Table 2. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 86 patients with neuroblastoma found clinically

Characteristics	No. of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
All patients	86	239 ± 357	
Age			
< 12 months	27	282 ± 471	
≥ 12 months	59	219 ± 294	0.7694 (MW)
Stage			
1 + 2 + 4s	21	154 ± 187	
3 + 4	65	266 ± 394	0.3900 (MW)
Primary site			
Mediastinal	11	124 ± 207	
Adrenal	46	285 ± 383	
Abdominal	26	220 ± 375	0.0982 (KW)
Others	3		
MYCN copy number			
1	59	157 ± 193	
> 3	27	418 ± 534	0.0028 (MW)
TrkA expression	63		
Medium + high	28	154 ± 189	
0 + low	35	296 ± 422	0.1865 (MW)
Ploidy	66		
Diploid	37	255 ± 436	
Hyperdiploid	27	234 ± 352	0.4304 (MW)

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test.

indicate that the serum NM23-H1 level serves as a useful prognostic factor for neuroblastoma, as well as the other well-known prognostic factors.

Subsequently, we classified the 86 patients into two groups according to the age of the patients, stage of the disease, or copy numbers of *MYCN*, and evaluated the influence of the serum NM23-H1 levels on the overall survival in each one of the six groups (Fig. 3). Of the 29 patients younger than 12 months of age, the seven patients with higher levels of NM23-H1 had a worse outcome than the 22 patients with the lower levels ($P = 0.0401$ according to the generalized Wilcoxon test and $P = 0.0273$ according to the log-rank test; Fig. 3a). The seven patients with higher levels of NM23-H1 had the following attributes: stage 1 + 2 + 4S ($n = 3$); stage 3 + 4 ($n = 4$); with non-amplified *MYCN* ($n = 4$); with more than three *MYCN* ($n = 3$). Likewise, of the 19 patients with a stage 3 tumor, four patients with higher levels had a worse outcome than the 15 patients with lower levels ($P = 0.0005$ and $P < 0.0001$; Fig. 3c). The four patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 0$); > 12 months of age ($n = 4$); with non-amplified *MYCN* ($n = 1$); with more than three *MYCN* ($n = 3$). Of the 59 patients with a single copy of *MYCN*, the 11 patients with higher levels had a worse outcome than the 48 patients with lower levels of serum NM23-H1 ($P = 0.0301$ and $P < 0.0366$; Fig. 3e). The 11 patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 4$); > 12 months of age ($n = 7$); stage 1 + 2 + 4S ($n = 2$); stage 3 + 4 ($n = 9$). In contrast, a higher serum NM23-H1 level did not influence overall survival in the 57 patients 12 months old or older, in the 46 patients with stage 4 disease, or in the 27 patients with *MYCN* amplification (Fig. 3b,d,f).

Four prognostic factors, including the age of the patients, stage of the disease, *MYCN* copy number, and the serum NM23-H1 level, were available for multivariate analysis in the 217 patients (Table 3a) and 86 patients (Table 3b). According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients (Table 3).

Discussion

The *NM23-H1* gene is overexpressed in various hematological malignancies and other neoplasms including neuroblastoma. Overexpression of *NM23-H1* mRNA is indicative of a poor prognosis in patients with neuroblastoma, and mutations and increased copy numbers of *NM23-H1* have been reported in advanced neuroblastoma.[6,24] In the present study, we found that the serum NM23-H1 level was significantly higher in patients with neuroblastoma than in the control children (Fig. 1), and that the serum NM23-H1 level predicted a poor outcome for patients with tumors (Fig. 2a). Furthermore, the higher level of NM23-H1 was correlated with a worse outcome in patients younger than 12 months of age, in those with stage 3 disease, or in those with a single *MYCN* copy (Fig. 3). In contrast, a higher serum NM23-H1 level did not influence overall survival in patients who were 12 months old or older, in those with stage 4 disease, or in those with *MYCN*

Table 3. Univariate and multivariate analysis for predictors of survival in neuroblastoma

Prognostic factors	Univariate (χ^2, log-rank)	*P*-value	Multivariate (relative risk & 95% CI)	*P*-value
Patients found by mass-screening or clinically ($n = 217$)				
Serum NM23-H1(< 250/> 250 ng/mL)	11.211	0.0008	1.7294 (0.7997–3.7398)	0.1639
Age (< 12/≥ 12 months)	32.353	< 0.00001	3.8979 (1.3818–10.996)	0.0101
Stage (1, 2, 4s/3, 4)	33.142	< 0.00001	8.2514 (1.8173–37.466)	0.0063
NMYC amplification (–/+)	43.997	< 0.00001	2.3253 (1.0541–5.1297)	0.0366
Patients found clinically ($n = 86$)				
Serum NM23-H1(< 250/> 250 ng/mL)	4.493	0.0340	1.6143 (0.7386–3.5282)	0.2299
Age (< 12/≥ 12 months)	5.825	0.0158	1.4742 (0.4877–4.4563)	0.4916
Stage (1, 2, 4s/3, 4)	6.994	0.0082	3.5721 (0.7158–17.826)	0.1206
NMYC amplification (–/+)	7.749	0.0054	1.9682 (0.9016–4.2967)	0.0892

CI, confidence interval.

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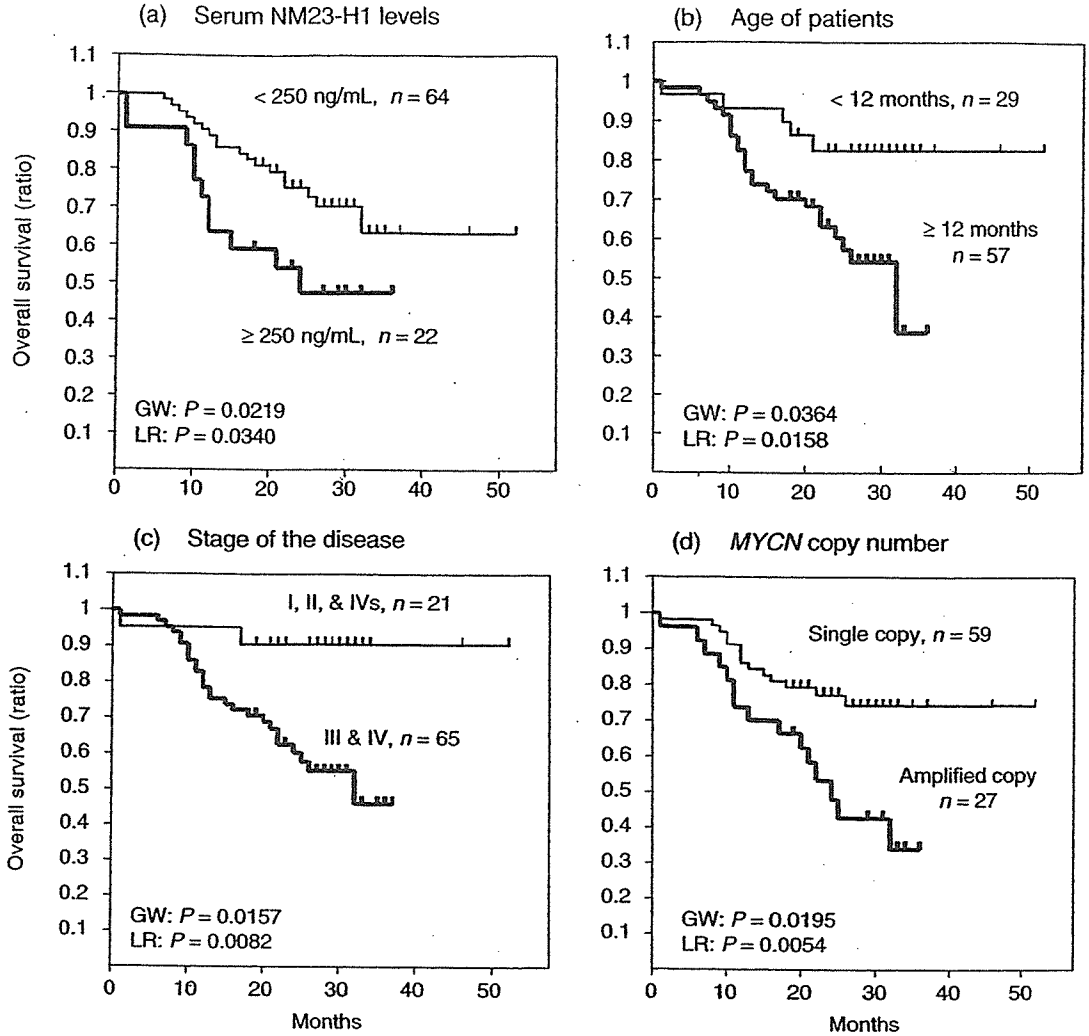

Fig. 2. Overall survival curves for 86 patients with neuroblastoma who were found clinically. (a) Overall survival curves for 22 patients with a serum NM23-H1 level ≥ 250 ng/mL, and for 64 patients with a level < 250 ng/mL. (b) Overall survival curves for 57 patients 12 months of age or older, and for 29 patients younger than 12 months. (c) Overall survival curves for 65 patients at stages 3 and 4 of the disease, and for 21 patients at stages 1, 2 and 4s. (d) Overall survival curves for 27 patients with *MYCN* amplification, and for 59 patients with a single copy of *MYCN*. GW, generalized Wilcoxon's test; LR, log-rank test.

amplification (Fig. 3). These findings suggest that the NM23-H1 level may be an important factor for predicting the outcome of patients in these low or intermediate risk groups (i.e. patients younger than 12 months of age, with stage 3 disease, or with a single copy of *MYCN*). In addition, the serum NM23-H1 level may be a clinically useful prognostic factor, because the measurement of serum NM23-H1 protein is easily and quickly carried out prior to treatment.

According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients shown in Table 3. These results might be due to the short observation time, the small number of cases, or the strong correlation between *MYCN* amplification and the elevated serum NM23-H1 level.

Although all the 131 patients found by MS were alive at the last follow-up (18–51 months) and were excluded from survival analysis, they contained 15 patients (the last follow-up: 19–37 months) with higher levels than 250 ng/mL of serum NM23-H1. It might be interesting to follow up these patients to clarify the clinical significance of serum NM23-H1 in the MS group.

Prognostic factors in neuroblastoma have been thoroughly investigated and include *MYCN* copy number, *TRKA* expression level, chromosomal ploidy, 1p loss, and 17q gain in tumor cells. Laborious and time-consuming work is required to examine these biological factors in tumor tissues. Therefore, serum markers that are easily measurable and can predict a clinical outcome are desired. Serum levels of lactate dehydrogenase (LDH) and ferritin are high in advanced stage neuroblastomas, but both may reflect a rapid cellular turnover or a large tumor burden.[25,26] Neuron-specific enolase (NSE) is a cytoplasmic protein that is associated with neural cells,

