\text{-}K^d$ or $TAP1^{-/-}/K^d$ ES-DC in the induction step exhibited reduced or no response to 129-derived BM-DC, thus

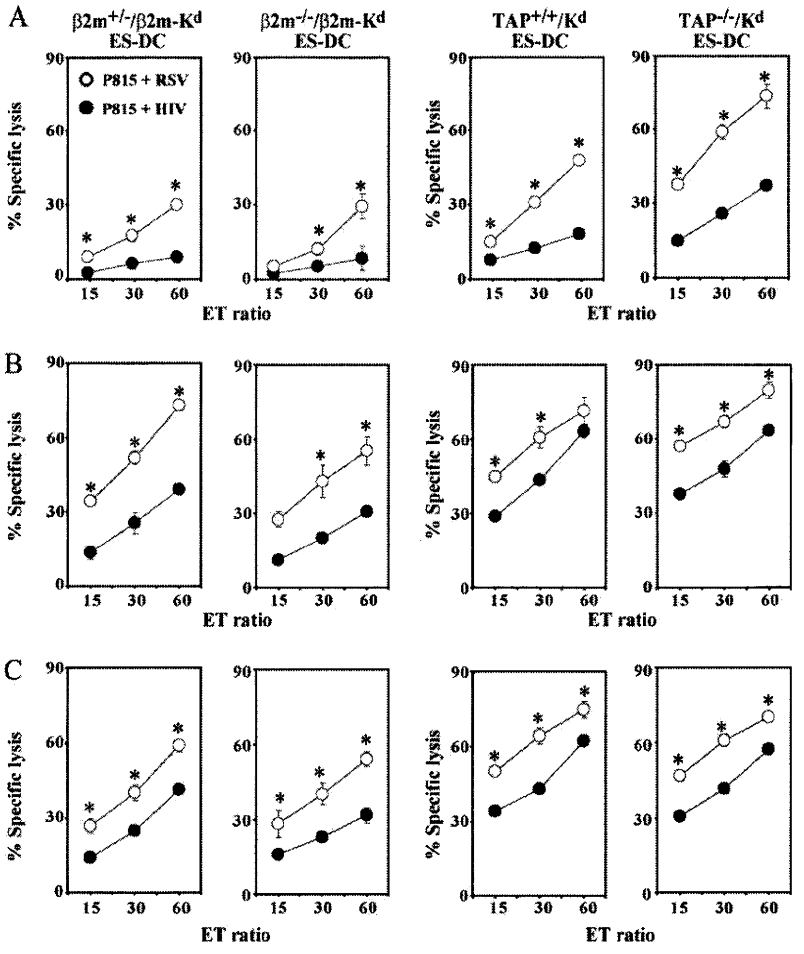

FIGURE 6. Priming of exogenous Ag-specific CTL by injection of genetically modified ES-DC loaded with antigenic peptide. *A*, RSV antigenic peptide-loaded ES-DC (1×10^5/injection/mouse) were injected i.p. into the naive mice twice with a 7-day interval. *B* and *C*, Non-Ag loaded ES-DC were injected 5 (*B*) or 10 (*C*) times with 7 days intervals before immunization with peptide-loaded ES-DC. The mice were sacrificed 7 days after the last injection of ES-DC and the spleen cells were isolated. The spleen cells were cultured in the presence of RSV peptide (1 μM) for 6 days and then analyzed on the RSV peptide-specific cytolytic activity by 5-h ^{51}Cr release assay. As target cells, P815 cells either pulsed with $M2_{82-90}$ peptide (○) or control H-2K^d-restricted HIV gag $p24_{199-207}$ peptide (●) were used. Data are representative of three experiments with similar results. The data are the mean specific lysis ± SD of triplicate assays. The asterisks indicate significant ($p < 0.05$, Student's t test) differences.

indicating that the H-2^b-reactive CD8$^+$ T cells had not been well primed. These results suggest that the in vitro priming of allo-MHC class I-reactive T cells by ES-DC can be reduced through the genetic modification of β_2m or TAP1.

Reduced priming of allo-MHC class I-reactive T cells in vivo by TAP1 or β_2m-deficient ES-DC

The next experiments assessed whether or not priming of alloreactive CD8$^+$ T cells upon in vivo administration of ES-DC could be avoided by the current strategy. The frequency of primed H-2^b-reactive CD8$^+$ T cells in mice was quantified by an ex vivo ELISPOT assay detecting the production of IFN-γ upon stimulation with 129-derived BM-DC. CD8$^+$ T cells from naive BALB/c mice showed little response to 129-derived BM-DC. CD8$^+$ T cells isolated from BALB/c mice injected 5 or 10 times with $\beta_2m^{+/-}$/β2m-K^d (Fig. 4*A*) or TAP1$^{+/+}$/K^d ES-DC (Fig. 4*B*) clearly responded, thus indicating the priming of H-2^b-reactive CD8$^+$ T cells. The magnitude of the response of the mice injected ten times was lower than those injected five times, in both the β2m$^{+/-}$/β_2m-K^d ES-DC and TAP1$^{+/+}$/K^d ES-DC-injected mice, thus suggesting that there is probably a limit in the frequency of alloreactive CD8$^+$ T cells. In contrast, the frequency of H-2^b-reactive CD8$^+$ T cells in mice inoculated with $\beta_2m^{-/-}$/β_2m-K^d or TAP1$^{-/-}$/K^d ES-DC was very low, indicating that alloreactive CD8$^+$ T cells were hardly primed in vivo by these ES-DC.

