aberrance between NB before and after discontinuation of MS was analyzed with the χ^2 and Fisher's Exact tests.

2. Results

2.1. Clinical features of 20 NBs detected in children older than 6 months sporadically in the cohort of children who did not undergo MS after the discontinuation of MS

The clinical characteristics of *20 NBs* detected sporadically in children older than 6 months in the cohort of children who did not undergo MS after discontinuation of MS are presented in Table 1. The age ranged from 7 to 67 months (median age, 13 months). There were 6 stage 1 or 2, 2 stage 3, and 12 stage 4 cases. Five free cases were detected by abdominal echo or chest x-ray conducted because of a nonrelated symptom. The primary tumor sites were the adrenal gland region in 15 cases and the nonadrenal gland region in 5 cases. In all 20 cases, the urinary VMA or HVA was elevated. Regarding the treatment of the patients younger than 12 months, in principle, the protocol of the Japanese Infantile Neuroblastoma study have been administered for patients younger than 12 months since 1994 [15]. Patients older than 1 year, in principle, have been treated based on the protocol of the Japan Study Group for Advanced Neuroblastoma for the patients older than 1 year with advanced neuroblastomas since 1998 [16]. Although the follow-up period was short, all of the patients are alive, and 11 of the 20 patients have demonstrated an event-free survival.

2.2. Biological features of 20 NBs detected sporadically in children older than 6 months in the cohort of children who did not undergo MS after the discontinuation of MS

The biological features of 20 NBs detected in children older than 6 months sporadically in the cohort of children who did not undergo MS after discontinuation of MS are presented in Table 2. Three (15%) of the 20 examined cases demonstrated an amplification of *MYCN* by FISH and quantitative PCR, in addition to the Southern blot method. Of the 20 cases, 14 (70%) had a diploid or tetraploid pattern based on flow cytometry. Of the 19 examined cases, 4 (21%) showed a 1p deletion by 2-color FISH method or SNP array. Fourteen cases were analyzed using an SNP array, and 4 showed 2p gain, 4 showed 3p loss, 7 showed 11q loss, and 6 showed 17q. Based on Shimada's histologic classification, 10 (53%) of the 19 examined tumors were considered to have an unfavorable histology. The BGT determined based on these genetic features showed that 6 patients were type 1 (low risk), 11 type 2A (intermediate risk), and 3 type 2B (high risk).

2.3. Comparison of NBs detected before and after the discontinuation of MS

Table 3 shows a comparison of the BGT for the NBs detected before and after the discontinuation of MS. The BGT for the 20 NBs detected after the discontinuation of MS was type 1 in 30% (6/20), type 2A in 55% (11/20),

Table 2 The biological features of NB detected after the discontinuation of MS

Case	The status of MYCN			DNA Ploidy	1p loss	2p gain	3p loss	11q loss	17q gain	Shimada histology	BGT
	SB (copy no.)	FISH	PCR								
1	1	No amp.	No amp.	TR	No	No	No	No	No	FH	Type 1
2	1	No amp.	No amp.	TR	No	No	No	No	No	FH	Type 1
3	1	No amp.	No amp.	D/T	No	No	Yes	Yes	Yes	UH	Type 2A
4	1	Gain	No amp.	D/T	No	Yes	Yes	Yes	No	FH	Type 2A
5	1	No amp.	No amp.	TR	No	No	No	No	No	FH	Type 1
6	40	Amp.	Amp.	D/T	Yes	No	No	No	Yes	UH	Type 2B
7	1	No amp.	No amp.	TR	No	No	No	No	No	UH	Type 1
8	1	No amp.	No amp.	TR	No	–	–	–	–	FH	Type 1
9	1	Gain	No amp.	D/T	No	Yes	No	Yes	Yes	UH	Type 2A
10	1	Gain	Slight increase	D/T	Yes	Yes	Yes	Yes	No	UH	Type 2A
11	1	No amp.	No amp.	D/T	No	No	No	Yes	Yes	UH	Type 2A
12	1	No amp.	No amp.	D/T	–	–	–	–	–	–	Type 2A
13	1	No amp.	No amp.	D/T	No	Yes	No	Yes	Yes	UH	Type 2A
14	1	No amp.	No amp.	D/T	No	No	No	No	No	FH	Type 2A
15	1	No amp.	No amp.	TR	No	–	–	–	–	FH	Type 1
16	1	No amp.	No amp.	D/T	No	No	Yes	Yes	Yes	UH	Type 2A
17	1	No amp.	No amp.	D/T	No	–	–	–	–	FH	Type 2A
18	18	Amp.	Amp.	D/T	No	–	–	–	–	UH	Type 2B
19	–	Amp.	Amp.	D/T	Yes	–	–	–	–	UH	Type 2B
20	–	Gain	Slight increase	D/T	Yes	–	–	–	–	FH	Type 2A

SB indicates Southern blot; amp., amplification; TR, triploid; D/T, diploid or tetraploid.

Table 3 Brodeur's genetic type of NB detected before or after the discontinuation of MS

	BGT		
	Type 1	Type 2A	Type 2B
Before stop of MS (n = 93)	56 (60%)	17 (18%)	20 (22%)
Screened cases (n = 82)	55 (67%)	11 (13%)	16 (20%)
MS-positive cases (n = 55)	49 (89%)	4 (7%)	2 (4%)
MS-negative cases (n = 27)	6 (22%)	7 (26%)	14 (52%)
Nonscreened cases (n = 11)	1 (9%)	6 (55%)	4 (36%)
After stop of MS (n = 20)	6 (30%)	11 (55%)	3 (15%)

Screened cases, NB detected in the cohort of children who underwent MS at 6 months of age; MS-positive cases, NB detected through MS at 6 months of age; MS-negative cases, NB detected at more than 6 months of age sporadically in the cohort of children who underwent MS at 6 months of age; nonscreened cases, NB detected at more than 6 months of age sporadically in the cohort of children who did not undergo MS at 6 months of age.

and type 2B in 15% (3/20). In contrast, in the 93 NBs detected before the discontinuation of MS, 60% (56/93) were type 1, 18% (17/93) type 2A, and 22% (20/93) type 2B. In the 55 MS-positive cases among these 93 NBs, 89% (49/55) were type 1, 7% (4/55) type 2A, and 4% (2/55) type 2B. In the 27 MS-negative cases among the 93 NBs, 22% (6/27) were type 1, 26% (7/27) type 2A, and 52% (14/27) type 2B. In the 11 nonscreened cases among these 93 NBs, 9% (1/11) were type 1, 55% (6/11) type 2A, and 36% (4/11) type 2B. In summary, there was a decrease of type 1 and an increase of type 2A NB in patients detected after MS was discontinued.