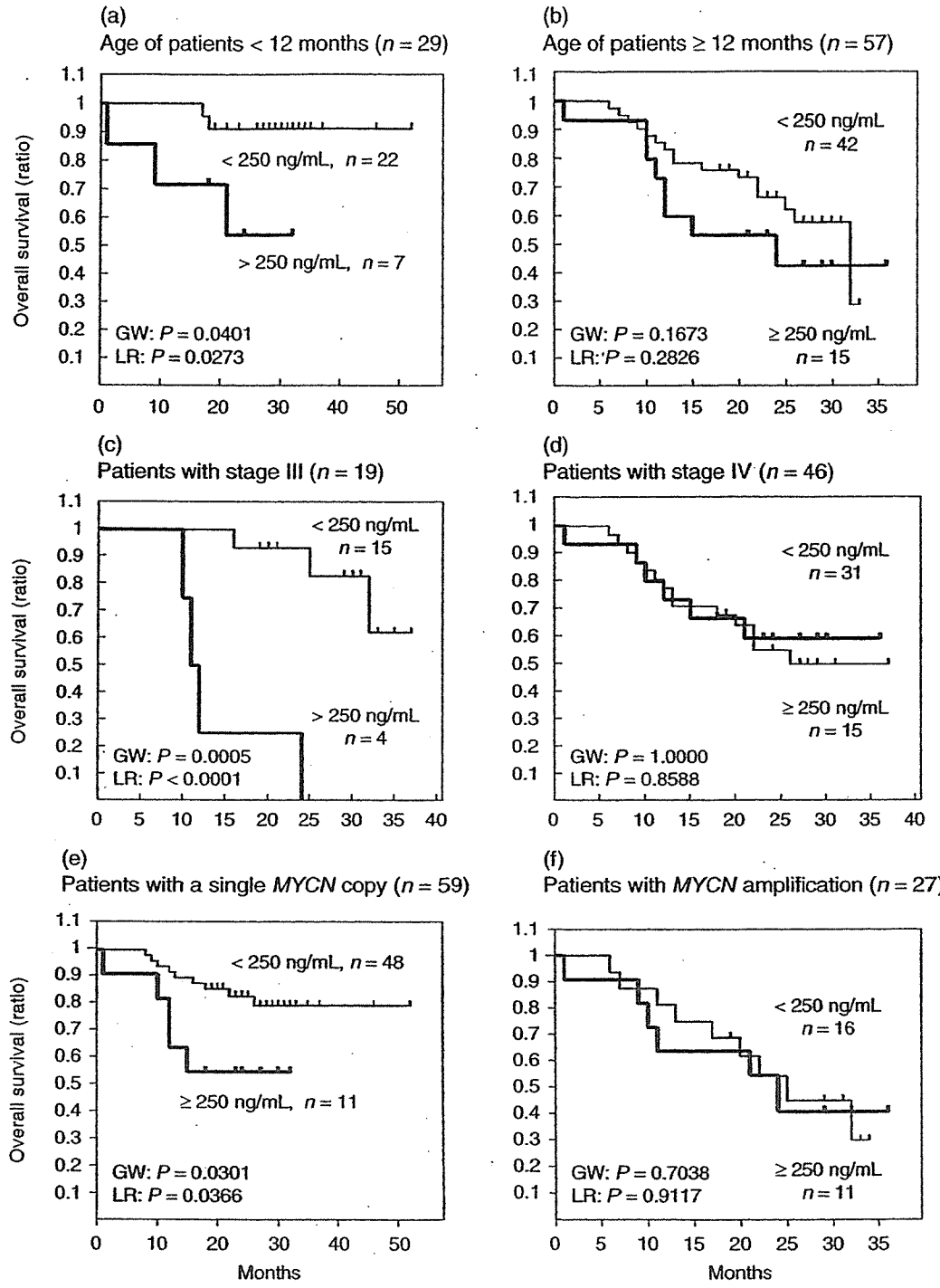

Fig. 3. Clinical significance of the serum NM23-H1 levels in the groups classified according to the age of the patients, or stage of the disease, or copy number of *MYCN*. (a) Survival curves for seven patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 22 patients with a level < 250 ng/mL. Both groups of patients were younger than 12 months of age. (b) Survival curves for 15 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 42 patients with a level < 250 ng/mL. Both groups of patients were 12 months old or older. (c) Survival curves for four patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 15 patients with a level < 250 ng/mL. Both groups of patients were at stage 3 of the disease. (d) Survival curves for 15 patients with the serum NM23-H1 level ≥ 250 ng/mL, and for 31 patients with the level < 250 ng/mL. Both groups of patients were at stage 4 of the disease. (e) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 48 patients with a level < 250 ng/mL. Both groups of patients had a single copy of *MYCN*. (f) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 16 patients with a level < 250 ng/mL. Both groups of patients had *MYCN* amplification in the tumor. GW, generalized Wilcoxon's test; LR, log-rank test.

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and serum NSE is a useful marker for patients with advanced neuroblastoma in whom the elevated levels are associated with a poor outcome.[27] The disialoganglioside GD2 is found on the surface of most neuroblastoma cells, and elevated plasma levels have been found in patients.[28] Nevertheless, none of these markers is used at present to predict clinical outcomes or to choose treatment protocols. Therefore, serum NM23-H1 levels might be useful for clinical purposes.

The elevated serum level of NM23-H1 was correlated with a poor prognostic feature, namely, *MYCN* amplification (Table 1). Godfrid *et al.* identified genes that are part of the *MYCN* downstream pathway using SAGE libraries of *MYCN* transfected and control neuroblastoma cell lines.[13] The chromosome 17q genes *NM23-H1* and *NM23-H2* were strongly induced in *MYCN*-expressing cells. A striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes was found in the cell lines. The present multivariate analysis showed no influence of serum NM23-H1 level on overall survival, and this finding might be caused by the overlap of patients with *MYCN* amplification with those with a high serum level of NM23-H1. However, within the group of patients with a single copy of *MYCN*, patients with a higher level of NM23-H1 had a worse outcome (Fig. 3e). The findings suggest that *NYCN* amplification may influence serum NM23-H1 levels as well as clinical outcome, and that neuroblastomas with a single copy of *MYCN* and a higher serum NM23-H1 level may have had a mutation or an increased copy number of the *NM23-H1* gene.[6,24,29] *MYCN* overexpression in some neuroblastomas with a single copy of *MYCN* may have resulted in higher serum NM23-H1 levels and a poor outcome; however, a recent study showed that *MYCN* overexpression did not affect the prognosis of advanced-stage neuroblastomas with a single *MYCN* copy.[30]

In patients with NHL and AML, it is thought that serum NM23-H1 protein is produced directly by the tumor cells, and its serum level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14] High concentrations of NM23 protein were found in the serum and body fluid of patients with lung cancer overexpressing the *NM23* genes.[31] Tumor cells may secrete this protein through some unknown mechanism, because there is no signal peptide sequence for secretion in the NM23 molecule. Serum NM23-H1 in patients with neuroblastoma might be derived from tumor cells and might be induced by *MYCN* amplification/overexpression or by *NM23-H1* overexpression independent of *MYCN*.

The serum level of NM23-H1 protein is clinically useful as an important prognostic factor in NHL or AML, and the present study showed that the protein could be a factor predicting an outcome of patients with neuroblastoma. It would be interesting to examine whether the serum NM23-H1 level generally predicts a poor outcome for patients with other tumors. The mechanisms by which the NM23-H1 protein is secreted into the serum and how it affects patient outcome are unclear. We are now studying the possibility that a high concentration of serum NM23-H1 may positively affect tumor cell growth or negatively affect normal cells.

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References

1 Steeg PS, Bevilacqua G, Kopper L *et al.* Evidence for a novel gene associated with low tumor metastatic potential. *J Natl Cancer Inst* 1988; **80**: 200–4.
2 Lacombe ML, Milon L, Munier A, Mehus JG, Lambeth DO. The human nm23/nucleoside diphosphate kinases. *J Bioenerg Biomembr* 2000; **32**: 247–58.
3 Lascu I, Gonin P. The catalytic mechanism of nucleoside diphosphate kinases. *J Bioenerg Biomembr* 2000; **32**: 237–46.
4 MacDonald NJ, Rosa ADL, Steeg PS. The potential roles of nm23 in cancer metastasis and cellular differentiation. *Eur J Cancer* 1995; **31A**: 1096–100.
5 Lacombe ML, Sastre-Garau X, Lascu I *et al.* Overexpression of nucleoside diphosphate kinase (Nm23) in solid tumors. *Eur J Cancer* 1991; **27**: 1302–7.
6 Leone A, Seeger RC, Hong CM *et al.* Evidence for nm23 RNA overexpression, DNA amplification and mutation in aggressive childhood neuroblastoma. *Oncogene* 1993; **8**: 855–65.
7 Chang C, Zhu X-X, Thoraval D *et al.* nm23–H1 mutation in neuroblastoma. *Nature (London)* 1994; **370**: 335–6.
8 Yokoyama A, Okabe-Kado J, Wakimoto N *et al.* Evaluation by multivariate analysis of the differentiation inhibitory factor nm23 as a prognostic factor in acute myelogenous leukemia and application to other hematological malignancies. *Blood* 1998; **91**: 1845–51.
9 Hailat N, Keim DR, Melhem RF *et al.* High levels of p19/nm23 protein in neuroblastoma are associated with advanced stage disease and with N-myc gene amplification. *J Clin Invest* 1991; **88**: 341–5.
10 Niitsu N, Okabe-Kado J, Okamoto M *et al.* Serum nm23-H1 protein as a prognostic factor in aggressive non-Hodgkin's lymphoma. *Blood* 2001; **97**: 1202–10.
11 Bown N, Cotterill S, Lastowska M *et al.* Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med* 1999; **340**: 1954–61.
12 Kaneko Y, Kobayashi H, Maseki N, Nakagawara A, Sakurai M. Disomy 1 with terminal 1p deletion was frequent in mass screening-negative/late-presenting neuroblastomas in young children, but not in mass screening-positive neuroblastomas in infants. *Int J Cancer* 1999; **80**: 54–9.
13 Godfried MB, Veenstra MV, Sluis P *et al.* The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. *Oncogene* 2002; **21**: 2097–101.
14 Okabe-Kado J. Serum nm23-H1 protein as a prognostic factor in hematological malignancies. *Leuk Lymphoma* 2002; **43**: 859–67.
15 Niitsu N, Okabe-Kado J, Nakayama M *et al.* Plasma levels of the differentiation inhibitory factor nm23-H1 protein and their clinical implication in acute myelogenous leukemia. *Blood* 2000; **96**: 1080–6.
16 Niitsu N, Nakamine H, Okamoto M *et al.* Clinical significance of intracytoplasmic nm23-H1 expression in diffuse large B-cell lymphoma. *Clin Cancer Res* 2004; **10**: 2482–90.
17 Sawada T, Hirayama M, Nakata T *et al.* Mass screening for neuroblastoma in infants in Japan. *Lancet* 1984; **2**: 271–3.
18 Brodeur GM, Pritchard J, Berthold F *et al.* Revision of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clinic Oncol* 1993; **11**: 1466–77.
19 Sawaguchi S, Kaneko M, Uchino J *et al.* Treatment of advanced neuroblastoma with emphasis on intensive induction chemotherapy. *Cancer* 1990; **66**: 1879–87.
20 Testa U, Thomopouls P, Vinci G *et al.* Transferrin binding to K562 cell line. *Exp Cell Res* 1982; **140**: 251–60.
21 Bowman LC, Castleberry RP, Cantor A *et al.* Genetic staging of unresectable or metastatic neuroblastoma in infants: a Pediatric Oncology Group Study. *J Nat Cancer Inst* 1997; **89**: 373–80.

22 Nakagawara A, Arima-Nakagawara M, Scavaruda NJ, Azar CG, Cantor AB, Brodeua GM. Association between high levels of expression of the *TRK* gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993; **328**: 847–54.
23 Willem R, Van Bockstaele DR, Lardon F *et al.* Decrease in nucleoside diphosphate kinase (NDPK/nm23) expression during hematopoietic maturation. *J Biol Chem* 1998; **273**: 13663–8.
24 Takeda O, Handa M, Uehara T *et al.* An increased NM23-H1 copy number may be a poor prognostic factor independent of LOH on 1p in neuroblastomas. *Br J Cancer* 1996; **74**: 1620–6.
25 Hann H-WL, Evans AE, Siegel SE *et al.* Prognostic importance of serum ferritin in patients with stages III and IV neuroblastoma: The Children's Cancer Study Group Experience. *Cancer Res* 1985; **45**: 2843–8.
26 Shuster JJ, McWilliams NB, Castleberry R *et al.* Serum lactate dehydrogenase in childhood neuroblastoma. A pediatric oncology group recursive partitioning study. *Am J Clin Oncol* 1992; **15**: 295–303.
27 Zeltzer PM, Marangos PJ, Evans AE, Schneider SL. Serum neuron-specific enolase in children with neuroblastoma. Relationship to stage and disease course. *Cancer* 1986; **57**: 1230–4.
28 Landisch S, Wu Z-L. Detection of a tumour-associated ganglioside in plasma of patients with neuroblastoma. *Lancet* 1985; **1**: 136–8.
29 Almgren MAE, Henriksson KCE, Fujimoto J, Chang CL. Nucleoside diphosphate kinase A/nm23-H1 promotes metastasis of NB69-derived human neuroblastoma. *Mol Cancer Res* 2004; **2**: 387–94.
30 Cohn SL, London WB, Huang D *et al. MYCN* expression is not prognostic of adverse outcome in advanced-stage neuroblastoma with nonamplified *MYCN*. *J Clin Oncol* 2000; **18**: 3604–13.
31 Huwer H, Kalweit G, Engel M, Welter C, Dooley S, Gams E. Expression of the candidate tumor suppressor gene nm23 in the bronchial system of patients with squamous cell lung cancer. *Eur J Cardiothorac Surg* 1997; **11**: 206–9.

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Superior Protective and Therapeutic Effects of IL-12 and IL-18 Gene-Transduced Dendritic Neuroblastoma Fusion Cells on Liver Metastasis of Murine Neuroblastoma[1]

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Fusion vaccine of dendritic cells (DCs) and tumor cells has the advantage of inducing an immune response against multiple tumor Ags, including unknown tumor Ags. Using the liver metastasis model of C1300 neuroblastoma cells, we assessed the protective and therapeutic effects of fusion cells transduced with the IL-12 gene and/or the IL-18 gene. Improving the fusion method by combining polyethylene glycol and electroporation increased loading efficiency. In the A/J mice vaccinated with fusion cells modified with the LacZ gene (fusion/LacZ), IFN-γ production and CTL activity increased significantly compared with that of DCs/LacZ, C1300/LacZ, or a mixture of the two (mixture/LacZ). With the transduction of IL-12 and IL-18 genes into the fusion cells (fusion/IL-12/IL-18), the level of IFN-γ increased more than five times that of other fusion groups. In addition, NK cell activity and CTL activity increased significantly compared with that of mixture/LacZ, fusion/LacZ, DC/LacZ, or C1300/LacZ. In the protective and therapeutic studies of fusion cell vaccine, mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18 showed a significant decrease in liver metastasis and a significant increase in survival compared with mice given a mixture/LacZ, DCs/LacZ, or C1300/LacZ. In particular, the mice receiving fusion/IL-12/IL-18 vaccine showed a complete protective effect and the highest therapeutic effects. The present study investigates the improved loading efficiency of fusion cells and suggests that the introduction of IL-12 and IL-18 genes can induce extremely strong protective and therapeutic effects on liver metastasis of neuroblastoma. *The Journal of Immunology,* **2006, 176: 3461–3469.**

Neuroblastoma, a common malignancy in children, is derived from sympathetic nerve lineage cells and is characterized by aggressive local growth, followed by metastasis to the regional lymph nodes, liver, bones, and bone marrow (1). The prognosis for patients with neuroblastoma relates to its dissemination through the body and has not greatly improved despite multimodal treatments (2). At the time of diagnosis, two-thirds of children with this tumor present with extensive local or distant metastatic disease. The overall survival rate of patients is reportedly only 25% (3, 4). In the absence of an effective conventional therapy for neuroblastoma, alternative biologically based strategies should be investigated. Furthermore, a distinctive scenario exists in patients <1 yr old with stage IV-S disease in which the disease is broadly disseminated but lacks amplification of the N-*myc* oncogene. Of these patients, 25% have spontaneous remissions, possibly mediated by the immune system (5). These findings prompted us to investigate immunotherapy for patients with neuroblastoma.