Surviving advantage of β_2m or TAP1 gene-modified ES-DC in vivo

According to a previous study by another group (23), BM-DC inoculated into allogeneic recipient mice are eliminated within a few days and the number of DC detected in the draining lymph node is lower than that of DC syngeneic to the recipient mice. In such circumstances, the rapid elimination of APC is mainly mediated by CD8$^+$ T cells reactive to allogeneic MHC class I (24). As described so far, the $\beta_2m^{-/-}$/β_2m-K^d ES-DC and TAP1$^{-/-}$/K^d ES-DC did not express intrinsic H-2^b-derived MHC class I molecule and escaped recognition by H-2^b-reactive CD8$^+$ T cells. Therefore, they were expected to have an advantage in surviving in allogeneic BALB/c mice, in comparison to ES-DC expressing H-2^b gene encoded MHC class I on the cell surface.

To examine the effect of genetic modification on the survival of ES-DC upon injection into allogeneic mice, equal numbers of CMTMR-labeled ES-DC and CMFDA-labeled BALB/c derived BM-DC, as a control, were mixed and injected in the right forelimb footpad of BALB/c mice. After 48 h, a single cell suspension was made from the axillary and brachial lymph nodes and the fluorochrome-labeled DC were detected by using flow cytometry (Fig. 5). The number of $\beta_2m^{-/-}$/β_2m-K^d ES-DC in the draining lymph nodes was ~3 times higher than that of $\beta_2m^{+/-}$/β_2m-K^d ES-DC. In the similar experiments, the number of detected TAP1$^{-/-}$/K^d ES-DC was ~4 times higher than that of TAP1$^{+/+}$/K^d ES-DC. These results suggest that ES-DC without cell surface expression of intrinsic MHC class I molecule can thus escape elimination by alloreactive CTL.

Priming of Ag-specific CTL by genetically modified ES-DC in allogeneic recipients

It has been noted that the CTL-mediated elimination of DC has a notable effect on the magnitude of immune responses in vivo (25) and the results so far described indicate that the β_2m or TAP1 gene-modified ES-DC expressing only recipient-matched MHC

class I could thus avoid elimination by CTL upon transfer into allogeneic recipients. Therefore, the ability of such ES-DC to elicit more robust Ag-specific immune responses in allogeneic recipients than ES-DC expressing intrinsic MHC class I was examined.

The priming of a RSV M2 protein epitope ($M2_{82-90}$)-specific and H-2K^d-restricted CTL by ES-DC administered into BALB/c mice was examined. $M2_{82-90}$ peptide-loaded ES-DC were injected i.p. into BALB/c mice twice with a 7-day interval. The spleen cells were isolated from the mice 7 days after the second injection and cultured in vitro in the presence of $M2_{82-90}$ peptide. After 6 days, the cultured spleen cells were recovered and assayed for their capacity to kill P815 mastcytoma cells (H-2^d) prepulsed with the M2 peptide. M2 peptide-specific and K^d-restricted CTL was primed in BALB/c mice immunized with either of the 4 types of genetically modified ES-DC (Fig. 6*A*). Therefore, ES-DC expressing K^d could prime K^d-restricted Ag-specific CTL, irrespective of cell surface expression of intrinsic MHC class I encoded by the H-2^b haplotype.

To determine whether or not ES-DC could prime $M2_{82-90}$ specific CTL in the presence of preprimed H-2^b-reactive CTL, ES-DC without peptide loading were injected in BALB/c mice five (Fig. 6*B*) or ten times (Fig. 6*C*) with 7-day intervals and then the same ES-DC loaded with $M2_{82-90}$ peptide were injected. In addition, in this case, the specific CTL were primed by all of the four types of ES-DC. These results indicate that even in the presence of preactivated alloreactive CTL, ES-DC expressing recipient-matched MHC class I are able to prime the Ag-specific CTL, whether or not the ES-DC express intrinsic MHC class I on their cell surface.

Induction of antitumor immunity by genetically modified ES-DC in allogeneic recipients

Next, antitumor immunity induced by the genetically modified ES-DC was assessed. To this end, a tumor cell line colon26/M2-Luc, a BALB/c-derived colon carcinoma cell line colon26 expressing RSV-M2 along with firefly luciferase, was generated. After the inoculation of the tumor cells, it was possible to quantify the number of cancer cells in mouse tissues by measuring the luciferase activity of tissue homogenates as reported (26). The luciferase activity in the homogenates of colon 26/M2-Luc cells was linearly correlated with the number of the cells in the range from 150 to 350,000 counts per second (Fig. 7*A*). In a pilot study, when the tumor cells were injected into the mice i.p., most tumor cells were detected in the greater omentum and the mesenterium, and the luciferase activity of these two organs were in parallel (data not shown). Therefore, the luciferase activity of the greater omentum was chosen to be measured in the following studies.

BALB/c mice were injected i.p. with ES-DC loaded with $M2_{82-90}$ peptide and other mice were injected with ES-DC prepulsed with irrelevant control peptide (HIV gag $p24_{199-207}$) with K^d-binding affinity. One week after the ES-DC injection, the mice were challenged i.p. with colon26/M2-Luc. After 10 days, the growth of tumor cells was evaluated by measuring the luciferase activity in the homogenate of the greater omentum. In the mice injected with either of the $\beta_2m^{-/-}/\beta2m$-K^d ES-DC (Fig. 7*B*) or TAP1$^{-/-}$/K^d ES-DC (Fig. 7*C*) loaded with M2 peptide, tumor growth was significantly reduced in comparison to the mice injected with the same ES-DC loaded with the control peptide. Therefore, the two types of genetically modified ES-DC could induce Ag-specific antitumor immunity. The antitumor effect induced by $\beta_2m^{-/-}/\beta_2m$-K^d ES-DC was significantly stronger than that induced by $\beta_2m^{+/-}/\beta_2m$-K^d ES-DC, indicating that the disruption of intrinsic β_2m gene and introduction of β_2m-linked MHC class I in ES-DC may provide an advantage in the induction of Ag-specific antitumor immunity.