The clinical stage of type 2A NB before or after the discontinuation of MS is shown in Table 4. Twenty-eight percent was stage I (5/18) NB before the discontinuation of MS, whereas only 1% (1/11) was stage I NB after the discontinuation of MS. However, 50% (9/18) was stage 4 NB before the discontinuation of MS, whereas 82% (9/11) was stage 4 NB after the discontinuation of MS.

The genetic aberrations of type 2A NB before or after the discontinuation of MS are summarized in Table 5. The genetic aberrations of 8 type 2A NBs before the discontinuation of MS and 8 type 2A NBs after discontinuation of MS were analyzed using an SNP array. The significant difference was not observed between NB treated before and after discontinuation of MS in each

Table 4 Clinical stage of type 2A NB

INSS	Before stop of MS (n = 18)	After stop of MS (n = 11)
Stage 1	5 (28%)	1 (1%)
Stage 2A	1	0
Stage 2B	1	0
Stage 3	2	1
Stage 4	9 (50%)	9 (82%)

Table 5 Genetic aberrations of type 2A NB

BGT	Before stop of MS (n = 8)	After stop of MS (n = 8)	*P*
1p loss	2 (25%)	1 (13%)	NS
2p gain	3 (38%)	3 (38%)	NS
3p loss	4 (50%)	4 (50%)	NS
11q loss	6 (75%)	7 (88%)	NS
17q gain	7 (88%)	5 (63%)	NS

NS indicates not significant.

genetic aberrance (1p loss, 2p gain, 3p loss, 11q loss, 17q gain), respectively.

3. Discussion

Most of the NBs detected by MS have a good prognosis. The purpose of MS at 6 months of age was to decrease the number of advanced-stage NB patients older than 1 year and mortality because of NB. Regrettably, this goal was not confirmed by several studies [6]. However, at least 17% of the NBs detected by MS have one or more biologically unfavorable factors and might thus have a higher risk of recurrence than in patients without such biologically unfavorable factors in our previous study [17]. Some of these tumors, which have one or more biologically unfavorable factors, may progress and eventually be fatal, if they are not detected by 6 months of age and, as a result, do not undergo any immediate surgical intervention with or without additional treatment. Furthermore, the analysis of large cohort in Japan recently reported that the mortality rate from NB in children who were screened at 6 months was lower than that in the prescreening cohort [18].

In the present study, 14 cases (60%) of 20 NBs detected sporadically in children older than 6 months in the cohort of children who did not undergo MS after the discontinuation of MS were advanced-stage NB (stage 3, 4). Furthermore, in all 20 cases, the urinary VMA or HVA was elevated. If the patients younger than 15 months already had a small tumor that secreted VMA or HVA at 6 months of age, 11 cases of 20 NBs would have been detected at 6 months of age if they underwent MS.

The analysis of BGT for NB before and after the discontinuation of MS in the present study showed a decrease of type 1 NB and an increase of type 2A NB in NB cases detected sporadically after the discontinuation of MS in Japan. Of type 2A NBs, the number of patients with stage 4 NB after the discontinuation of MS was more than that of patients with stage 4 NB before the discontinuation of MS. The rate of type 2A NB (55%) in the 20 NBs diagnosed after the discontinuation of MS was higher than that (26%) of the 27 MS-negative cases detected before the discontinuation of MS and almost equal to that (55%) of the 11 nonscreened cases before the discontinuation of MS. These results suggest

that most of the type 1 NBs detected by MS regressed and were not detected sporadically, and most of the type 2A NBs detected by MS were detected sporadically as advanced NB at more than 1 year of age after the discontinuation of MS. Another possibility is that the type 1 NBs detected by MS have intratumoral genetic heterogeneity, which is associated with some type 2A NB cells. In the present study, the pattern of genetic aberrations for type 2A NBs before discontinuation of MS and type 2A NBs after discontinuation of MS are quite similar. If type 1 NBs with the intratumoral genetic heterogeneity of type 2B NB cells are not detected during MS at 6 months of age, the type 2A NB cells of these tumors may progress and present sporadically as type 2A NB with advanced stage at more than 1 year of age.

As the present study is not a population-based study, and includes only a short follow-up period, the difference in the mortality rate and accurate incidence rate of NBs detected before and after the discontinuation of MS is unclear. This study is a preliminary report of the experience in Japan of managing NB after the discontinuation of MS. A future analysis of the population-based incidence of sporadically detected NB after the discontinuation of MS and the detailed evaluation for the biology of a large number of NB detected after the discontinuation of MS are necessary to elucidate the risks and benefits of MS at 6 months of age in Japan.