Dendritic cells (DCs),[3] the most potent of APCs, induce a primary antitumor immune response via direct cell-cell interactions and/or cytokine production (6, 7). This antitumor immune response of DCs has resulted in the development of DC-based tumor vaccines, which are used clinically as a form of immunotherapy (8, 9). Despite the focus on DCs, this approach has not yet resulted in any significant therapeutic benefit in neuroblastoma. DCs have been loaded with tumor-derived material in several ways, such as pulsing of synthetic peptides, tumor cell lysate, and transducing tumor-derived RNA (8–11). In many tumor cells tumor-specific Ags remain unidentified. In neuroblastoma, a few tumor-associated Ags, such as survivin, NY-ESO, and MYC-N, have been reported in humans (12–14). Most clinical tumor samples have shown heterogeneity of tumor Ag and tumor cells with immunogenicity-evading immunological surveillance. Recently, an interesting new vaccine therapy that uses a fusion of DCs and tumor cells was reported (15–21). Fusion vaccine has the advantage of inducing an immune response against multiple tumor Ags, including unknown ones. With its use, the heterogeneity of tumor cells can be overcome. When making fusion vaccines, whole tumor cells are loaded with DCs, using polyethylene glycol (PEG) or electroporation. However, the loading efficiencies of these methods are insufficient and must be improved to increase the therapeutic effect of fusion vaccine (17–21).

IL-12 is a 70-kDa (p70) heterodimer protein in which the 40-kDa (p40) and 35-kDa (p35) subunits are connected by one S-S bond (22, 23). IL-12, a potent proinflammatory cytokine, is produced primarily by professional APCs, such as DC and macrophages, and exerts pleiotropic effects on immune effector cells

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[3] Abbreviations used in this paper: DC, dendritic cell; PEG, polyethylene glycol; MOI, multiplicity of infection.

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(24). IL-12 induces Th1 differentiation from naive Th0 cells, stimulates IFN-γ production, promotes proliferation of T and NK cells, and enhances CTLs, NK cells, and lymphokine-activated T cells (25, 26). IL-18, an 18.3-kDa glycoprotein, was initially identified as a cytokine that facilitates the production of IFN-γ induced by endotoxin (27). IL-18 plays an essential role in inducing a Th1 response in vivo. It stimulates T cell proliferation, augments CTL activation, and enhances NK cell cytolytic activation, mediated primarily via the FasL-Fas mechanism (28, 29). Interestingly, IL-12 with IL-18 was shown to induce the highest level of IFN-γ in vitro and in vivo (30, 31). Cumulative evidence has shown the importance of IFN-γ in the induction of native and acquired immunity.

The aim of this study is to clarify the role of fusion vaccine for treating neuroblastoma with liver metastasis, and to evaluate the antitumor effect of the transduction of both IL-12 and IL-18 genes to fusion vaccine.

Materials and Methods

Mice and cell lines

Pathogen-free A/J ($H\text{-}2^a$) female mice, 8- to 10-wk-old, were purchased from Japan SLC. During the experiments, they were kept in pathogen-free animal facilities at a controlled temperature and humidity, according to the guidelines of the university. Murine neuroblastoma C-1300 originating in A/J mice was maintained in RPMI 1640 (Nissui Pharmaceutical) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MC38 adenocarcinoma cells ($H\text{-}2^b$) were provided by Dr. J. Primus (Vanderbilt University Medical Center, Nashville, TN), and YAC-1 lymphoma was purchased from the DNA Bank, BioResource Center, RIKEN.

Generation of bone marrow-derived DCs and phenotype of cell surface

DCs were prepared from bone marrow as described previously (32). To analyze the surface phenotype, DCs were stained with PE or FITC-conjugated mAb, including $H\text{-}2K^k$, $I\text{-}A^k$, CD11, CD80, CD83, and CD86 (BD Pharmingen), and expression of the surface markers was examined by FACSCalibur (BD Biosciences).

Recombinant adenoviral vectors

A mature IL-18 cDNA molecule was isolated by PCR, using full-length IL-18 cDNA expression vector plasmid (provided by Dr. M. Okamura of Hyogo College of Medicine, Hyogo, Japan). First, the PCR fragment of the mature IL-18 was cloned into a *HindIXho*I site of the pSecTag2B eukaryotic expression plasmid vector, which contains the Igκ leader sequence as described previously (Invitrogen Life Technologies) (33). Then, mature IL-18 gene combined with a leader sequence was cloned by PCR and inserted into the cosmid vector pAxCAwt using an adenovirus expression vector kit (Takara Biomedicals) to generate the pAxCAmIL-18 constructs. The subunit of the IL12p35 and IL12p40 genes was inserted into the pIRES vector (BD Biosciences). Then, the IL-12 gene (p35 and p40) was cloned by PCR and inserted into the cosmid vector pAxCAwt to generate the pAxCAmIL-12 constructs. pAxCALacZ-encoding β-galactosidase was also constructed using the same cosmid vector. To isolate the recombinant adenovirus, each construct was cotransfected with restriction enzyme-digested DNA-terminal protein complex into 293 cells (DNA Bank, BioResource Center, RIKEN). The recombinant virus was purified through ultracentrifugation in cesium chloride step gradients, and its titer was determined by PFU on the 293 cells, according to standard protocols (34). The resultant adenovirus vectors were named Ad-IL-18, Ad-IL-12, and Ad-LacZ.

Transduction of IL-12 and IL-18 genes by adenovirus vector

DCs were cultured with Ad-IL-12, or Ad-LacZ adenovirus (multiplicity of infection (MOI) 100), and C1300 neuroblastoma cells were cultured with Ad-IL-18, or Ad-LacZ adenovirus (MOI 100). The gene was transduced by centrifuging the culture plates for 2 h at 700 × *g*. The gene transduction efficacy of DCs and C1300 neuroblastoma was assessed by intracellular staining, using the X-gal staining assay kit (Gene Therapy System). After gene transduction, DCs and tumor cells were prepared for cell fusion. Forty-eight hours after culture, the production of IL-12 and IL-18 protein by the gene-transduced DCs (5×10^5), C1300 tumor cells (5×10^5), and fusion cells (5×10^5) was measured with an ELISA Kit (p70 IL-12; BD Pharmingen) (IL-18; Medical and Biological Laboratories).

Loading of tumor Ags

Three fusion methods of PEG (Sigma-Aldrich) treatment, electrofusion, and a combination of PEG and electrofusion were examined. DCs and irradiated (100 Gy) C1300 neuroblastoma were mixed at a ratio of 2:1 (DC:tumor cells) and centrifuged at1500 rpm for 5 min. For fusion by PEG, 50% PEG solution was added to the cell pellet and treated for 1 min, then diluted PEG was added and incubated at 37°C, 5% CO_2. For electrofusion, a mixture of DCs and tumor cells were resuspended in hybrid medium (0.25 M glucose with 0.1 mM $Ca2^+$ and 0.1 mM $Mg2^+$) and dielectrophoretically aligned to form cell-cell conjugates by alternating current (100 V/cm, 20 s). Then, a fusion pulse of direct current was applied to break down the membrane and to form hybrid cells (1.2 kV/cm, 30 μs), and finally, an alternating current of postfusion was applied to solidify the conformation of the hybrid cells (100 V/cm, 3s) (Nepa Gene). To improve the loading efficiency, we examined a two-step fusion procedure combining PEG treatment and electrofusion. In the first step, the mixture of DCs and irradiated tumor cells was treated with 50% PEG as described previously, and the cells were incubated at 37°C, 5% CO_2. In the second step, nonadherent cells, which are mostly unfused cells, were collected and refused by electrofusion. PEG fusion cells and electrofusion cells were gathered and prepared to examine loading efficiency. To determine the loading efficiency, DCs and tumor cells were prestained with the fluorescent dyes DiO (green fluorescence; Molecular Probes) and DiI (red fluorescence; Molecular Probes), respectively, and analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The form of the loading cell was confirmed by an inverted system fluorescence microscope (Olympus).

Animal studies

Experimental groups for the immunization of each cell were as follows: 1) PBS; 2) DCs transduced with the LacZ gene (DC/LacZ); 3) C1300 tumor cells transduced with the LacZ gene (C1300/LacZ); 4) C1300 tumor cells transduced with the IL-12 gene (C1300/IL-12); 5) C1300 tumor cells transduced with the IL-18 gene (C1300/IL-18); 6) C1300 tumor cells transduced with the IL-12 and IL-18 gene (C1300/IL-12/IL-18); 7) a mixture of DCs/LacZ and C1300/LacZ (mixture/LacZ); 8) a mixture of DCs/IL-12 and C1300/IL-18 (mixture/IL-12/IL-18); 9) fusion of DC/LacZ and C1300/LacZ (fusion/LacZ); 10) fusion of IL-12 gene-transduced DCs and LacZ gene-transduced C1300 tumor cells (fusion/IL-12); 11) fusion of LacZ gene-transduced DCs and IL-18 gene-transduced C1300 tumor cells (fusion/IL-18); and 12) fusion of IL-12 gene-transduced DCs and IL-18 gene-transduced C1300 tumor cells (fusion/IL-12/IL-18). To examine the protective effect, each vaccine (5×10^5 cells/mouse) was administered s.c. to the inguinal region on days 7 and 14, before the i.v. administration of C1300 tumor cells (1×10^6 cells/mouse). To examine the therapeutic effect, each vaccine (5×10^5 cells/mouse) was administered s.c. to the inguinal region on days 3 and 10 after i.v. inoculation of the C1300 tumor cells. At 21 days after the tumor injection, the livers were collected and the metastases enumerated. Survival time and rates were observed until 90 days after tumor inoculation.

Cytokine production and cytotoxicity assay

The spleens were collected 2 wk after the administration of each vaccine, and cytokine production and cytotoxicity assay (NK and CTL) were examined. Splenic NK cells were isolated from spleen cells with the NK cell isolation kit (Miltenyi Biotec) for NK assay, and T cells were separated using a T Cell Immunocolumns Kit (Cedarlane Laboratories) for cytokine production and CTL assay. To characterize the effector cells, CD4- and/or CD8-positive cells were eliminated by AutoMACS separation system (Miltenyi Biotec). Splenic T cells were restimulated in vitro with irradiated (50 Gy) fusion cells at 37°C under 5% CO_2 for 48 h, and levels of IFN-γ, IL-4, and IL-10 were detected using a Mouse CBA Kit (BD Pharmingen). For cytotoxicity assay, NK or T cells were restimulated in vitro with irradiated (50 Gy) fusion cells in the presence of 10 U of IL-2 (Sionogi) and 10 U of IL-7 (Genzyme) for 5 days. Viable cells were then separated with Lympholyte-M (Cedarlane Laboratories) and prepared as effector cells. As target cells, YAC-1 cells and C1300 were used for the NK assay, and C1300 and allogeneic MC38 cells were used for the CTL assay. The target cells labeled with $Na_2\ ^{51}Cr\ O4$ (DuPont-NEN) were cultured with each of the effector cells for 4 h in NK assay and 6 h in CTL assay. Spontaneous and maximum release was measured by adding medium and 0.045 M NH4OH instead of effector cells. Each sample was assayed in triplicate.

The percentage of cytotoxicity was determined by calculating the percentage of specific ^{51}Cr release according to the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. All determinations were made in triplicate.

In vivo depletion of T cell subsets and NK cells

Mice received injections i.v. with rat azide-free anti-mouse CD4 mAb (YTS191.1; Serotec), rat anti-mouse CD8 mAb (YTS169.4; Serotec), rabbit anti-mouse Asialo GM1 polyclonal Ab (Wako Chemical), or normal rat IgG (Sigma-Aldrich) at 1 mg/mouse 1 day before fusion/IL-12/IL-18 vaccine inoculation and once every 5 days thereafter for an additional 20 days. Depletion of the corresponding NK, CD4, or CD8 T cells was >80%, as confirmed by FACS analysis of spleen cells from treated mice.

Statistical analysis

Values were presented as means ± SD. Multiple groups were evaluated by ANOVA and the posthoc Scheffe multirange test. Survival estimates were determined using the Kaplan-Meier method, and data were compared by the log-rank test. A probability of <0.05 was considered significant.