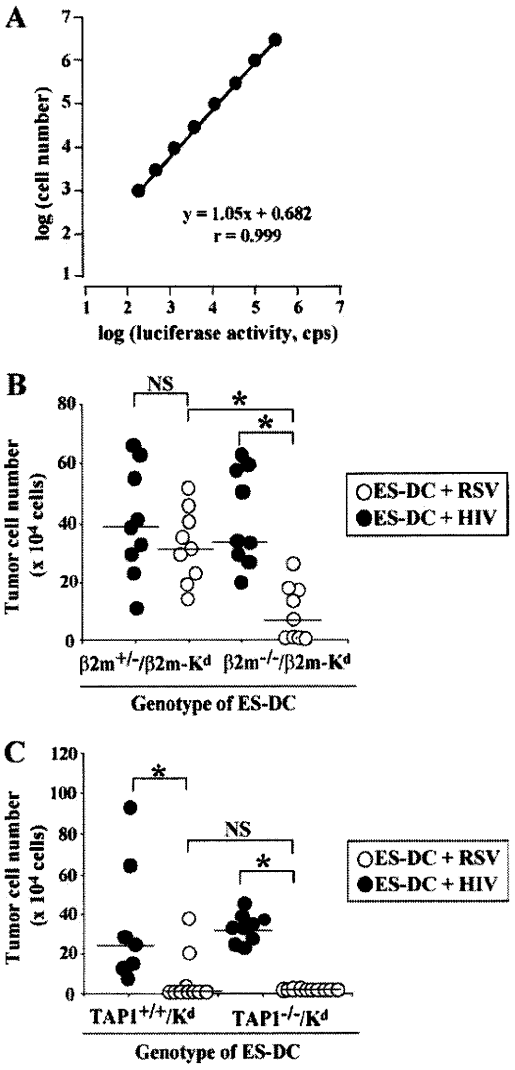

FIGURE 7. Induction of protective immunity by Ag-loaded genetically modified ES-DC against peritoneally disseminated tumor cells in allogeneic recipients. *A*, Homogenates were made from the indicated numbers of in vitro cultured colon26/M2-Luc cells and the luciferase activity was measured. r, correlation coefficient. *B* and *C*, BALB/c mice were injected with $M2_{82-90}$ peptide- or control HIV $p24_{199-207}$ peptide-loaded ES-DC (1×10^5/mouse) i.p. on day –7 and challenged with colon26/M2-Luc (1×10^6/mouse) i.p. on day 0. On day 10, the mice were sacrificed and luciferase activity of the greater omentum was measured. Luciferase activity of tissue lysates was converted to tumor cell number in the greater omentum based on the standard curve shown in *A*. The results of mice treated with $\beta_2m^{+/-}/\beta_2m$-K^d ES-DC or $\beta_2m^{-/-}/\beta_2m$-K^d ES-DC are shown in *B*. The results of mice treated with TAP1$^{+/+}$/K^d ES-DC or TAP1$^{-/-}$/K^d ES-DC are shown in *C*. All data are representative of at least two experiments with similar results. Values for individual mice injected with $M2_{82-90}$ peptide-loaded ES-DC (○) and HIV $p24_{199-207}$ peptide-loaded ES-DC (●) are shown; bars indicate median values. The asterisks indicate significant ($p < 0.05$) differences between two groups based on the Mann-Whitney *U* test. NS, not significant.

Discussion

To induce T cell-mediated anticancer immunity, vaccination with DC loaded with tumor Ag-derived peptides or tumor cell lysates are being clinically tested (27, 28). For such purposes, DC are generated from monocytes obtained from peripheral blood of the patients. However, monocytes are not easily propagated in vitro and apheresis, a procedure sometimes invasive for the patients, is necessary to obtain a sufficient number of monocytes as the source of DC. In addition, the culture to generate DC should be done

separately for each patient and for each treatment and thus the presently used method is too labor-intensive and costly to be broadly applied.

ES cells exhibit the remarkable properties of self-renewal and pluripotency. This capacity allows for the production of sizeable quantities of therapeutic cells of the hematologic lineage, including DC (29). If the ES-DC method is clinically applied, it will be possible to generate genetically engineered DC-expressing target Ags or immunostimulatory molecules, without use of virus-based vectors. However, considering the medical application, one drawback of the ES-DC method is the unavailability of human ES cells genetically identical to the patients to be treated (12). Specifically, an HLA allele mismatch between ES cells and patients is a crucial problem.

In previous studies, it was shown that allogeneic BM-DC are rapidly eliminated from the draining lymph nodes during the course of a primary alloreactive responses (23, 30). Another study revealed that the elimination of transferred APC in allogeneic recipients is mainly mediated by T cells reactive to allogeneic MHC class I but not MHC class II (24). Therefore, if the expression of the intrinsic MHC class I by ES-DC could be blocked, then ES-DC inoculated into allogeneic recipients would escape elimination by the alloreactive T cells of the recipients. However, the genomic region including the MHC class I genes spans more than 1,000 kb in both the mouse and human genome and complete elimination of such a large genomic region by gene-targeting technique is currently infeasible. The feasible candidates for genetic modification are the genes that encode β_2m and TAP, which regulate MHC class I expression (31, 32). Therefore, the present study adopted a strategy to block the cell surface expression of the MHC class I molecules by elimination of the β_2m or TAP1 gene.