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ORIGINAL ARTICLE

Complete elimination of established neuroblastoma by synergistic action of γ-irradiation and DCs treated with rSeV expressing interferon-β gene

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Dendritic cell (DC)-based immunotherapy has been investigated as a new therapeutic approach to intractable neuroblastomas; however, only limited clinical effect has been reported. To overcome the relatively low sensitivity of neuroblastomas against immunotherapy, we undertook a preclinical efficacy study to examine murine models to assess the combined effects of γ-irradiation pretreatment and recombinant Sendai virus (ts-rSeV/dF)-mediated murine interferon-β (mIFN-β) gene transfer to DCs using established c1300 neuroblastomas. Similar to intractable neuroblastomas in the clinic, established c1300 tumors were highly resistant to monotherapy with either γ-irradiation or DCs activated by ts-rSeV/dF without transgene (ts-rSeV/dF-null) that has been shown to be effective against other murine tumors, including B16F10 melanoma. In contrast, immunotherapy using DCs expressing mIFN-β through ts-rSeV/dF (ts-rSeV/dF-mIFNβ-DCs) effectively reduced tumor size, and its combination with γ-irradiation pretreatment dramatically enhanced its antitumor effect, resulting frequently in the complete elimination of established c1300 tumors 7–9 mm in diameter, in a high survival rate among mice, and in the development of protective immunity in the mice against rechallenge by the tumor cells. These results indicate that the combination of ts-rSeV/dF-mIFNβ-DCs with γ-irradiation is a hopeful strategy for the treatment of intractable neuroblastomas, warranting further investigation in the clinical setting.
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Keywords: *dendritic cell; recombinant Sendai virus; irradiation; neuroblastoma*

Introduction

Neuroblastoma, with its many clinical and molecular faces, is the most common extracranial malignant solid tumor seen in children.[1] When occurring in infants less than 1 year old, neuroblastoma shows a relatively good prognosis, whereas only 30% of children above that age with advanced cases of the disease do not experience disease progression for at least 3 years after treatment.[2] Recent efforts by physicians have demonstrated that surgical intervention, irradiation and intensive chemotherapy followed by stem cell transplantation improved the survival of such patients.[3,4] However, in a large number of these children, especially in cases with *MYCN* amplification, the disease remains intractable.[3,4]

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As an alternative potential therapy, clinical evaluation of dendritic cell (DC)-based immunotherapy was initiated several years ago. The first reported clinical study, which enrolled 15 children with advanced solid tumors including three individuals with neuroblastoma, demonstrated modest antitumor responses.[5] Subsequently, another group reported the results of tumor RNA-loaded DC vaccination for 11 patients with stage 4 neuroblastoma.[6] Even though these challenging clinical studies showed specific antitumor immune reactions, the clinical outcome is still far from the level required for a standard therapy.

Very importantly, these frontier studies suggested that the immunosuppressed condition of these patients after intensive chemotherapy might limit the efficacy of DC-based immunotherapy.[6] In addition, neuroblastoma is shown to be less immunogenic,[7] in association with a suppressed expression of major histocompatibility complex class I, which can be caused by *MYCN* amplification.[8–10] DC-based cancer immunotherapy, moreover, is now also a developing technology and has shown limited clinical outcome in other malignancies. Therefore, scientists and physicians should elucidate (1) the

most effective DC subtypes, (2) the optimal conditions and activation stimuli to generate activated DCs showing optimal antitumor effects *in vivo*, (3) the optimal route for administration and (4) the optimal dose and frequency of DC vaccinations.[11–13] The lack of such information may explain the limited efficacy of DC-based immunotherapy for advanced neuroblastoma in the clinical setting.

Recently, we have demonstrated a dramatic improvement in the efficacies of DCs activated by recombinant Sendai virus (rSeV), namely 'immunostimulatory virotherapy', on multiple syngeneic mouse models bearing highly malignant tumors, including B16F10 melanoma,[14] MH134 hepatocellular carcinoma[14] and SCCVII squamous cell carcinoma.[15] rSeV is a novel and powerful gene transfer modality as a cytoplasmic gene expression system[16–18] that leads DCs to highly activated/mature state through a DExD/H-box RNA helicase, retinoic acid-inducible gene-I (RIG-I).[19,20] Therefore, we hypothesized that rSeV-activated DCs might enhance antitumor immunity against less immunogenic c1300 neuroblastoma.

With this background in mind, we here examined and optimized the antitumor effect of DC immunotherapy activated by a 'temperature-sensitive mutant' and F-gene-deleted non-transmissible rSeV (ts-rSeV/dF), an advanced vector design showing a less cytopathic effect,[18,21,22] that is now available for mass production according to the good manufacturing practice guidelines.

Our goal was to develop therapeutics based on ts-rSeV/dF-DCs that could completely eliminate, rather than merely shrink, established tumors as well as induce protective antitumor immunity against recurrence. Importantly, although here we confirmed that the c1300 tumor was still highly resistant to DC-based immunotherapy, even with the use of DCs activated by ts-rSeV/dF, we here found that γ-irradiation pretreatment accompanying murine interferon-β (mIFN-β) gene transfer dramatically and synergistically enhanced the antitumor immunity induced by intratumoral (i.t.) injection of ts-rSeV/dF-DCs without any antigen loading *ex vivo*. We here show that this new regimen resulted not only in the complete elimination of a high proportion of established c1300 tumors 7–9 mm in diameter, but also in the induction of tumor-specific protective immunity.

Results

c1300 neuroblastoma is highly resistant to DC-based immunotherapy

Throughout this study, DCs were not pulsed by tumor antigen *ex vivo*, because antigen loading did not enhance the antitumor effect of ts-rSeV/dF-DCs that were administered i.t. to c1300 tumors (data not shown). These findings were similar to those of our earlier studies using B16 melanoma[14] and SCCVII squamous cell carcinoma.[15]

To optimize the dose of DC-based immunotherapy that was activated by ts-rSeV/dF-null (ts-rSeV/dF-DCs), we first determined the effective dose of DCs to dermally implanted c1300 neuroblastomas in the abdominal wall of A/J female mice. We here administered ts-rSeV/dF-DCs without antigen pulsation i.t., because this injection route showed an optimal antitumor effect against both B16F10 melanoma[14] and SCCIIV squamous cell carcinoma[15] in our earlier studies. At the same time, the therapeutic effect against c1300 was directly compared with that against B16F10 melanoma.