Results

Comparing the loading efficiency and antitumor effect after improving the fusion method

The loading efficiency of electrofusion, PEG, and PEG followed by electrofusion (the two-step method) was compared using the DCs and C1300 tumor cells, which were labeled with DiO and DiI intracellular fluorescent dyes, respectively (Fig. 1). DCs loaded by whole tumor cells were characterized by the emission of both colors in the *upper right* of the dot plot analysis. Double-positive cells increased from 12.9 ± 3.2% using electrofusion or 34.1 ± 2.6% using PEG treatment to 51.6 ± 2.1% using the two-step method (Fig. 1*A*). This suggests that the loading efficiency of the two-step fusion method increased 1.5 times and is 4 times higher than with PEG treatment and electrofusion, respectively. In the two-step method, the loading efficiency of gene-transduced fusion cells was 45.2 ± 2.3% in fusion/LacZ, 48.1 ± 2.2% in fusion/IL-18, 49.7 ± 4.1% in fusion/IL-12, and 50.7 ± 3.5% in fusion/IL-12/IL-18, and there were no significant differences among them (data not shown).

The same samples of FACS analysis were observed under a fluorescent microscope (Fig. 1, *A* and *B*). In fluorescent micrographs of fusion cells, a unity of cell membranes of multinuclear cells was recognized. Using this method, cell viability was >80%, which was achieved by the trypan blue dye exclusion test. In contrast, simple repetition of PEG treatment or electrofusion caused a significant decrease in cell viability (<50%; data not shown).

Next, we compared the ability for CTL production of these fusion cells generated by the different protocols (Fig. 1*C*). The cytotoxicity of splenic lymphocytes from mice vaccinated with two-step fusion cells was significantly higher than that of PEG treatment or electroporation alone. These results show the usefulness as cancer vaccines of fusion cells generated by the two-step method.

Cell surface phenotypes of DCs, tumor cells, and fusion cells

The cell surface markers of each vaccine were analyzed by FACS (Fig. 2). C1300 tumor cells transduced with the LacZ gene (C1300/LacZ) showed only the expression of MHC class I Ags (H-2K) but not MHC class II (I-A), CD11c, or costimulatory molecules (CD80, CD83, CD86). DCs transduced with the LacZ gene (DCs/LacZ) expressed high levels of MHC class II Ags and CD11c, CD80, and CD86, and a low level of CD83. Almost the same levels of DC-derived markers were identified on the surface of the fusion cells transduced with the LacZ gene (fusion/LacZ) or the IL-18 gene (fusion/IL-18). In contrast, transduction with the IL-12 genes enhanced the expression of CD83 molecule on the surface of the fusion cells, and a high level of CD83 was shown in fusion/IL-12 and fusion/IL-12/IL-18.

Cytokine production by gene-transduced DCs and tumor cells

At a MOI 100, the gene transduction efficiency for IL-12 in DCs was 61%, and that for IL-18 in C1300 tumor cells was 68% (data not shown). Fig. 3*A* shows the IL-12 protein levels in the medium of each group. Significant levels of p70 IL-12 proteins were produced in DCs or fusion cells following transduction with the IL-12 gene. The p70 IL-12 levels of the DCs/LacZ, fusion/LacZ, DCs/

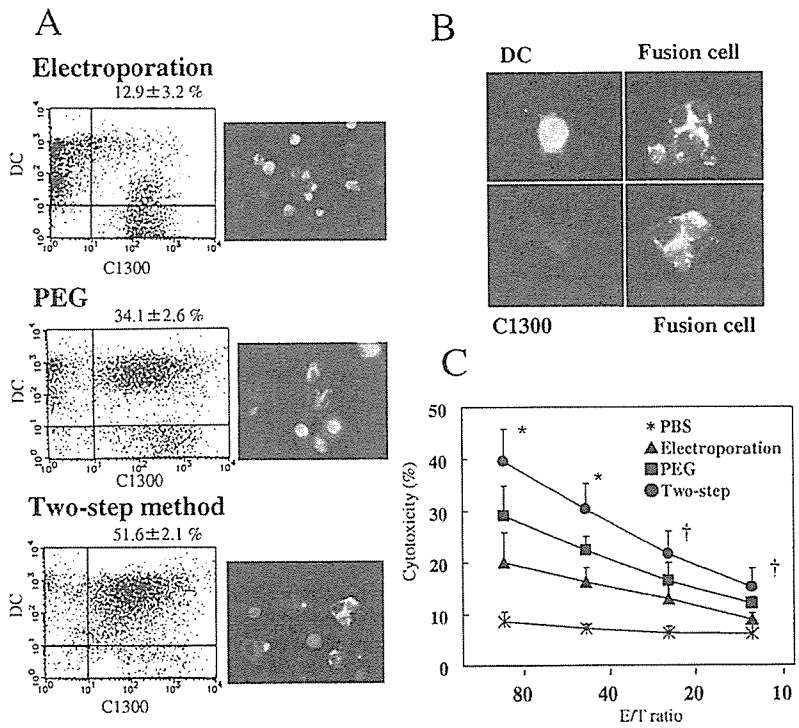

FIGURE 1. Comparison of loading method of C1300 neuroblastoma to DCs and CTL activities of fusion cells generated by different protocols. *A*, DiI-labeled C1300 neuroblastoma was loaded to DiO-labeled DCs by electroporation, PEG-treatment, or a combination of PEG and electroporation (two-step method), and loading efficiency was examined by FACS analysis. Each experiment was done in triplicate. The figures in the *upper right panel* show the mean percentages of double-positive cells (mean ± SD). Morphology of cells analyzed by FACS was observed by fluorescent microscopy (×100). *B*, Magnified fluorescent micrographs show the DC, C1300 tumor cells, and fusion cells (×250). *C*, Splenic T cells were collected from mice vaccinated with DC/1300 fusion cells prepared by electroporation, PEG treatment, or the two-step method, and cytotoxicity was measured against C1300 tumor cells. *, $p < 0.05$ (two-step vs PEG, electroporation, PBS). †, $p < 0.05$ (two-step vs electroporation, PBS. Mean ± SD ($n = 5$).

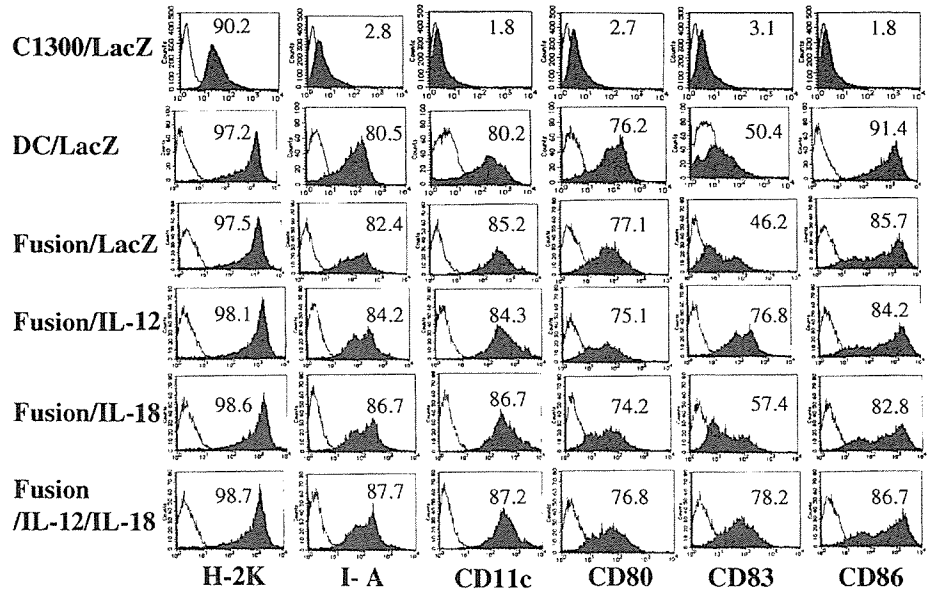

FIGURE 2. Cell surface phenotypes of DCs, C1300 tumor cells, and fusion cells. LacZ gene-transduced C1300 tumor cells (C1300/LacZ), LacZ gene-transduced DCs (DCs/LacZ), LacZ gene-transduced fusion cells (fusion/LacZ), IL-12 gene-transduced fusion cells (fusion/IL-12), IL-18 gene-transduced fusion cells (fusion/IL-18), and IL-12 and IL-18 gene-transduced fusion cells (fusion/IL-12/IL-18) were stained with PE or FITC-labeled mAb (anti-H-2K, anti-I-A, anti-CD11c, anti-CD80, anti-CD83, and anti-CD86). Surface markers were analyzed by FACS. Numerical values show the percentage of positive cells.

IL-12, fusion/IL-12, and fusion/IL-12/IL-18 were 166 ± 51 pg/ml, 185 ± 65 pg/ml, 3210 ± 702 pg/ml, 2820 ± 568 pg/ml, and 2672 ± 468 pg/ml, respectively. There were no significant differences in IL-12 levels between the DCs/IL-12, fusion/IL-12, and fusion/IL-12/IL-18. Fig. 3*B* shows the IL-18 protein levels in the medium of each group. Significant levels of IL-18 proteins were produced in C1300 cells or fusion cells by transduction with the IL-18 gene. The IL-18 levels of C1300/LacZ, fusion/LacZ, C1300/IL-18, fusion/IL-18, and fusion/IL-12/IL-18 cells were 58 ± 25 pg/ml, 78 ± 35 pg/ml, 1280 ± 308 pg/ml, 1036 ± 202 pg/ml, and 1006 ± 282 pg/ml, respectively. No significant differences in IL-18 levels were found between the C1300/IL-18, fusion/IL-18, and fusion/IL-12/IL-18.

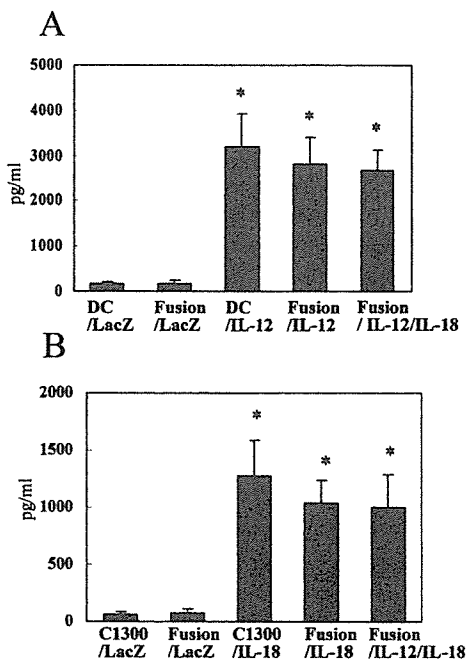

FIGURE 3. Cytokine production by gene-transduced DCs, C1300, and fusion cells. DCs and C1300 tumor cells were infected with LacZ, IL-12, or IL-18 recombinant adenovirus vector and fused. Forty-eight hours after infection, IL-12 (*A*) and IL-18 (*B*) protein levels in the supernatant were examined by ELISA. *, $p < 0.01$ (DCs/IL-12, fusion/IL-12, fusion/IL-12/IL-18 vs DCs/LacZ, fusion/LacZ). *, $p < 0.01$ (C1300/IL-18, fusion/IL-18, fusion/IL-12/IL-18 vs C1300/LacZ, fusion/LacZ). Mean ± SD ($n = 5$).

Cytokine production by splenic T cells

Production of IFN-γ by splenic T cells collected from mice immunized with each vaccine and cell surface phenotypes of effector cells in IFN-γ production were examined (Fig. 4). IFN-γ levels in the culture supernatant of splenic T cells were significantly higher in the mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18 than with the mixture of DC/LacZ and C1300/LacZ (mixture/LacZ), DCs/LacZ, C1300/LacZ, or PBS (Fig. 4*A*). The fusion/IL-12/IL-18-vaccinated group showed the highest levels of IFN-γ compared with that of fusion/LacZ, fusion/IL-12, and fusion/IL-18. In contrast, IL-4 and IL-10 levels from splenic T cells were very low in all groups, and there were no significant differences between these groups (data not shown). To characterize the T cells that produce the IFN-γ, $CD8^+$ T cells and/or $CD4^+$ T cells were depleted by negative selection of MACS (Fig. 4*B*). In the mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18, production of IFN-γ was significantly inhibited by depletion of splenic T cells expressing not only $CD4^+$ but also $CD8^+$ T cells. These results suggest that a fusion vaccine itself has the ability to induce a Th1 immune response, and cotransduction of the IL-12 and IL-18 genes causes a strong shift to a Th1 response by markedly increasing production of IFN-γ by $CD4^+$ and $CD8^+$ T cells.