Both alleles of the TAP1 or β_2m gene were disrupted in 129-derived ES cells (H-2^b) and subsequently expression vectors for the recipient (BALB/c)-matched K^d or β_2m-linked form of K^d were introduced. The genetically modified ES cells were subjected to the differentiation culture to generate TAP1$^{-/-}$/K^d ES-DC and $\beta_2m^{-/-}$/β_2m-K^d ES-DC. As intended, the $\beta_2m^{-/-}$/β_2m-K^d ES-DC expressed only K^d molecule as MHC class I molecules on the cell surface. TAP1$^{-/-}$/K^d ES-DC hardly expressed any classical MHC class I and a low level of cell surface expression of K^d was observed after incubation with K^d-binding peptide. In vitro, $\beta_2m^{-/-}$/β_2m-K^d and TAP1$^{-/-}$/K^d ES-DC were not recognized by pre-activated H-2^b-reactive CD8 T^+ cells and the ES-DC did not prime H-2^b-reactive $CD8^+$ T cells (Fig. 3). When these cells were inoculated into BALB/c mice, they did not prime H-2^b reactive $CD8^+$ T cells in vivo (Fig. 4).

Consistent with these results, $\beta_2m^{-/-}$/β2m-K^d ES-DC and TAP1$^{-/-}$/K^d ES-DC had a survival advantage in comparison to $\beta_2m^{+/-}$/β_2m-K^d ES-DC and TAP1$^{+/+}$/K^d ES-DC, when transferred into BALB/c mice (Fig. 5). The results suggest that ES-DC deficient in β_2m or TAP1 and expressing only recipient-matched MHC class I were resistant to elimination by alloreactive CTL. It has been shown that CTL-mediated elimination of DC has a notable effect on the magnitude of immune responses in vivo (25, 33). Therefore, β_2m- or TAP1-deficient ES-DC should be able to elicit more robust priming of Ag-specific CTL in allogeneic recipients than ES-DC expressing intrinsic MHC class I. When loaded with RSV-derived peptide and inoculated into BALB/c mice, not only $\beta_2m^{-/-}$/β_2m-K^d and TAP1$^{-/-}$/K^d ES-DC but also $\beta_2m^{+/-}$/β_2m-K^d and TAP1$^{+/+}$/K^d ES-DC primed K^d-restricted, RSV peptide-specific CTL (Fig. 6). Unexpectedly, there was no significant difference in the magnitude of priming of Ag-specific CTL among these ES-DC. CTL-mediated allogeneic DC elimination is mainly dependent on the perforin/granzyme B pathway (24, 30). Therefore, the result shown in Fig. 6 may be due to resistance of ES-DC to killing by CTL that is attributed partly to the high level of expression of SPI-6, the granzyme B-specific protease inhibitor, in ES-DC (34). In addition, in the experiments shown in Fig. 6, we cultured spleen cells isolated from immunized mice for 5 days in the presence of RSV-M2 peptide to amplify RSV-specific CTL before the cytotoxicity assay, because we could not detect RSV-specific CTL activity in a direct ex vivo killing assay. Probably, in the data shown in Fig. 6, difference in the CTL activity induced in vivo by the different genotype of ES-DC may have been masked by this culture procedure.

In the present study, the MHC class II haplotype of ES-DC was always b while that of the recipient mice (BALB/c) was d. Therefore, there was mismatch in MHC class II haplotype between ES-DC and the recipient mice in all of the experiments. Because the mismatch of the MHC class II allele has been reported to not cause any acute elimination of transferred APC (24), the class II mismatch may not have negatively affected the priming of Ag-specific CTL in the present study. Instead, alloreactive helper T cells are expected to enhance the CTL response via cytokine production, although we did not experimentally address this issue in the present study.

Theoretically, the issue of histocompatibility related to the ES cell-based medical technology may be resolved by the recent development of induced pluripotent stem (iPS) cells that can be generated by introduction of several defined genes into somatic cells (35–38). However, the medical application of iPS cells nevertheless has some drawbacks. The use of virus vectors is necessary to generate iPS cells and generation of iPS cells for individual patients may be too costly, time consuming, and labor-intensive to be broadly applied. The genetic modification of ES cells or iPS cells to modify cell surface HLA class I by the presently reported methods may be more economical, faster and thus, more realistic than the individual generation of "fully personalized iPS cells".

Although targeted gene disruption of OCT4 and HPRT in human ES cells has been reported (39), the methodology of gene targeting for human ES cells has not been well established at present. Therefore, as an alternative strategy, we are planning to generate iPS cells from patients with Type I bare lymphocyte syndrome caused by mutation of the TAP 1 or TAP 2 gene (40, 41). Once a clone of TAP- or β_2m-deficient human ES or iPS cells is established, a premade library of pluripotent stem cell clones expressing various types of HLA class I can be generated by the introduction of various HLA class I genes. Such a pluripotent stem cell bank may serve as a source of not only DC but also of various kinds of differentiated cells that may be useful in the field of regenerative medicine.

Disclosures

The authors have no financial conflict of interest.

References

1. Banchereau, J., and A. Palucka. 2005. Dendritic cells as therapeutic vaccines against cancer. *Nat. Rev. Immunol.* 5: 296–306.
2. Brossart, P., S. Wirths, G. Stuhler, V. Reichardt, L. Kanz, and W. Brugger. 2000. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood* 96: 3102–3108.
3. Banchereau, J., and R. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
4. Fairchild, P., F. Brook, R. Gardner, L. Graça, V. Strong, Y. Tone, M. Tone, K. Nolan, and H. Waldmann. 2000. Directed differentiation of dendritic cells from mouse embryonic stem cells. *Curr. Biol.* 10: 1515–1518.
5. Senju, S., S. Hirata, H. Matsuyoshi, M. Masuda, Y. Uemura, K. Araki, K. Yamamura, and Y. Nishimura. 2003. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. *Blood* 101: 3501–3508.
6. Zhan, X., G. Dravid, Z. Ye, H. Hammond, M. Shamblott, J. Gearhart, and L. Cheng. 2004. Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro. *Lancet* 364: 163–171.