As shown in Figure 1, in the 'early treatment regimen',[14] three-times weekly administration of ts-rSeV/dF-DCs, the highest efficacy against B16F10 melanoma occurred at 10^6 cells per dose of ts-rSeV/dF-DCs, resulting in the elimination of 50% of the tumors. In contrast, ts-rSeV/dF-DCs showed a modest suppression of tumor growth of c1300 neuroblastomas without eliminating any of the tumors, suggesting that ts-rSeV/dF-DC-based immunotherapy was more effective against B16F10 than against c1300.

Gene transfer of mIFN-β by ts-rSeV/dF to DCs enhances the antitumor effect against c1300

To overcome the limited efficacy of ts-rSeV/dF-DCs against c1300, we next examined the effect of mIFN-β gene transfer by ts-rSeV/dF to DCs (ts-rSeV/dF-mIFNβ-DCs), which has been shown to enhance antitumor immunity to B16 melanoma effectively.[14]

As shown in Figure 2a, recombinant mIFN-β or human IFN-β was effective in upregulating major histocompatibility complex class I antigen expression not only in murine (c1300) but also in human (SK-N-SH and IMR32) neuroblastoma cell lines, irrespective of predefined *N-myc* amplification. However, direct cytoxicity had no or only a modest effect on them (Figure 2b). These findings were similar to those of our earlier study obtained by the use of melanomas[14] and indicated the modest susceptibility of c1300 neuroblastomas to mIFN-β.

In turn, the use of ts-rSeV/dF-mIFNβ, instead of the ts-rSeV/dF-null vector, as a DC activator dramatically enhanced the antitumor effect on c1300, resulting in the elimination of 60% of the tumors in the 'early' treatment regimen *in vivo*, as expected (Figure 3a, left three panels and Figure 3b, left graph).

We then asked whether or not established c1300 tumors, 7–9 mm in diameter, could respond to ts-rSeV/dF-mIFNβ-DCs through the 'later' treatment regimen starting at 10 days after tumor inoculation. Although ts-rSeV/dF-mIFNβ-DCs significantly suppressed the growth of established c1300 tumors, the percentage of tumors eliminated was still and unexpectedly low (one of six animals showed complete elimination; Figure 3a, right three panels and Figure 3b, right graph). In this experiment, no animal except the one showing tumor elimination survived over 120 days (data not shown; the representative data are shown in Figure 5b), indicating that rSeV/dF-mIFNβ-DCs contributed to the significant suppression of tumor growth, though not enough to prolong the survival of tumor-bearing mice.

Synergistic sensitization of c1300 tumor to ts-rSeV/dF-IFNβ-DCs by γ-irradiation pretreatment

Together, these data confirmed the limited efficacy of ts-rSeV/dF-DC-based immunotherapy against less-immunogenic c1300 neuroblastoma. We therefore next looked for a possible sensitizer that might enhance the effect of DC immunotherapy. Recent studies have demonstrated that radiotherapy induces an 'abscopal effect' against distant tumors,[23,24] probably due to the enhancement of antitumor immunity.[25] We therefore examined the combined effect of a clinically available dose of γ-irradiation (4 Gy day^{-1} for 3 days) followed by weekly