Cytotoxicity of splenic NK cells and splenic T cells

Productivity of cytotoxic splenic NK cells against NK-sensitive YAC-1 cells and C1300 cells was examined (Fig. 5*A*). Splenic NK cells of fusion/LacZ-vaccinated mice showed a significant increase of cytotoxicity against YAC-1 cells compared with mice vaccinated with DC/LacZ, C1300/LacZ, and PBS. Furthermore, NK activities were increased by transduction of IL-12 and/or IL-18 genes. Splenic NK cells of the mice vaccinated with fusion IL-12/IL-18, fusion/IL-12, fusion/IL-18, or nonfused mixture/IL-12/IL-18 showed a significant increase of cytotoxicity against YAC-1 cells and C1300 cells compared with that of mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS-administered mice. The group vaccinated with fusion/IL-12/IL-18 showed the highest NK activity in

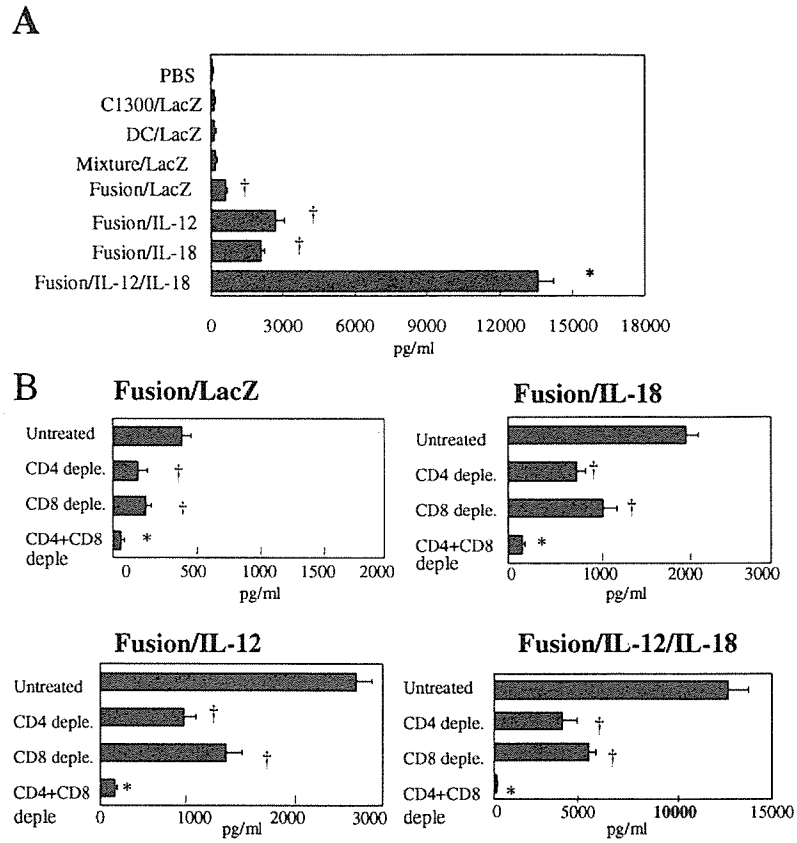

FIGURE 4. Production of IFN-γ by splenic T cells from mice immunized with each vaccine, and cell surface phenotypes of effector cells in IFN-γ production. *A*, IFN-γ production. Mice were immunized with each vaccine (PBS, C1300/LacZ, DC/LacZ, mixture/LacZ, fusion/LacZ, fusion/IL-12, fusion/IL-18, fusion/IL-12/IL-18). On day 14 after immunization, splenic T cells were separated and incubated for 48 h with irradiated DC/C1300 fusion cells. IFN-γ levels in culture medium were measured by ELISA. *, $p < 0.0001$ (fusion/IL-12/IL-18 vs other all groups). †, $p < 0.01$ (fusion/LacZ, fusion/IL-12, fusion/IL-18 vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). Mean ± SD ($n = 5$). *B*, Cell surface phenotypes of effector cells in IFN-γ production. Mice were immunized with fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18, which have the ability to induce IFN-γ. CD4 and/or CD8 T cells were depleted by MACS, and the productivity of IFN-γ was compared with the untreated group. *, $p < 0.01$ ($CD4^+CD8$ depletion vs untreated). †, $p < 0.05$ (CD4 depletion, CD8 depletion vs untreated). Mean ± SD ($n = 5$).

all groups. The NK activity against YAC-1 cells was higher than that of C1300 cells. These results suggest that the fusion vaccine has the ability to induce NK activity, and that transduction of IL-12 and IL-18 genes greatly increase the NK activities.

Next, we examined the CTL activities of splenic T cells against parental C1300 tumor cells and allogeneic MC38 tumor cells (Fig. 5*B*). In the mice transduced with the LacZ gene, CTL activity against C1300 tumor cells was significantly higher in the mice vaccinated with fusion cells compared with those vaccinated with mixture/LacZ, DCs/LacZ, C1300/LacZ, and PBS. The CTL activities of splenic T cells in mice vaccinated with fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18 were higher than that of mice vaccinated with fusion/LacZ, and mice vaccinated with fusion/IL-12/IL-18 showed the highest level of activity in these groups. In contrast, mice vaccinated with nonfused mixture/IL-12/IL-18 or mixture/LacZ did not show a significant level of CTL activity. These results suggest that the formation of fusion cells is important for CTL induction. Cytotoxic activity against allogeneic MC38 adenocarcinoma was not detected in any group.

We then determined the surface phenotype and the location of MHC restriction of the cytotoxic effector cells induced by each fusion vaccine (Fig. 5*C*). The cytotoxic activity in all fusion groups (fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18) was significantly inhibited by the depletion of $CD8^+$ T cells but not by $CD4^+$ T cells. In the blocking assay of MHC Ags, CTL activities of all fusion groups were significantly decreased by treatment in anti- $H\text{-}2K^K$ mAb. However, treatment with anti-$I\text{-}A^K$ mAb did not affect cytotoxicity. These results indicate that fusion cells could induce MHC class I-restricted $CD8^+$ CTLs.

Protective effects of fusion vaccine

The protective effect induced by each vaccine was assessed by the number of liver metastases at 21 days and the survival rates 90 days after tumor inoculation (Table I and Fig. 6*A*). Each vaccine was administered on days 7 and 14, before the i.v. inoculation of C1300 tumor cells. In mice vaccinated with fusion/LacZ, a significant decrease in the number of liver metastases was observed compared with that of mice vaccinated with mixture/LacZ, DCs/LacZ, C1300/LacZ, or PBS. Transduction with IL-12 or IL-12 and IL-18 gene into fusion cells led to a significantly lower liver metastasis number compared with mice vaccinated with C1300/IL-12, C1300/IL-18, mixture/LacZ, DCs/LacZ, C1300/LacZ, or PBS. In particular, fusion/IL-12/IL-18 vaccine showed a dramatic decrease of liver metastasis, and all mice were tumor-free.

In a comparison of survival rates, the mice vaccinated with fusion/LacZ showed a significant increase compared with mice vaccinated with mixture/LacZ, C1300/LacZ, DC/LacZ, or PBS (Fig. 6*A*). Transduction with the IL-12 and/or IL-18 gene into fusion cells led to a higher survival rate compared with that of fusion/LacZ. In particular, fusion/IL-12/IL-18 vaccine showed a complete protection, and all mice remained tumor-free for at least 90 days. In contrast, mice vaccinated with mixture/IL-12/IL-18, C1300/IL-12/IL-18, C1300/IL-12, and C1300/IL-18 showed partial protection compared with that of fusion/IL-12/IL-18. These results demonstrate that the fusion cells transduced with the IL-12 and IL-18 genes lead to a dramatic protective effect and that conformation of fusion cells is important for strong tumor protection.

Therapeutic effects of fusion vaccine

To assess the therapeutic effects, each vaccine was administered after tumor inoculation, and the number of liver metastasis and survival rates were examined (Table II and Fig. 6*B*). In the study of liver metastasis, the mice vaccinated with fusion/LacZ, fusion/IL-18, C1300/IL-12/IL-18, or mixture/IL-12/IL-18 demonstrated a significant decrease in liver metastasis number compared with that

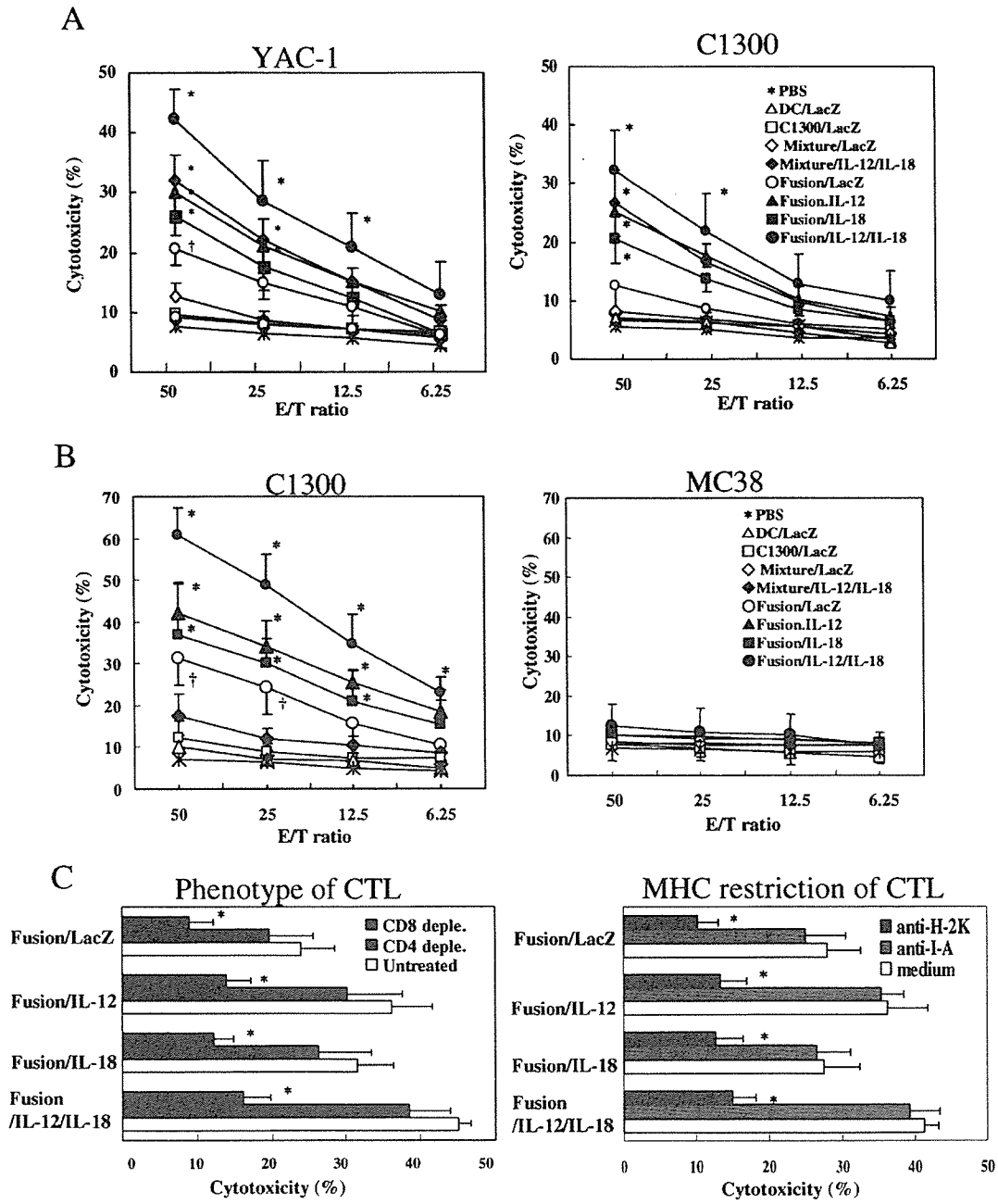

FIGURE 5. NK and cytotoxic T cell activities of splenic NK cells or T cells from mice immunized with each vaccine. *A*, NK activity. On day 14 after vaccination by DC/LacZ, C1300/LacZ, mixture/LacZ, mixture/IL-12/IL-18, fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18, NK cells were isolated from the spleen and cocultured with irradiated DC/C1300 fusion cells, and cytotoxicity was measured against YAC-1 and C1300 tumor cells. *, $p < 0.05$ (fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, mixture/IL-12/IL-18 vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). †, $p < 0.05$ (fusion/LacZ vs DC/LacZ, C1300/LacZ, PBS). Mean ± SD ($n = 5$). *B*, CTL activity. On day 14 after vaccination by each vaccine, T cells were separated from spleen cells and cocultured with irradiated DC/C1300 fusion cells, and cytotoxicity was measured against parental C1300 tumor cells and MC38. *, $p < 0.05$ (fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18 vs mixture/IL-12/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). †, $p < 0.05$ (fusion/LacZ vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). Mean ± SD ($n = 5$). *C*, Phenotype and MHC restriction of CTL induced by fusion vaccines. On day 14 after immunization of fusion vaccines (fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18), splenic T cells were collected and cocultured with irradiated DCs/C1300 fusion cells, and phenotype and MHC restriction were examined. To examine the phenotype of CTL, $CD8^+$ and/or $CD4^+$ cells were depleted by MACS, and CTL activity was compared with that of undeleted T cells. The ratio of E:T cells was 25:1. *, $p < 0.01$ (CD8 deple vs CD4 deple, untreated). Mean ± SD ($n = 5$). To examine the MHC restriction of effector cells, C1300 target cells were pretreated with anti-H-$2K^k$ mAb, anti-I-A^k mAb or medium, and cytotoxicities were measured. The ratio of E:T cells was 25:1. *, $p < 0.01$ (anti-H-$2K^k$ vs anti-I-A^k, medium). Mean ± SD ($n = 5$).

of mice vaccinated with mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS. Fusion/IL-12 showed a significant decrease in the number of liver metastases compared with C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS. In contrast, transduction of the IL-12 and IL-18 genes into fusion cells showed the lowest number of liver metastases in all groups, and this level differed significantly from that of C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS.