7. Slukvin, I., M. Vodyanik, J. Thomson, M. Gumenyuk, and K. Choi. 2006. Directed differentiation of human embryonic stem cells into functional dendritic cells through the myeloid pathway. *J. Immunol.* 176: 2924–2932.
8. Senju, S., H. Suemori, H. Zembutsu, Y. Uemura, S. Hirata, D. Fukuma, H. Matsuyoshi, M. Shimomura, M. Haruta, S. Fukushima, et al. 2007. Genetically manipulated human embryonic stem cell-derived dendritic cells with immune regulatory function. *Stem Cells* 25: 2720–2729.
9. Hirata, S., S. Senju, H. Matsuyoshi, D. Fukuma, Y. Uemura, and Y. Nishimura. 2005. Prevention of experimental autoimmune encephalomyelitis by transfer of embryonic stem cell-derived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand. *J. Immunol.* 174: 1888–1897.
10. Motomura, Y., S. Senju, T. Nakatsura, H. Matsuyoshi, S. Hirata, M. Monji, H. Komori, D. Fukuma, H. Baba, and Y. Nishimura. 2006. Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16–F10. *Cancer Res.* 66: 2414–2422.
11. Matsuyoshi, H., S. Hirata, Y. Yoshitake, Y. Motomura, D. Fukuma, A. Kurisaki, T. Nakatsura, Y. Nishimura, and S. Senju. 2005. Therapeutic effect of α-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells. *Cancer Sci.* 96: 889–896.
12. Bradley, J., E. Bolton, and R. Pedersen. 2002. Stem cell medicine encounters the immune system. *Nat. Rev. Immunol.* 2: 859–871.
13. Robertson, N., F. Brook, R. Gardner, S. Cobbold, H. Waldmann, and P. Fairchild. 2007. Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance. *Proc. Natl. Acad. Sci. USA* 104: 20920–20925.
14. Kulkarni, A., H. R. Morse, J. Bennink, J. Yewdell, and B. Murphy. 1993. Immunization of mice with vaccinia virus-M2 recombinant induces epitope-specific and cross-reactive Kd-restricted $CD8^+$ cytotoxic T cells. *J. Virol.* 67: 4086–4092.
15. Doe, B., M. Selby, S. Barnett, J. Baenziger, and C. Walker. 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. USA* 93: 8578–8583.
16. Zijlstra, M., E. Li, F. Sajjadi, S. Subramani, and R. Jaenisch. 1989. Germ-line transmission of a disrupted β 2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature* 342: 435–438.
17. Senju, S., K. Iyama, H. Kudo, S. Aizawa, and Y. Nishimura. 2000. Immunocytochemical analyses and targeted gene disruption of GTPBP1. *Mol. Cell. Biol.* 20: 6195–6200.
18. Mortensen, R. M., D. A. Conner, S. Chao, A. A. Geisterfer-Lowrance, and J. G. Seidman. 1992. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* 12: 2391–2395.
19. Joyce, S., I. Negishi, A. Boesteanu, A. D. DeSilva, P. Sharma, M. J. Chorney, D. Y. Loh, and L. Van Kaer. 1996. Expansion of natural ($NK1^+$) T cells that express $\alpha\beta$ T cell receptors in transporters associated with antigen presentation-1 null and thymus leukemia antigen positive mice. *J. Exp. Med.* 184: 1579–1584.
20. Hirata, S., H. Matsuyoshi, D. Fukuma, A. Kurisaki, Y. Uemura, Y. Nishimura, and S. Senju. 2007. Involvement of regulatory T cells in the experimental autoimmune encephalomyelitis-preventive effect of dendritic cells expressing myelin oligodendrocyte glycoprotein plus TRAIL. *J. Immunol.* 178: 918–925.
21. Raulet, D., P. Gottlieb, and M. Bevan. 1980. Fractionation of lymphocyte populations with monoclonal antibodies specific for LYT-2.2 and LYT-3.1. *J. Immunol.* 125: 1136–1143.
22. Ritchie, D. S., I. F. Hermans, J. M. Lumsden, C. B. Scanga, J. M. Roberts, J. Yang, R. A. Kemp, and F. Ronchese. 2000. Dendritic cell elimination as an assay of cytotoxic T lymphocyte activity in vivo. *J. Immunol. Methods* 246: 109–117.
23. Wells, J. W., C. J. Cowled, D. Darling, B. A. Guinn, F. Farzaneh, A. Noble, and J. Galea-Lauri. 2007. Semi-allogeneic dendritic cells can induce antigen-specific T-cell activation, which is not enhanced by concurrent alloreactivity. *Cancer Immunol. Immunother.* 56: 1861–1873.
24. Loyer, V., P. Fontaine, S. Pion, F. Hetu, D. C. Roy, and C. Perreault. 1999. The in vivo fate of APCs displaying minor H antigen and/or MHC differences is regulated by CTLs specific for immunodominant class I-associated epitopes. *J. Immunol.* 163: 6462–6467.
25. Hermans, I. F., D. S. Ritchie, J. Yang, J. M. Roberts, and F. Ronchese. 2000. $CD8^+$ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. *J. Immunol.* 164: 3095–3101.
26. Hyoudou, K., M. Nishikawa, Y. Kobayashi, Y. Kuramoto, F. Yamashita, and M. Hashida. 2006. Inhibition of adhesion and proliferation of peritoneally disseminated tumor cells by pegylated catalase. *Clin. Exp. Metastasis* 23: 269–278.
27. Chang, A., B. Redman, J. Whitfield, B. Nickoloff, T. Braun, P. Lee, J. Geiger, and J. Mulé. 2002. A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. *Clin. Cancer Res.* 8: 1021–1032.
28. Wierecky, J., M. Mueller, and P. Brossart. 2006. Dendritic cell-based cancer immunotherapy targeting MUC-1. *Cancer Immunol. Immunother.* 55: 63–67.
29. Olsen, A., D. Stachura, and M. Weiss. 2006. Designer blood: creating hematopoietic lineages from embryonic stem cells. *Blood* 107: 1265–1275.
30. Laffont, S., J. Coudert, L. Garidou, L. Delpy, A. Wiedemann, C. Demur, C. Coureau, and J. Guéry. 2006. $CD8^+$ T-cell-mediated killing of donor dendritic cells prevents alloreactive T helper type-2 responses in vivo. *Blood* 108: 2257–2264.
31. Van Kaer, L., P. Ashton-Rickardt, H. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4-8^+$ T cells. *Cell* 71: 1205–1214.
32. Zijlstra, M., M. Bix, N. Simister, J. Loring, D. Raulet, and R. Jaenisch. 1990. β2-microglobulin deficient mice lack $CD4-8^+$ cytolytic T cells. *Nature* 344: 742–746.
33. Yang, J., S. Huck, R. McHugh, I. Hermans, and F. Ronchese. 2006. Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific $CD8^+$ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 103: 147–152.
34. Fukuma, D., H. Matsuyoshi, S. Hirata, A. Kurisaki, Y. Motomura, Y. Yoshitake, M. Shinohara, Y. Nishimura, and S. Senju. 2005. Cancer prevention with semi-allogeneic ES cell-derived dendritic cells. *Biochem. Biophys. Res. Commun.* 335: 5–13.
35. Takahashi, K., and S. Yamanaka. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126: 663–676.
36. Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861–872.
37. Yu, J., M. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. Frane, S. Tian, J. Nie, G. Jonsdottir, V. Ruotti, R. Stewart, et al. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318: 1917–1920.
38. Lowry, W., L. Richter, R. Yachechko, A. Pyle, J. Tchieu, R. Sridharan, A. Clark, and K. Plath. 2008. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc. Natl. Acad. Sci. USA* 105: 2883–2888.
39. Zwaka, T., and J. Thomson. 2003. Homologous recombination in human embryonic stem cells. *Nat. Biotechnol.* 21: 319–321.
40. de la Salle, H., D. Hanau, D. Fricker, A. Urlacher, A. Kelly, J. Salamero, S. Powis, L. Donato, H. Bausinger, and M. Laforet. 1994. Homozygous human TAP peptide transporter mutation in HLA class I deficiency. *Science* 265: 237–241.
41. Furukawa, H., S. Murata, T. Yabe, N. Shimbara, N. Keicho, K. Kashiwase, K. Watanabe, Y. Ishikawa, T. Akaza, K. Tadokoro, et al. 1999. Splice acceptor site mutation of the transporter associated with antigen processing-1 gene in human bare lymphocyte syndrome. *J. Clin. Invest.* 103: 755–758.