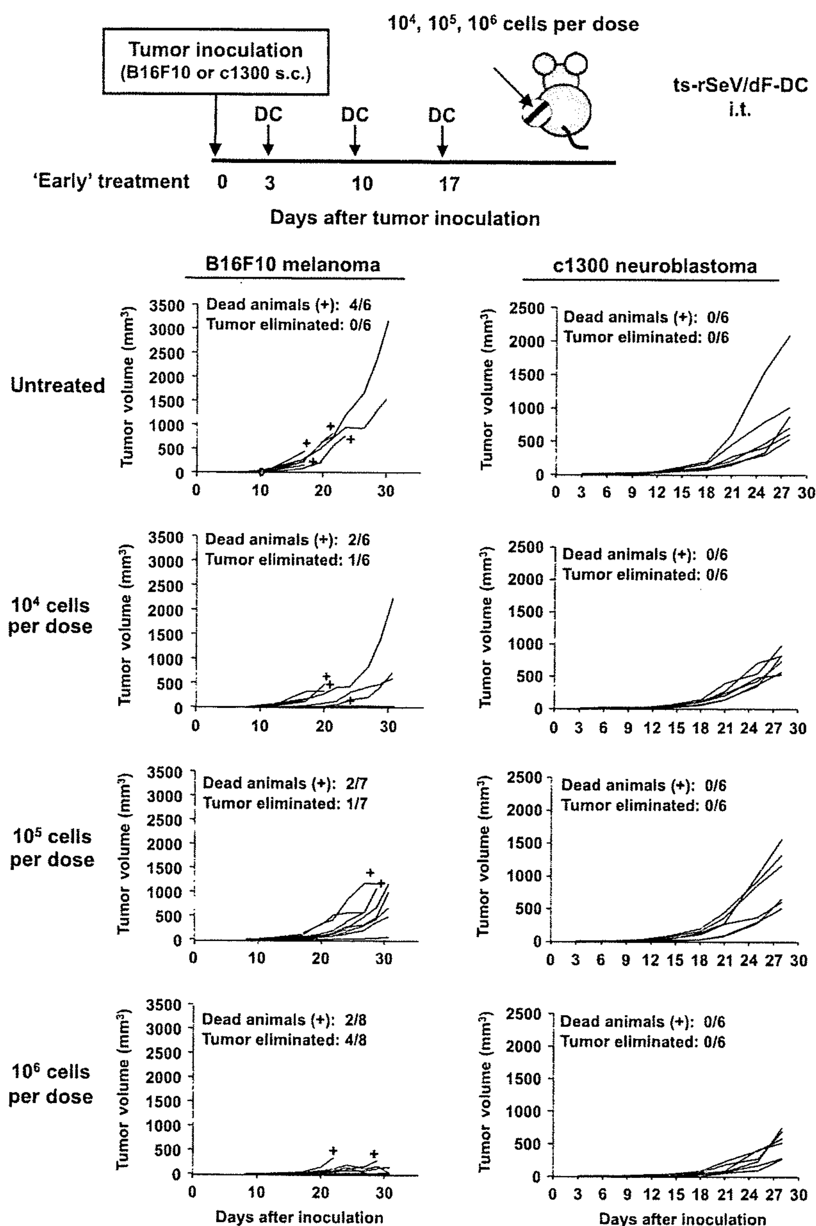


Figure 1 Direct comparison of antitumor effect by direct and repeated i.t. injection of DCs activated by temperature-sensitive mutant and F-gene-deleted non-transmissible recombinant Sendai virus (ts-rSeV/dF-DCs) without any therapeutic gene. Three days after intradermal inoculation of B16F10 melanoma (left panels) or c1300 neuroblastoma (right panels), various amounts of ts-rSeV/dF-DCs were injected weekly through an i.t. route as the 'early' treatment regimen. Thereafter, the tumor volume was measured. Lines on the panels indicate time courses of tumor volume in individual animals. Apparent dose-efficacy response was seen in the tumor volume of B16F10 melanoma, and four of eight animals that received 10^6 DCs per dose showed complete tumor elimination (left panels). In contrast, no animals inoculated with c1300 neuroblastoma demonstrated either complete elimination or significant dose–response on tumor size at any dose. The + symbol indicates animals that died during observation. DC, dendritic cell; i.t., intratumoral; rSeV, recombinant Sendai virus.

i.t. administration of rSeV/dF-mIFNβ-DCs in the 'later' treatment regimen (Figure 4, scheme). In this experiment, the tumor was intradermally implanted in the thigh to avoid radiation-induced toxicity to vital organs.

As shown in Figures 4a and b, monotherapy consisting of either γ-irradiation or rSeV/dF-mIFNβ-DCs effectively reduced the tumor volume. However, the established tumors were rarely eliminated (γ-irradiation: 0/6 animals; rSeV/dF-mIFNβ-DCs: 2/8 animals). In contrast, when these therapies were combined, six of eight animals showed the complete elimination of all established tumors at 38 days after inoculation. As the result, 5 of the 8 animals treated with both γ-irradiation and rSeV/dF-mIFNβ-DCs survived over 200 days in tumor-free condition ($P<0.001$ vs other groups) (Figure 4c).

a

Murine

c1300
(*N-myc* amp-)

FL2-H: H-2Kk PE

c1300 mIFN-β(+) 92.5%
c1300 (–) 89.1%
Isotype matched 0%

Human

SK-N-SH
(*N-myc* amp-)

FL2-H: classI-PE

SK-N-SH hIFN-β(+) 22.6%
SK-N-SH (–) 0%
Isotype matched 0%

IMR32
(*N-myc* amp+)

FL2-H: classI-PE

IMR32 hIFN-β(+) 99.6%
IMR32 (–) 23.4%
Isotype matched 0%

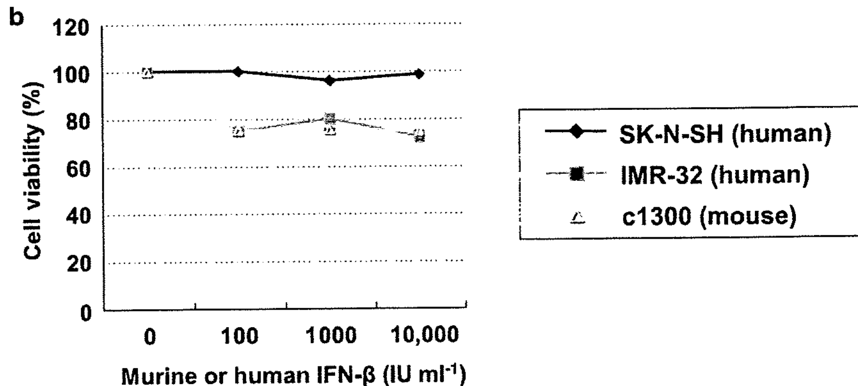


Figure 2 Interferon-β strongly upregulates MHC class I molecules of, and shows no or modest cytotoxicity against, murine and human neuroblastomas. c1300 (mouse), SK-N-SH (human) or IMR32 (human) neuroblastoma was treated with IFN-β at $1000\,\mathrm{U\,ml^{-1}}$ for corresponding species (c1300 for mIFN-β and SK-N-SH or IMR32 for hIFN-β, respectively) for 48 h. (**a**) Expression of MHC class I molecule. The panels are the typical FACS patterns among three independent experiments. C1300 cells abundantly expressed MHC class I, and mIFN-β upregulated mean fluorescence intensity (MFI). SK-N-SH cells show no expression of MHC class I, and hIFN-β stimulated its expression. IMR32 indicated dual peaks of MHC class I expression, suggesting that this cell line was composed of heterogeneous populations, and hIFN-β treatment strongly induced MHC class I expression. (**b**) *In vitro* cytotoxicity assay for IFN-βs to murine neuroblastoma (yellow line) and human neuroblastomas (SK-N-SH: blue line and IMR32: red line). The viability of SK-N-SH cells was not affected by hIFN-β, and c1300 and IMR32 showed modest (~20%) cytotoxicity by IFN-β treatment. hIFN-β, human IFN-β; MHC, major histocompatibility complex; mIFN-β, murine interferon-β. (See online version for color figure.)

Immunotherapy using ts-rSeV/dF-IFNβ-DCs was required to establish protective immunity for the second challenge of c1300 tumor cells

Finally, we asked whether or not the complete elimination of c1300 tumors might contribute to the establishment of long-lasting protective immunity.