Table I. *Protective effects of each vaccine on liver metastasis*[a]

Immunogen	Tumor-Free Mice	No. of Liver Metastasis
PBS	0/10	43.5 ± 9.2
DC/LacZ	0/10	37.3 ± 9.8
C1300/LacZ	0/10	40.6 ± 7.1
C1300/IL-18	1/10	24.5 ± 14.2[b]
C1300/IL-12	1/10	20.3 ± 11.9[b]
C1300/IL-12/IL-18	2/10	10.8 ± 9.6[c]
Mixture/LacZ	0/10	34.0 ± 10.5
Mixture/IL-12/IL-18	3/10	7.0 ± 5.3[c]
Fusion/LacZ	2/10	12.2 ± 9.6[d]
Fusion/IL-18	3/10	7.3 ± 7.8[c]
Fusion/IL-12	5/10	4.1 ± 5.1[e]
Fusion/IL-12/IL-18	10/10	0.0 ± 0.0[e]

[a] Mean ± SD (n = 10). The data presented were pooled from two separated experiments, and the reproducibility of results was shown.
[b] $p < 0.05$ (C1300/IL-12, C1300/IL-18 vs. C1300/LacZ, PBS).
[c] $p < 0.05$ (C1300/IL-12/IL-18, mixture/IL-12/IL-18, fusion/IL-18 vs. C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).
[d] $p < 0.05$ (fusion/LacZ vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).
[e] $p < 0.05$ (fusion/IL-12, fusion/IL-12/IL-18 vs. C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

In the study of survival rates, all of the mice vaccinated with fusion/LacZ died within 57 days of tumor inoculation (Fig. 6*B*). The mice vaccinated with fusion/IL-12, fusion/IL-18, or mixture/IL-12/IL-18 had increased survival rates; however, their levels were <20%. In contrast, mice vaccinated with fusion/IL-12/IL-18 showed a significant increase of survival rates compared with that of all other groups, and 60% of mice remained tumor-free for at least 90 days. The survival rates of mice vaccinated with mixture/IL-12/IL-18 decreased significantly compared with that of mice vaccinated with fusion/IL-12/IL-18, suggesting the significance of conformation of fusion cell for strong therapeutic effect. These results demonstrate that the therapeutic effect of the fusion cells transduced with the IL-12 and IL-18 genes is superior to that of any other group.

Next, we investigated the participation of immune cell subsets in the generation of the therapeutic effects of the fusion/IL-12/IL-18 vaccine (Fig. 7). The NK, CD8 T cells, or CD4 T cells of mice were depleted by the administration of anti-asialoGM1, anti-CD4, or anti-CD8 mAb. The depleted NK cells, CD8 T cells, or CD4 T cells showed a significant increase of liver metastasis compared with normal IgG-injected mice. These results suggest that NK, CD8 T cells, and CD4 T cells are necessary and are associated with the therapeutic effects of the fusion vaccine transduced with the IL-12 and IL-18 genes.

Discussion

In the present study, we demonstrated the protective and therapeutic effect of DC/tumor fusion cells on neuroblastoma with liver metastasis. The transduction of both the IL-12 and the IL-18 genes to fusion cells induced the highest levels of IFN-γ, NK cell activity, and CTL activity. Furthermore, the fusion vaccine transduced with IL-12 and IL-18 genes showed complete protective and highly significant therapeutic effects on liver metastasis of neuroblastoma in mice.

Immunotherapy has been put forward as a feasible strategy for treating neuroblastoma based upon the observation that some aggressive neuroblastomas spontaneously regress (5). Although clinical trials of DC-based immunotherapy were investigated, its effects on several carcinomas were limited by the low number of defined tumor-associated Ags and the heterogeneity of tumor cells. In contrast, a new strategy using a DC-based tumor vaccine reported that DCs loaded with whole tumor cells or tumor lysate have the advantage of inducing antitumor immunity to multiple

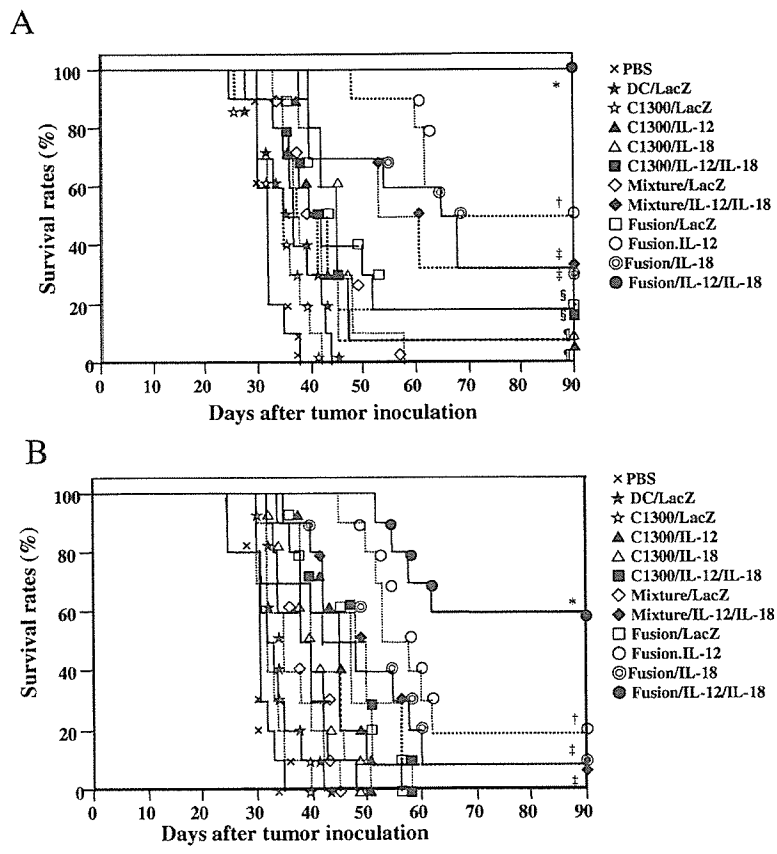

FIGURE 6. Protective and therapeutic effects of each vaccine on survival rates. *A*, Protective effect. On day 7 and day 14 before the i.v. inoculation of C1300, mice were vaccinated with fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, fusion/LacZ, mixture/IL-12/IL-18, mixture/LacZ, C1300/IL-12/IL-18, C1300/IL-12, C1300/IL-18, C1300/LacZ, DCs/LacZ, or PBS. The data presented were pooled from two separate experiments, and the reproducibility of results was demonstrated. *, $p < 0.05$ (fusion/IL-12/IL-18 vs other all groups). †, $p < 0.05$ (fusion/IL-12 vs all groups except fusion/IL-18 and mixture/IL-12/IL-18). ‡, $p < 0.05$ (fusion/IL-18, mixture/IL-12/IL-18 vs fusion/IL-12/IL-18, C1300/IL-12, C1300/IL-18, mixture/LacZ, C1300/LacZ, DCs/LacZ or PBS). §, $p < 0.05$ (fusion/LacZ, C1300/IL-12/IL-18 vs fusion/IL-12/IL-18, fusion/IL-12, mixture/LacZ, C1300/LacZ, DCs/LacZ or PBS). ¶, $p < 0.05$ (C1300/IL-12, C1300/IL-18 vs fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, mixture/IL-12/IL-18, C1300/LacZ or PBS). *B*, Therapeutic effect. On days 3 and 10 after i.v. inoculation of C1300, mice were vaccinated with fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, fusion/LacZ, mixture/IL-12/IL-18, mixture/LacZ, C1300/IL-12/IL-18, C1300/IL-12, C1300/IL-18, C1300/LacZ, DCs/LacZ, or PBS. The data presented were pooled from two separate experiments, and the reproducibility of results was demonstrated. *, $p < 0.05$ (fusion/IL-12/IL-18 vs other all groups). †, $p < 0.05$ (fusion/IL-12 vs all groups expect fusion/IL-18). ‡, $p < 0.05$ (fusion/IL-18, mixture/IL-12/IL-18 vs C1300/IL-12, C1300/IL-18, mixture/LacZ, C1300/LacZ, DCs/LacZ, or PBS)

Table II. *Therapeutic effects of each vaccine on liver metastasis*[a]

Immunogen	Tumor-Free Mice	No. of Liver Metastasis
PBS	0/10	48.0 ± 23.2
DC/LacZ	0/10	46.8 ± 21.6
C1300/LacZ	0/10	47.9 ± 21.4
C1300/IL-18	0/10	38.5 ± 10.0
C1300/IL-12	0/10	30.2 ± 11.2
C1300/IL-12/IL-18	0/10	19.6 ± 5.9[b]
Mixture/LacZ	0/10	41.3 ± 14.5
Mixture/IL-12/IL-18	1/10	16.8 ± 9.3[b]
Fusion/LacZ	0/10	20.1 ± 5.0[b]
Fusion/IL-18	1/10	18.7 ± 14.1[b]
Fusion/IL-12	2/10	9.7 ± 6.2[c]
Fusion/IL-12/IL-18	6/10	2.6 ± 3.4[d]

[a] Mean ± SD (n = 10). The data presented were pooled from two separated experiments, and the reproducibility of results was shown.
[b] $p < 0.05$ (fusion/LacZ, fusion/IL-18, C1300/IL-12/IL-18, mixture/IL-12/IL-18 vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).
[c] $p < 0.05$ (fusion/IL-12 vs. C1300/LL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).
[d] $p < 0.05$ (fusion/IL-12/IL-18 vs. C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

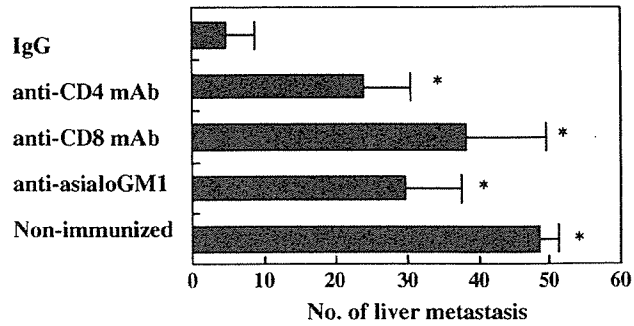

FIGURE 7. Participation of CD4, CD8, and NK cells in fusion/IL-12/IL-18 vaccine-induced immunity. After C1300 tumor inoculation, mice were administered fusion/IL-12/IL-18 vaccine or PBS (nonimmunized). Anti-CD4 mAb, anti-CD8 mAb, anti-Asialo GM1 Ab, or normal rat IgG was injected i.v. before vaccination and once every 5 days thereafter for an additional 20 days. On day 21, the number of liver metastasis was counted. *, $p < 0.01$ (nonimmunized, anti-asialo GM1, anti-CD8 mAb, anti-CD4 mAb vs IgG). Mean ± SD (n = 10).

tumor Ags, including unidentified tumor Ags. A phase I clinical trial of tumor lysate-pulsed DC treatment for patients with neuroblastoma has been conducted and a stable clinical response demonstrated (35). In contrast, our preliminary data have shown that the fusion vaccine of DCs and tumor cells induce a stronger antitumor immunity than that of tumor lysate (our unpublished observations). In previous experimental models using mice, Ag presentation and the antitumor effects of the fusion vaccine were reported in adenocarcinoma, plasmacytoma, hepatocellular carcinoma, and melanoma (15–23). Recently, we reported not only an increase in the antitumor effect, but also protective and therapeutic effects of an IL-2 gene-modified fusion vaccine in mice with pulmonary metastasis of fibrosarcoma (36). In clinical trials of the fusion vaccine in patients with melanoma, glioma, and renal cell carcinoma, complete remission in melanoma, a partial clinical response in glioma, and stability in renal cell carcinoma were demonstrated (37–39).

Although the chemical agent PEG and electrofusion have been used for the fusion vaccine, the fusion process of these methods is different. In PEG treatment, the lipid bilayer of the cell membrane is thought to be broken down through the dehydration action of PEG, followed by an increase in the fluidity of the cell membrane. In contrast, the fundamental step in electrofusion is reversible membrane breakdown. When short-duration, direct current electric impulses applied to the cell membrane exceed a critical threshold, that membrane will become transiently but highly permeable through the formation of micropores. Moreover, the adjacent process of touching cells may form channels and lead to the formation of new spherical hybrid cells. The loading efficiency of PEG and electrofusion was reported to be 17.0–35.0% and 5.0–25.0%, respectively (15–21). Cell fusion is known to be influenced by the characteristics of the cell membrane, and loading efficiency differs markedly among tumor cells. To increase and stabilize loading efficiency, we improved the fusion method by combining PEG and electrofusion. By this two-step method, tumor-loading efficiency was increased 1.6 times that of PEG treatment and 4 times that of electrofusion, and allowed higher CTL activities. We expect that the combination method of PEG and electrofusion may have beneficial effects on the stable loading of various types of tumor cells.