特集：特異抗原をターゲットとした Immunotherapy
総　説

新規癌胎児性抗原 Glypican-3 の肝細胞癌の診断と免疫療法への応用

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Usefulness of a novel oncofetal antigen, Glypican-3, for diagnosis and immunotherapy of hepatocellular carcinoma

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summary

We identified glypican-3 (GPC3), as a novel oncofetal antigen, overexpressed specifically in hepatocellular carcinoma (HCC) and melanoma in humans by utilizing genome-wide cDNA microarray analyses of HCC tissues and normal fetal and adult tissues. We also found that GPC3 is a novel tumor marker for HCC and melanoma, and that the pre-immunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse GPC3 298-306 (EYILSLEEL) peptide prevented the growth of tumor expressing mouse GPC3. Because of similarities in the binding peptide motifs between H-2K^d and HLA-A24 (*A*2402*), the H-2K^d-restricted GPC3 298-306 peptide thus seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. We investigated whether the GPC3 298-306 peptide could induce GPC3 reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A24 (*A*2402*)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice (Tgm) to identify the HLA-A2 (*A*0201*)-restricted GPC3 epitopes to expand the applications of GPC3 based immunotherapy to the HLA-A2$^+$ HCC patients. We found that the GPC3 144-152 (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) Tgm without inducing autoimmunity. In 5 out of 8 HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3 144-152 peptide-reactive CTLs were generated from PBMCs by in vitro stimulation with the peptide and the GPC3 298-306 peptide-reactive CTLs were also generated from PBMCs in 4 of 6 HLA-A24$^+$ GPC3$^+$ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into NOD/SCID mice. We have recently started a phase I clinical trial of GPC3 peptide vaccine-based immunotherapy of patients with advanced HCC.

We have also succeeded in inhibition of growth of tumors expressing mouse GPC3 by immunization of mice with dendritic cells differentiated in vitro from mouse embryonic stem cells and pulsed with the GPC3 peptides. Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of patients with HCC and melanoma.