At first, we assessed the cytotoxic T-lymphocyte (CTL) activity using splenocytes from mice with DC treatment. A strong and c1300-specific cell lytic activity of stimulated splenocytes with tumor antigen was found only in the case of mice with combined treatment, but not with other treatment groups (Figure 5a, left graph). Such a cell lytic activity could not be found when MuSS (a third-party tumor: A/J mouse-derived malignant fibrous histiocytoma)[26] was used as the target (Figure 5a, right graph).

Next, we performed an additional experiment for the second challenge by the simultaneous inoculation of c1300 and MuSS on day 186. The overdose irradiation group (34 Gy × 3 days) that showed a high percentage of c1300 tumor elimination (>70%, according to our repeated preliminary study) was also included as a control group.

As shown in Figure 5b, none of the animals bearing established c1300 tumors without any treatment or with a clinically available dose of radiation (4 Gy × 3 days) survived over 120 days after tumor inoculation. Only one of four animals treated with rSeV/dF-mIFNβ-DCs

showed tumor-free survival over 120 days, but this mouse accepted not only third-party MuSS but also c1300 at the second challenge. Three of the four tumor-bearing mice that were treated with overdose irradiation survived over 120 days in tumor-free condition, but no mouse could reject both MuSS and c1300, indicating that

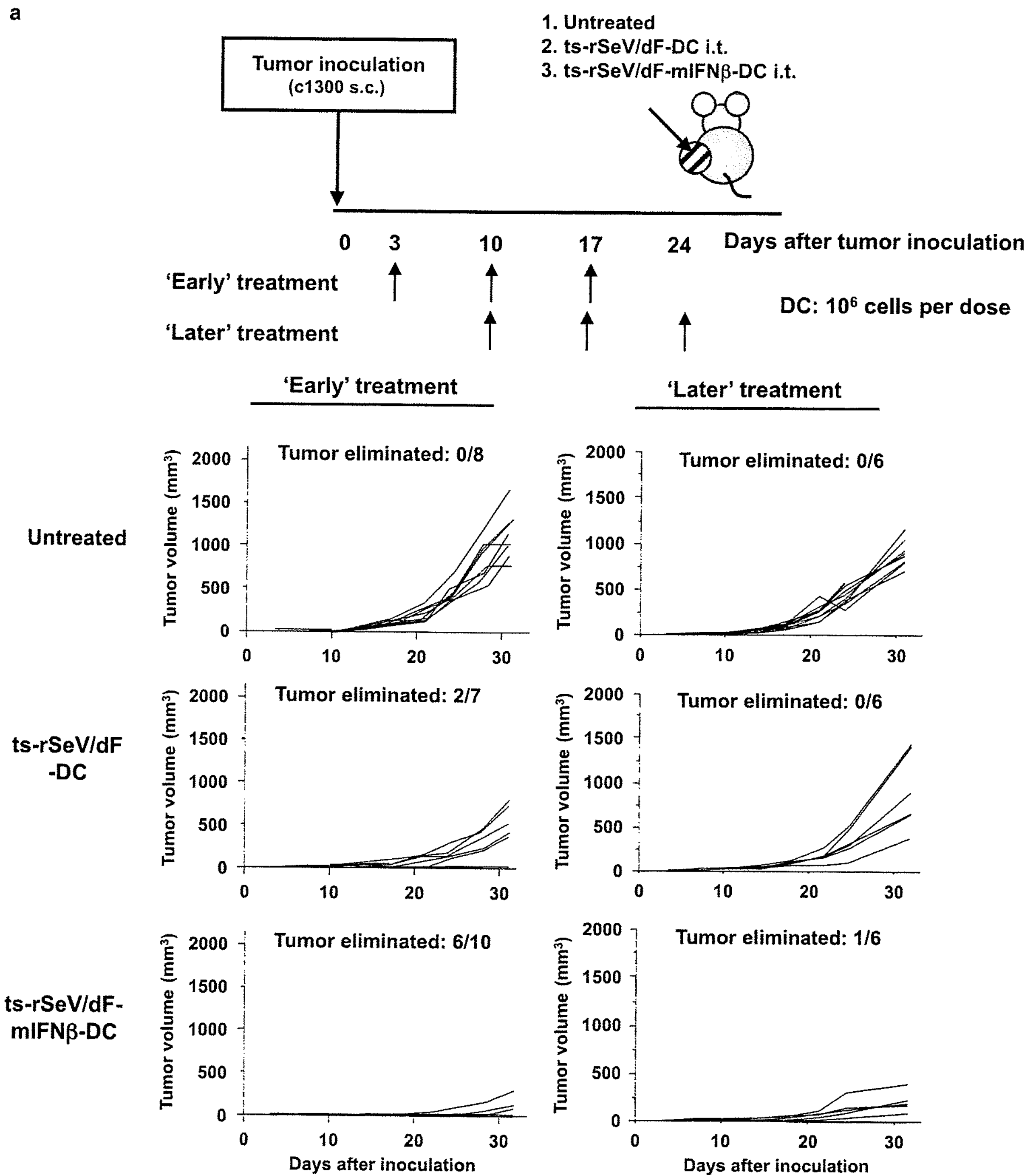


Figure 3 Enhanced antitumor activity against established c1300 neuroblastoma *in vivo* by ts-rSeV/dF-DCs expressing mIFN-β. Three days ('early' treatment regimen) or 10 days ('later' treatment regimen) after intradermal inoculation of c1300 neuroblastomas, 10^6 cells of ts-rSeV/dF-DCs with or without exogenous mIFN-β expression were injected weekly through the i.t. route. Thereafter, the tumor volume was measured. Note that all tumors treated in the 'later' regimen were over 7 mm in diameter at day 10. (**a**) Time courses of tumor volume in individual animals by 'early' (left panels) and 'later' (right panels) treatment regimens. The c1300 tumors were still resistant to the weekly treatment with ts-rSeV/dF-DCs in both regimens (middle two panels), and apparent efficacies with regard to volume reduction and tumor elimination were found in the use of ts-rSeV/dF-DC-associated exogenous mIFN-β. (**b**) Panels showing c1300 tumor volume on day 31 in the animals demonstrated in (**a**). Note that animals showing a complete elimination of tumors were excluded from these analyses. Treatment with ts-rSeV/dF-DCs significantly inhibited tumor growth, and the expression of exogenous mIFN-β strongly reduced the tumor size in both regimens. $*P<0.001$. DC, dendritic cell; mIFN-β, murine interferon-β; rSeV, recombinant Sendai virus.