Previous immunotherapeutic approaches to neuroblastoma have focused on the use of IL-2 to activate both T cell-dependent and -independent cytotoxic immune responses (40, 41). Recent strategies for treating neuroblastoma have incorporated advances in molecular biology to engineer a tumor cell for the induction of a more efficient immune response. Yoshida et al. (42) showed that murine neuroblastoma retrovirally transduced with the IL-2 or GM-CSF gene exhibited tumor-specific acquired immunity. Shimizu et al. (43) and Redlinger et al. (44) reported that DCs transduced with the IL-12 gene by adenovirus could induce an antitumor immune response in an established murine neuroblastoma. However, the efficacy of DC transduced with IL-18 gene has not been reported in the murine neuroblastoma model. It has been reported that IL-18 could induce high-level expression of IFN-γ by T cells, NK cells, B cells, and monocytes and play an important role in CTL activation and enhancement of NK cell cytotoxic activity (27, 28). Furthermore, IL-18 acts synergistically with IL-12, but not IL-2 or GM-CSF, in inducing IFN-γ production by T cells (30, 31). Both IL-12 and IL-18 may be required for an effective differentiation into Th1 cells. In this study, we evaluated the Th1 immune response, CTL activity, and NK activity using fusion cells modified with both IL-12 and IL-18 genes. The production of IFN-γ by $CD4^+$ and $CD8^+$ T cells was markedly increased by the vaccination of fusion cells transduced with IL-12 and IL-18 genes. Moreover, transduction with the IL-12 and IL-18 genes in fusion cells showed a significant increase of MHC class I-restricted $CD8^+$ CTL activity and NK activity.

Our results showed that fusion cells of DCs and neuroblastoma, but not a mixture of DCs and neuroblastoma, significantly reduced liver metastasis and significantly increased survival rates compared with DCs or tumor cells alone, suggesting that the formation of fusion cells is important in inducing strong antitumor immunity. Orentas et al. (17) reported the protective effect of electrically prepared fusion cells on mice with Neuro-2a neuroblastoma cells. However, in his paper the loading efficiency by electrofusion was only 5–10%. In contrast, the loading efficiency of our two-step fusion method showed 45–52% efficiency. By improving loading efficiency, we demonstrated not only the protective effects but also the therapeutic effects of the fusion cells themselves. Furthermore, our study showed that the transduction of the fusion cells with both IL-12 and IL-18 genes produced complete protective effects and highly significant therapeutic effects on liver metastasis and survival. The relative importance of the NK cell, $CD4^+$, and $CD8^+$ T cell subsets for the therapeutic effect of the fusion/IL-12/IL-18 vaccine was demonstrated. To the best of our knowledge, this is the first study to demonstrate the protective and therapeutic effects

of an IL-12 and IL-18 gene-modified fusion vaccine on murine neuroblastoma with liver metastasis.

In conclusion, we demonstrated that improved loading efficiency may provide a basis for using a fusion vaccine and that introducing both IL-12 and IL-18 genes can induce extremely strong protective and therapeutic effects.

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Disclosures

The authors have no financial conflict of interest.

References

1. Isaacs, H., Jr. 1997. Neuroblastoma. *In* Tumors of the fetus and newborn. K.T. Oldham, P. M. Colombani, and R. P. Foglia, eds. *Scientific Principles and Practice*, Lippincott-Raven, Philadelphia, pp. 130–149.
2. Young, J. L., L. G. Ries, E. Silverberg, J. W. Horm, and R. W. Miller. 1986. Cancer incidences, survival and mortality for children younger than age 15 years. *Cancer* 58: 598–602.
3. Nagabuchi, E., and M. M. Ziegler. 1997. Neuroblastoma. *In* Surgery of Infants and Children. K. T. Oldham, P. M. Colombani, and R. P. Foglia, eds. *Scientific Principles and Practice*, Lippincott-Raven, Philadelphia, PA, pp. 593–613.
4. . Grosfeld, J. L., F. J. Rescorla, K. W. West, and J. Goldman. 1993. Neuroblastoma in the first year of life: clinical and biological factors influencing outcome. *Semin. Pediatr. Surg.* 2: 37–46.
5. Cooper, M. J., G. M. Hutchins, R. J. Mennie, and M. A. Israel. 1990. β2-microglobulin expression in human embryonal neuroblastoma reflects its developmental regulation. *Cancer Res.* 50: 3694–3700.
6. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9: 271–296.
7. Reid, D. C. 2001. Dendritic cells and immunotherapy for malignant disease. *Br. J. Haematol.* 112: 874–887.
8. Nestle, F. O., S. Aliagic, M. Gilliet, Y. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* 4: 328–332.
9. Chang, A. E., B. G. Redman, J. R. Whitfield, B. J. Nickoloff, T. M. Braun, P. P. Lee, J. D. Geiger, and J. J. Muller. 2002. A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. *Clin. Cancer Res.* 8: 1021–1032.
10. Matsuda, K., T. Tsunoda, H. Tanaka, Y. Umano, H. Tanimura, I. Nukaya, and K. Takesako. 2004. Enhancement of cytotoxic T-lymphocyte responses in patients with gastrointestinal malignancies following vaccination with CEA peptide-pulsed dendritic cells. *Cancer Immunol. Immunother.* 53: 609–616.
11. Nair, S. K., M. Morse, D. Boczkowski, R. I. Cumming, L. Vasovic, E. Gilboa, and H. K. Lyerly. 2002. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. *Ann. Surg.* 235: 540–549.
12. Coughlin, C. M., B. A. Vance, S. A. Grupp, and R. H. Vonderheide. 2004. RNA-transfected CD40-acivated B cells induce functional T-cell responses against viral and tumor antigen targets: implications for pediatric immunotherapy. *Blood* 103: 2046–2054.
13. Rodolfo, M., R. Luksch, S. Elisabeth, Y. T. Chen, P. Collini, T. Ranzani, C. Lombardo, P. Dalerba, L. Rivoltini, F. Arienti, et al. 2003. Antigen-specific immunity in neuroblastoma patients: antibody and T-cell recognition of NY-ESO-1 tumor antigen. *Cancer Res.* 63: 6948–6955.
14. Sarkar A. K., and J. G. Nuchtern. 2000. Lysis of MYCN-amplified neuroblastoma cells y MYCN peptide-specific cytotoxic T lymphocytes. *Cancer Res.* 60: 1908–1913.
15. Gong, J., D. Chen, M. Kashiwaba, and D. Kufe. 1997. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat. Med.* 3: 558–561.
16. Homma, S., G. Toda, J. Gong, D. Kufe, and T. Ohno. 2001. Preventive antitumor activity against hepatocellular carcinoma (HCC) induced by immunization with fusions of dendritic cells and HCC cells in mice. *J. Gastroenterol.* 36: 764–771.
17. Orentas, R., D. Schauer, Q. Bin, and B. D. Johnson. 2001. Electrofusion of a weakly immunogenic neuroblastoma with dendritic cells produces a tumor vaccine. *Cellular Immunol.* 213: 4–13.
18. Wang, J., S. Saffold, X. Cao, J. Krauss, and W. Chen. 1998. Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines. *J. Immunol.* 161: 5516–5524.
19. Gottfried, E., R. Krieg, C. Eichelberg, R. Andreesen, A. Mackensen, and S.W. Krause. 2002. Characterization of cells prepared by dendritic cell-tumor cell fusion. *Cancer Immunity* 2: 15–36.
20. Kao, J. Y., Y. Gong, C. M. Chen, Q. D. Zheng, and J. J. Chen. 2003. Tumor-derived TGF-βreduces the efficacy of dendritic cell/tumor fusion vaccine. *J. Immunol.* 170: 3806–3811.
21. Trevor, K. T., C. Cover, Y. W. Ruiz, E. T. Akporiaye, E. M. Hersh, D. Landais, R. R. Taylor, A. D. King, and R. E. Walters. 2004. Generation of dendritic cell-tumor cell hybrids by electrofusion for clinical vaccine application. *Cancer Immunol. Immunother.* 53: 705–774.
22. Gubler, U., A. O. Chua, D. S. Schoenhaut, C. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA* 88: 4143–4147.
23. Wolf, S. F., P. A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, et al. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* 146: 3074–3081.
24. Kobayashi, M., L. Fitz, M. Ryan, R. Hewick, S. C. Clark, S. Chang, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170: 827–845.
25. Gately, M. K., A. G. Wolitzky, P. N. Quinn, and R. Chizzonite. 1992. Regulation of human cytotoxic lymphocytes response by interleukin-12. *Cell Immunol.* 143: 127–142.
26. Robertson, M. J., R. J. Soiffer, S. F. Wolf, T. J. Manley, C. Donahue, D. Young, S. H. Herrmann, and J. Ritz. 1992. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J. Exp. Med.* 175: 779–788.
27. Okamura, H., H. Tsutsi, T. Komatsu, M. Yitsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, et al. 1995. Cloning a new cytokine that induces IFN-γ production by T cells. *Nature* 378: 88–91.
28. Okamura, H., K. Nagata, T. Komatsu, T. Tanimoto, Y. Nukata, F. Tanabe, K. Akita, K. Torigoe, T. Okura, S. Fukuda, and M. Kurimoto. 1995. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxin shock. *Infect. Immun.* 63: 3966–3972.
29. Tsutsui, H., K. Matsui, and N. Kawada. 1997. IL-18 accounts for both TNF-α and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. *J. Immunol.* 159: 3961–3973.
30. Fukao T., S. Matsuda, and S. Koyasu. 2000. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-γ production by dendritic cells. *J. Immunol.* 164: 64–71.
31. Kishida, T., H. Asada, E. Satoh, S. Tanaka, M. Shinya, H. Hirai, H. Tahara, J. Imanishi, and O. Mazda. 2001. In vivo electroporation-mediated transfer of interleukin-12 and interleukin-18 genes induces significant antitumor effects against melanoma in mice. *Gene Ther.* 8: 1234–1240.
32. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
33. Yoshimura, K., S. Hazama, N. Iizuka, S. Yoshino, K. Yamamoto, M. Muraguchi, Y. Ohmoto, T. Noma, and M. Oka. 2001. Successsful immunogene therapy using colon cancer cells (colon 26) transduced with plasmid vector containing mature interleukin-18 cDNA and the Igκ leader sequence. *Cancer Gene Ther.* 8: 9–16.
34. Miyake, S., M. Makimura, M. Kanegae, S. Harada, Y. Sato, K. Takamori, C. Tokuda, and I. Saito. 1996. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl. Acad. Sci. USA* 93: 1320–1324.
35. Geiger, J. D., R. J. Hutchinson, L. F. Hohenkirk, E. A. McKenna, G. A. Yanik, J. E. Levine, A. E. Chang, T. M. Braun, and J. J. Mule. 2001. Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cell mediate tumor regression. *Cancer Res.* 61: 8513–8519.
36. Ogawa, F., H. Iinuma, and K. Okinaga. 2004. Dendritic cell vaccine therapy by immunization with fusion cells of IL-2-gene-transduced dendritic cells and tumor cells. *Scan. J. Immunol.* 59: 432–439.
37. Kikuchi, T., Y. Akasaki, M. Irie, S. Homma, T. Abe, and T. Ohno. 2001. Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. *Cancer Immunol. Immunother.* 50: 337–344.
38. Märten, A., S. Renoth, T. Heinicke, P. Albers, A. Pauli, U. Mey, R. Casparo, D. Flieger, P. Hanfland, A. V. Ruecker, et al. 2003. Allogeneic dendritic cells fused with tumor cells: preclinical results and outcome of a clinical phase I/II trial in patients with metastatic renal cell carcinoma. *Hum. Gene Ther.* 14: 483–494.
39. Trefzer, U., G. Herberth, K. Wohlan, A. Milling, M. Thiemann, T. Sharav, K. Sparbier, W. Sterry, and P. Walden. 2005. Tumour-dendritic hybrid cell vaccination for the treatment of patients with malignant melanoma: immunological effects and clinical results. *Vaccine* 23: 2367–2373.
40. Bauer, M., G. H. Reaman, J. A. Hank, M. S. Cairo, P. Anderson, B. R. Blazar, S. Frierdich, and P. M. Condel. 1995. A phase II trial of human recombinant interleukin-2 administered as a 4-day continuous infusion for children with refractory neuroblastoma, non-Hodgkin's lymphoma, sarcoma, renal cell carcinoma, and malignant melanoma: a Childrens Cancer Group study. *Cancer* 75: 2959–2965.
41. Rueda, F., F. Marti, N. Pardo, I. Badell, M. Peiro, E. Bertran, E. Villen, J. Garcia, and J. Cubells. 1996. Interleukin-2 in neuroblastoma: clinical perspectives based on biological studies. *Cancer Biother. Radiopharm.* 11: 303–308.
42. Yoshida H., H. Enomoto, M. Tagawa, K. Takenaga, K. Tasaki, K. Nakagawara, N. Ohnuma, H. Takahashi, and S. Skiyama. Impaired tumorgenicity and decreased liver metastasis of murine neuroblastoma cells engineered to secrete interleukin-2 or granulocyte macrophage colony-stimulating factor. 1998. *Cancer Gene Ther.* 5: 67–73.
43. Shimizu, T., A. Berhanu, R. E. Redkinger, Jr., W. Simon, M. T. Lotze, and E. M. Barksdale, Jr. 2001. Interleukin-12 transduced dendritic cells induce regression of established murine neuroblastoma. *J. Pediatr. Surg.* 36: 1285–1292.
44. Redlinger, R. E., Jr., B. Mailliard, and E. M. Barksdale, Jr. 2003. Neuroblastoma and dendritic cell function. *Semin. Pediatr. Surg.* 13: 61–71.