Key words——肝細胞癌（HCC）；癌特異抗原；Glypican-3（GPC3）；癌免疫療法；ES-DC

抄　録

ヒトの肝細胞癌組織と正常組織における cDNA マイクロアレイ解析により，肝細胞癌に高発現する遺伝子として Glypican-3（GPC3）を同定した．GPI アンカー膜蛋白質である GPC3 は，肝細胞癌患者の約 40％の血清中に検出される新規癌胎児性抗原であり，αフェト蛋白，PIVKA-II につぐ肝細胞癌の第 3 の腫瘍マーカーとして有用であることを示した．また，マウスに GPC3 ペプチドを負荷した樹状細胞を投与した後に，マウス GPC3 を発現する癌細胞株を移植すると，自己免疫現象を伴うことなく著明な腫瘍の増殖抑制と生存期間の延長を誘導できた．さらに，HLA-A2 トランスジェニックマウスや，癌患者の血液検体を利用して，HLA-A2 あるいは A24 によりヒト・キラー T 細胞に提示される GPC3 ペプチドを同定した．これらのペプチドで癌患者のリンパ球を刺激することにより，GPC3 発現ヒト肝細胞癌細胞株を傷害するヒト・キラー T 細胞を誘導できた．これらの GPC3 ペプチドを用いた，肝細胞癌の免疫療法に関する臨床試験を開始した．また，我々はマウス胚性幹（ES）細胞から樹状細胞（ES-DC）を分化誘導する方法を開発し，マウス GPC3 を発現する ES-DC をマウスに免疫したところ，GPC3 発現マウス癌細胞株に対する in vivo 抗腫瘍効果の誘導が観察された．

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はじめに

癌細胞にのみ発現する抗原を免疫することにより，癌細胞を攻撃して破壊するT細胞を誘導する免疫療法を確立するために，様々な癌抗原ワクチンの開発が試みられている．従来，正常組織に発現を認めず癌細胞に特異的に高発現する癌抗原を同定することは困難であったが，cDNAマイクロアレイ解析による癌組織と正常組織におけるゲノムワイドの遺伝子発現プロフィール解析により，癌特異抗原の同定が飛躍的に進んだ．我々は，この手法を用いて多数の癌特異抗原を同定し，これを用いた癌免疫療法の臨床試験を開始している．本稿では，肝細胞癌に高発現する新規癌胎児性抗原であるGlypican-3（GPC3）の発見と，その癌免疫療法への応用について紹介する．

さらに，筆者らは，細胞ワクチンとして用いる樹状細胞の供給源として胚性幹（ES）細胞に着目し，ES細胞由来の樹状細胞（ES-DC）を用いた免疫療法の開発に関する基礎研究を行っている．本稿では，マウスの腫瘍モデルを用いたES細胞から分化誘導したES-DCによる腫瘍免疫の誘導に関する研究成果，ならびに，最近開発したヒトES-DCの分化誘導法についても紹介する．

I. 肝細胞癌（HCC）に対する免疫療法の現況

肝細胞癌（HCC）の患者数は，欧米およびアジア諸国において依然として増加している．HCCは治療後も高頻度に再発を繰り返すため予後不良な癌であり，B型およびC型肝炎と，それに引き続いて発症する肝硬変から発生する，ごく初期の癌に対する早期治療法や，治療後の再発予防のために有効な補助療法の確立が望まれている．

慢性肝炎，肝硬変患者におけるHCCの発症予防や，HCC術後における術後化学療法は，いまだ開発途上にある．HCCに対する免疫療法についても，1990年代よりlymphokine-activated killer（LAK）cells，tumor-infiltrating lymphocytes（TIL），peripheral blood mononuclear cell（PBMC）を用いた養子免疫療法，DCワクチン療法，αフェト蛋白質由来のペプチドワクチン療法などが試みられている．またHCCにおいて高発現する癌特異的抗原も複数報告されており，各施設でその有用性が検討されている[1]．

II. 新規癌胎児性抗原Glypican-3（GPC3）の発見

1. cDNAマイクロアレイ解析によるHCC特異的な癌胎児性抗原の発見

我々は，東京大学医科学研究所ヒトゲノムセンターの中村祐輔博士らとの共同研究により，癌部と非癌部におけるcDNAマイクロアレイ解析データ[2]を用いて肝細胞癌（HCC）特異的に高発現する遺伝子としてGlypican-3（GPC3）を同定した[3]．図1Aに示すように，GPC3は正常な肝細胞と比較してHCCの約80％の症例のHCC組織において高発現しているが，成人の正常組織には，ほとんど発現していない．いっぽうGPC3は胎盤や胎児期の肝臓，肺あるいは腎臓に高発現しており，いわゆる癌胎児性抗原（Carcinoembryonic antigenあるいはOncofetal antigen）の範疇に入る蛋白質である．

2. GPC3の構造と機能

膜結合型糖蛋白質であるGlypicanファミリーは，現在までのところ6種類が報告されている[4]．GPC3は，580アミノ酸からなる60 kDのコア蛋白質にヘパラン硫酸糖鎖修飾が加わった膜蛋白質で，C末端がGPIアンカーにより形質膜に結合している．Piliらは，X染色体（Xq26）連鎖疾患である巨人症の一つである，Simpson-Golabi-Behmel症候群において，GPC3の遺伝子変異を報告している．また，GPC3ノックアウトマウスでも，Simpson-Golabi-Behmel症候群と同様に体の巨大化などの表現型を示すことが報告されている．

GPC3は，ある種の腫瘍細胞では増殖を抑制したり，あるいはアポトーシスの誘導に関連があると報告されている[5]．近年，GPC3コア蛋白質が直接Wntと結合することにより，Wntシグナルを活性化し，肝細胞癌の増殖を促進することが報告されている[6]．

III. HCC癌組織におけるGPC3の発現と腫瘍マーカーとしての有用性

我々は，GPC3遺伝子の発現量の差が，その遺伝子産物である蛋白質量の差として反映されているか否かについてRT-PCR法，ならびに組織切片における免疫組織化学的解析を用いて確認した（図1B，C）．その結果，GPC3は蛋白質レベルにおいても，胎児期の肝臓組織に発現するが出生後発現しなくなり，HCCにおいて再び発現することを確認し