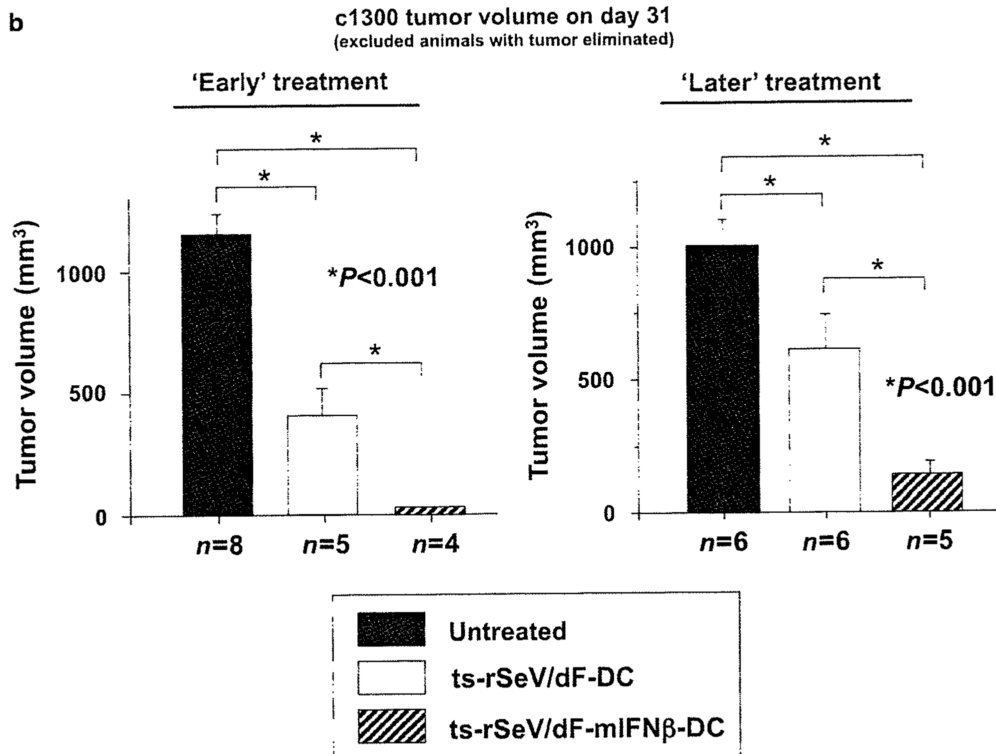


Figure 3 Continued.

tumor elimination by γ-irradiation itself does not significantly contribute to the establishment of protective immunity. In contrast, combination therapy of radiation and rSeV/dF-mIFNβ-DCs also resulted in tumor elimination and tumor-free survival over 120 days in three of the four animals, and all the four mice demonstrated complete and tumor-specific rejection to c1300 tumor inoculation, but none of them showed such a rejection of third-party, MuSS cells.

These results indicate that the synergism of combining radiation and rSeV/dF-mIFNβ-DCs contributes greatly not only to tumor reduction but also to the establishment of long-lasting and protective antitumor immunity.

Discussion

Since the early reports describing the efficacy of DC-based immunotherapy on subjects with malignancies,[27,28] this therapeutic mode has been evaluated all over the world. Recurrent neuroblastoma, which is highly resistant to currently available surgery, chemotherapy and radiotherapy, has also been a target of DC-based immunotherapy; but recent clinical studies have failed to show significant improvements in outcome.[5,6] To overcome the current limitation of this mode, we recently developed a new concept, 'immunostimulatory virotherapy', using rSeVs, which are recombinant virus-based immune boosters for DCs.[14,15] DCs activated by an rSeV have shown apparently superior antitumor effects on several tumor types compared with those seen by DCs treated with conventional stimuli, including lipopolysaccharide; they have also been shown not to lose their phago/pinocytotic activity,[15] and therefore an i.t. injection of rSeV-DCs without exposure to tumor antigen *ex vivo* evoked tumor-specific antitumor immunity.[14,15] On the basis of these findings, the present experimental study was performed to examine the potential of DC-based immunotherapy boosted by a newly developed rSeV/dF, the less cytotoxic, clinically available vector ts-rSeV/dF,[18] to treat less-immunogenic murine c1300 neuroblastoma.

The key observations obtained in this study were as follows: (1) c1300 tumors were highly resistant to ts-rSeV/dF-DC therapy by the 'early' treatment regimen that was sufficiently effective against highly malignant B16F10 melanoma, indicating that c1300 should be less immunogenic than such melanoma; (2) the use of ts-rSeV/dF-mIFNβ as the activating modality for DCs and expressing murine IFN-β dramatically attenuated the antitumor effect on c1300 tumors through the 'early' treatment regimen, similar to a finding of our earlier study;[14] (3) when established c1300 tumors were treated through the 'later' treatment regimen, however, the antitumor effect of ts-rSeV/dF-mIFNβ-DCs was not sufficient; (4) radiation pretreatment at a clinically reasonable dose (4 Gy × 3 days) demonstrated a dramatically improved antitumor effect, resulting in a high percentage of elimination of established tumors; and (5) the elimination of established c1300 tumors by radiation followed by ts-rSeV/dF-mIFNβ-DCs contributed to the development of long-lasting tumor-specific immunity, whereas the antitumor effect through overdose irradiation did not. These results indicate the potential utility of the combination of radiotherapy and ts-rSeV/dF-mIFNβ-DC immunogene therapy in the clinical setting.