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IRINOTECAN FOR CHILDREN WITH RELAPSED SOLID TUMORS

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□ *Irinotecan is expected to become a new drug for childhood solid tumors. Sixteen children with relapsed solid tumors received irinotecan 180 mg/m^2/day for 3 consecutive days, repeated once after 25 days off. Their original tumors were neuroblastoma in 7, rhabdomyosarcoma in 3, nephroblastoma and undifferentiated sarcoma in 2 each, and primitive neuroectodermal tumor and leiomyosarcoma in 1 each. The average age at trials was 6 years. Partial response was achieved in 5 (31.3%) (neuroblastoma, rhabdomyosarcoma, nephroblastoma, undifferentiated sarcoma, and leiomyosarcoma), and decrease in tumor marker in the other 2. Irinotecan appears promising, and could become included in the first-line treatment.*

Keywords clinical trials, irinotecan, nephroblastoma, neuroblastoma, rhabdomyosarcoma

Camptothecin (CPT), an alkaloid with a novel ring structure, was first isolated from the Chinese tree *Camptotheca acuminate*, and a water-soluble derivative of CPT, 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyl-camptothecin hydrochloride trihydrate (irinotecan, CPT-11), was subsequently synthesized [1]. Irinotecan as well as topotecan [2] inhibits DNA topoisomerase I, which is an essential nuclear enzyme that relaxes torsionally strained duplex DNA, enabling replication and transcription [3]. Irinotecan has been reported to be effective against various human malignancies, including lymphoma, gastric cancer, small cell lung cancer, non-small cell lung cancer, cervical cancer, epithelial ovarian cancer, colorectal cancer, and desmoplastic round blue cell tumor [4–13]. Four phase I trials of irinotecan in children were conducted in the United States, France, and Japan [14–17], and investigators in these four groups have proposed their own appropriate drug doses and administration schedules for the use in phase II trials.

A variety of approaches to the treatment of advanced pediatric solid tumors such as neuroblastoma have been employed, and the clinical results have improved in recent years [18, 19]. Nevertheless, the prognosis is dismal, particularly once patients with such tumor relapse after myeloablative therapy followed by stem cell transplantation, and it appears appropriate to investigate the activity of new agents such as irinotecan for these patients [3]. We therefore started clinical trials of the use of irinotecan in our patients who relapsed. We were also strongly encouraged to do so with our promising results in preclinical studies of irinotecan, which were conducted against several pediatric solid tumor xenograft models [20–22].

While other investigators [23] are reporting mediocre phase II results, much better clinical results were obtained in our trials of irinotecan in Japan, prompting us to write this article.

PATIENTS AND METHODS

This study was conducted by selected members of the Study Group of Japan for Treatment of Advanced Neuroblastoma [19]. Patients with

histologically confirmed, measurable solid tumors that were deemed to be treatment failures on conventional treatment (relapsed or refractory) were eligible for this trial. Other eligibility criteria included a life expectancy of at least 3 months, Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1, at least 4 weeks since and recovery from the toxic effects of previous chemo- and radiotherapy, hemoglobin concentration $\geq$8.0 g/dL, granulocyte count $\geq$1000/mm^3, and platelet count $\geq$50,000/mm^3. Other requirements included normal liver function (total bilirubin <1.5 mg/dL, aspartate transaminase and alanine transaminase less than twice the normal level), adequate renal function (serum creatinine <1.2 mg/dL). Patients with active infection, diarrhea, intestinal obstruction, pleural fluid or ascites, pneumonitis or pulmonary fibrosis, uncontrollable diabetes, and allergic reaction were excluded from this study, according to the recommendation of the Society of Japanese Pharmacopoeia [24]. Written informed consent for participation was obtained from all patients or their guardians, and the study protocol approved by the institutional review boards of participating institutions.

Patients were evaluated for nonhematopoietic toxicity with the ECOG common toxicity criteria [25] and for hematopoietic toxicity with a table of hematopoietic toxicities [17] modified by the current study group from the ECOG criteria (Table 1). The idea for this modification derived from the fact that all of the patients in the current study were recipients of prolonged high-dose chemotherapy and their granulocyte and platelet counts were only slightly more than 1000/mm^3 and 50,000/mm^3, respectively. The tumors were measured on the longest diameter, and the response was evaluated by the guidelines developed by Therasse and others [26]. Responses such as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were centrally reviewed, evaluated at 4 weeks after

TABLE 1 Nonhematopoietic Toxicities of the ECOG Common Toxicity Criteria [25] and Hematopoietic Toxicities Modified from the ECOG Common Toxicity Criteria for the Present Study [17]

	Grade				
	0	1	2	3	4
Serum aspartate transaminase (× nL)	<1.5	1.5–2	2.1–5	>5	—
Alkaline phosphatase (× nL)	<1.5	1.5–2	2.1–5	>5	—
Bilirubin (× nL)	<1.5	1.5–2	2.1–5	>5	—
Nausea and vomiting	None	Nausea	Controllable	Intractable	—
Diarrhea	None	2–3 times increase in stool	4–6 times increase in stool	7–9 times increase in stool	≥10 times increase in stool, bloody stool
White blood cell (×10^3/mm^3)	≥4.0	3.9–2.5	2.4–1.5	1.4–0.5	≤0.4
Granulocyte count (×10^3/mm^3)	≥2.0	1.9–1.3	1.2–0.8	0.7–0.3	≤0.2
Platelet count (×10^3/mm^3)	≥100	99–70	69–40	39–20	≤19

TABLE 2 Summary of the Cases Studied

Patient	Age (years)	Sex	Disease	Clinical response
1	12	F	Leiomyosarcoma	PR
2	9	M	Neuroblastoma	SD
3	4	M	Neuroblastoma	PD
4	11	F	PNET	PD
5	8	M	Neuroblastoma	SD*
6	9	F	Neuroblastoma	SD*
7	4	F	Neuroblastoma	PR
8	1	F	Undifferentiated sarcoma	PR
9	2	F	Nephroblastoma	PR
10	5	F	Nephroblastoma	PD
11	5	F	Neuroblastoma	SD
12	3	F	Rhabdomyosarcoma	PD
13	6	M	Rhabdomyosarcoma	PR
14	11	M	Undifferentiated sarcoma	PD
15	1	M	Neuroblastoma	SD
16	6	F	Rhabdomyosarcoma	PD

Note. Dose, 180 mg/m^2/day for 3 consecutive days. PNET, primitive neuroectodermal tumor; SD*, stable disease but with transient decrease in tumor marker levels.

the 2nd course of treatment, and 4 weeks was a minimum time period for confirmation of response [26].

The expected response (CR + PR) ratio was set to 20%.

RESULTS

Results of the study, performed from June 2001 to November 2004, are shown in Tables 2 and 3. The original tumors were neuroblastoma in 7, rhabdomyosarcoma in 3, nephroblastoma and undifferentiated sarcoma in 2 each, and primitive neuroectodermal tumor (PNET) and leiomyosarcoma in 1 each. One patient had a refractory tumor, and the remaining 15 had relapsed tumors. Age of the patients at the start of trials ranged from 1 year

TABLE 3 Adverse Effects Observed in 32 Courses of Irinotecan Treatment for 16 Patients

	Grade 0	Grade 1	Grade 2	*Grade 3*	*Grade 4*
Myelosuppression					
White blood cell count	0	1	8	22	*1*
Granulocyte count	0	1	5	13	*13*
Platelet count	5	6	8	11	*2*
Gastrointestinal tract					
Diarrhea	3	3	15	*4*	*7*
Vomiting	1	4	18	8	1

Note. Italic type indicates severe adverse effects.

to 12 years, and the average age was 6 years. Thirty-two treatment courses were administered to these 16 patients. All patients were given irinotecan 180 mg/m^2/day by 120-min drip infusion for 3 consecutive days repeated once after 25 days off. A partial response (PR) was achieved in 5 (neuroblastoma, rhabdomyosarcoma, nephroblastoma, undifferentiated sarcoma, and leiomyosarcoma), and transient decrease in tumor marker levels was observed in the other 2 patients with neuroblastoma. Therefore, PR was observed in 31.3% of the relapsed/refractory patients with the current administration schedule of irinotecan, which is different from the results of the other investigators. The response (CR + PR) ratio was much higher than expected, and exceeded the response rate of 21.1% by Cosetti et al. [27].

Of 7 children with relapsed neuroblastoma, PR was achieved in 1, SD but with transient decrease in urinary tumor marker levels in 2, SD alone in 3, and PD in 1. One of 3 patients with rhabdomyosarcoma had a PR, but the response was PD in the remaining 2. Similarly, one of 2 patients with nephroblastoma showed PR, but the response was PD in the remaining one, and 1 of 2 patients with undifferentiated sarcoma showed a PR, but the response was PD in the remaining 1.

Grade 3 suppression of white blood cell count was observed in 69% of the patient courses, grade 3 to 4 suppression of granulocyte count in 81%, and grade 3 to 4 suppression of platelet count in 41%, whereas diarrhea of grade 3 or greater was seen in 34% (Table 3), but these side effects were all managed well using routine clinical methods, with recombinant human granulocyte colony-stimulating factor (rhG-CSF), oral or intravenous antibiotics, and/or central line fluid administration. Prophylactic administration of loperamide were not recommended, but in patients who developed diarrhea, loperamide and/or antibiotics were given with subsequent courses to decrease gastrointestinal toxicity, according to the judgment of each investigator.

DISCUSSION

Among the camptothecin derivatives, topotecan and irinotecan are most widely used in clinical trials [3]. As to the use in children, irinotecan appears to be promising in the treatment of such tumors as rhabdomyosarcoma, neuroblastoma, and desmoplastic round blue cell tumor [13–17]. The recommended dose and administration schedule of irinotecan differ among researchers [14–17]. Furman and coworkers [14] recommend administration of irinotecan 20 mg/m^2/day for 5 consecutive days, repeated once after 2 days off (10 days' administration altogether) based on their results of irinotecan experiments in an in vivo system. On the other hand, Vassal and coworkers [16] reported that the maximum tolerated dose (MTD) of irinotecan for children was 600 mg/m^2 when given as a 120-min intravenous infusion every 21 days. Mugishima and others from Japan [17] determined that the

TABLE 4 Recommended Administration Schedules for Phase II Trials of Irinotecans and Some Results of Phase II Trials

Authors	Results of phase I (MTD) and recommended schedules for phase II	Results of phase II trials
Mugishima et al. [17] (Japan)	180 mg/m^2/day for 3 consecutive days; repeated every 4 weeks	31.3% response rate; 5 PR in 16 patients (present report)
Furman et al. [14] (USA)	20 mg/m^2/day for 5 consecutive days; repeated after 2 days off; repeated every 4 weeks	21.1% response rate; 2 CR/2 PR in 19 patients (Cosetti et al. [27])
Blaney et al. [15] (USA)	50 mg/m^2/day , daily for 5 days, beginning every 3 weeks	Ongoing
Vassal et al. [16] (France)	600 mg/m^2/day; repeated every 3 weeks	Disappointing in neuroblastoma (Vassal et al. [23])

Note. MTD, maximum tolerated dose.

MTD of irinotecan for children should be between 160 and 180 mg/m^2/day administered over 3 consecutive days, repeated once after 25 days off. These MTDs are currently recommended for phase II trials in the respective groups (Table 4), but these four regimens have both merits and demerits [14–17]. Cosetti et al. [27] reported 4 objective responses (2 CR and 2 PR) (21.1%) in 19 evaluable patients treated on the administration schedules developed by Furman et al. [14], but the authors' response rate of 31.3% (5 PR in 16 cases) was superior to their results [27].

Although it might be too premature to refer to the relationship between the nature of the tumor and the efficacy of irinotecan, Cosetti et al. [27] already observed CP + PR in 6 of 7 patients with relapsed rhabdomyosarcoma on the schedule of Furman et al. [14], and this observation coincides with our results in rhabdomyosarcoma with 1 PR in 3 cases, although they used a different mode of administration. On the other hand, the use of irinotecan for neuroblastoma in a single day by Vassal et al. [23] met with disappointing results with no clinically useful activity in their phase II trials. They noted that since the majority of children had received very intensive induction treatments and retinoids it made it unlikely that a single agent in a phase II setting would demonstrate activity. They considered that they needed to evaluate neuroblastoma in a different setting in the future to prevent clinically important agents from being overlooked [23]. The authors observed one PR and two cases of transient decrease in tumor marker levels in 7 relapsed neuroblastomas, and the patient who showed PR (Table 2) at the evaluation is now disease-free without treatment for 46 months since the second remission [28].

It is also hard to conclude the relationship between the administration schedule and the effectiveness of irinotecan. Protracted per oral use of irinotecan might be recommended because of its consistent effectiveness [22, 29], but the use of irinotecan over 12 days could be somewhat burdensome for patients and clinicians. Vassal et al. prefer the use of it in a single