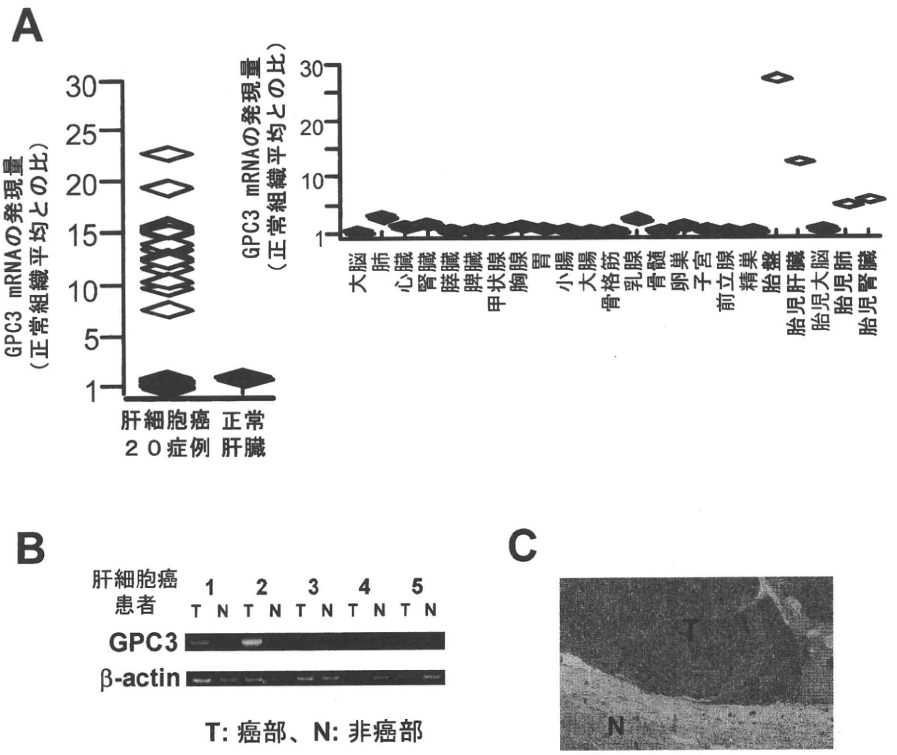

図1　HCC 20 症例の癌部，非癌部および多様な正常臓器における GPC3 遺伝子発現の cDNA マイクロアレイ解析データ[2]と HCC 組織における GPC3mRNA および蛋白質の発現

A：HCC 患者 20 症例の癌部と非癌部における 23,040 種類の遺伝子の発現を比較検討し，さらに胎生期の 4 臓器を含む 23 臓器の正常組織において，各遺伝子の発現プロフィールを解析した．GPC3 は，肝臓癌患者 20 症例中 16 症例で癌部/非癌部の発現の比が 5 以上（平均 396.2）で，胎盤や胎生期の肝臓および腎臓に発現する以外は，ほとんどの成人の正常臓器に発現を認めない，癌胎児性抗原をコードする遺伝子であった．B：HCC 組織の癌部（T）と非癌部（N）における GPC3 mRNA の発現の有無を RT-PCR 法にて検討したところ，癌部においてのみ GPC3 の発現を認めた．C：HCC 組織切片における GPC3 蛋白質の発現を，抗 GPC3 抗体を用いた免疫組織学的解析により確認した．

た．

さらに HCC 患者の約 40％の血清中に可溶性 GPC3 が検出されるが，健常人，慢性肝炎，その他の肝疾患では全く検出されず，HCC の血清腫瘍マーカーとして有用であることを発見した[3]．また HCC の外科的な治療後に，血清 GPC3 が消失あるいは減少することから，治療効果の判定などの臨床への応用が期待される．

IV．癌免疫療法のターゲットとしての GPC3 の有用性

1．マウスにおける抗腫瘍免疫の解析

発現の組織特異性が優れていることから，我々は癌胎児性抗原 GPC3 が，理想的な腫瘍拒絶抗原になり得るかどうかについてマウスを用いて検討した．日本人の約 60％が所有する HLA-A24 と，BALB/c マウスのクラス I 分子の K^d に結合するペプチドの構造モチーフは，非常に類似していることがわかっている．さらに，ヒトとマウスの GPC3 では，アミノ酸配列のレベルで 95％以上のホモロジーを認めることから，ヒトとマウスの GPC3 でアミノ酸配列が完全に一致し，HLA-A24 および K^d のいずれにも結合すると予測される GPC3 由来のペプチドを合成した．このペプチドを骨髄由来樹状細胞に負荷し，BALB/c マウスに免疫して解析することにより，K^d 分子に結合して細胞傷害性 T 細胞（CTL）に提示される K^d 拘束性 CTL エピトープペプチドを同定した[7]．

このエピトープペプチドを負荷した骨髄由来樹状細胞ワクチンを腹腔内に予防的に投与した BALB/c マウスでは，コントロール群に比べてマウス GPC3 遺伝子を強制発現させたマウス大腸癌細胞株の増殖は著明に抑制され，さらにマウスの生存期間の延長が確認された[7]．このエピトープペプチドは HLA-A24 によっても提示され，ヒトでも同様に CTL エピトープとなる可能性があると思われた．

2．HCC 患者における GPC3 特異的 CTL の誘導

日本人の HLA-クラス I 対立遺伝子のうち，HLA-A24（*A*2402*）は日本人の約 60％が所有し，