It has been suggested that radiotherapy for malignancies might stimulate antitumor immunity as a systemic bystander effect called the 'abscopal effect'. However, the molecular and cellular mechanisms underlying this effect are largely unknown. In addition, the concept of combining radiation therapy with immunotherapy is not new, and some publications suggest the beneficial effect of irradiation on antitumor immunity. Radiation induces cell death through apoptosis and necrosis. In turn, necrotic and apoptotic cells could induce DC-mediated antitumor immunity.[29–31] These types of cell death are also shown not only to induce the release of inflammatory cytokines,[32] but also to stimulate tumor vasculature to upregulate the expression of adhesion molecules, and to facilitate the trafficking of immune cells to cancer foci; thus, these types of cell

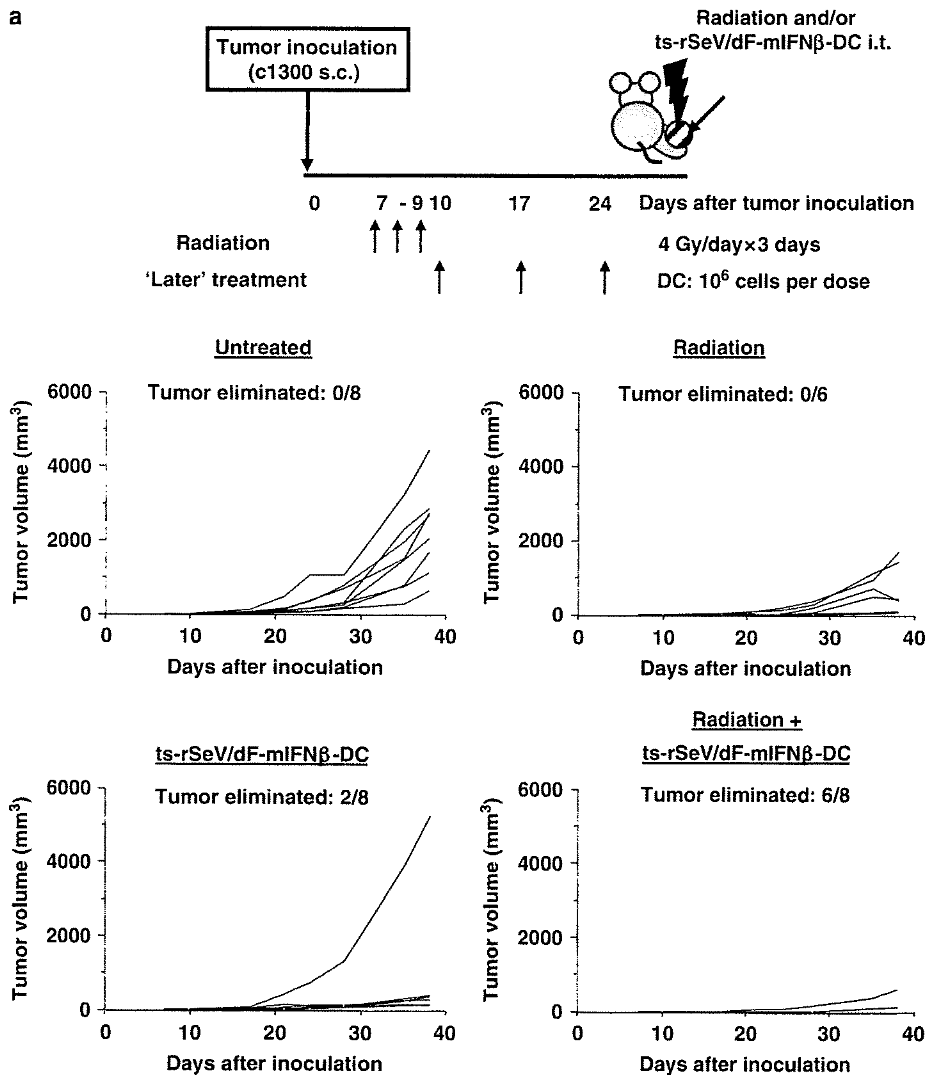


Figure 4 Pretreatment of clinically available dose of irradiation dramatically enhanced antitumor effect of ts-rSeV/dF-mIFNβ-DCs seen in the 'later' treatment regimen. Seven days after s.c. tumor inoculation into the left thigh, the tumors were irradiated three times daily at 4 Gy day^{-1}. At day 10 ('later' treatment regimen), 10^6 cells of ts-rSeV/dF-DCs with exogenous mIFN-β expression were injected weekly through the i.t. route. Thereafter, the tumor volume was measured. Note that all tumors treated in the 'later' regimen were over 7 mm in diameter at day 10. (**a**) Time courses of tumor volume in individual animals. No tumor disappeared in the group receiving radiation only, but tumor growth was suppressed (right upper panel) compared with those without any treatment (left upper). In contrast, the ts-rSeV/dF-mIFNβ-DC group showed a strong suppression of tumor growth (representative findings seen in Figure 3), and this effect was dramatically enhanced by the combination therapy, which resulted in the elimination of a high percentage of tumors (6/8 = 75%). (**b**) Direct comparison of tumor size. Panels showing c1300 tumor volume on day 38 in the animals demonstrated in (**a**). Note that animals showing a complete elimination of tumors were excluded in these analyses. Treatment with either irradiation or ts-rSeV/dF-mIFNβ-DCs significantly inhibited tumor growth, and synergism was found by the combination therapy. $*P<0.001$ and $^{\#}P<0.05$. (**c**) Long-term survival of animals demonstrated in (**a**). Two animals in the rSeV/dF-mIFNβ-DC group that showed the complete disappearance of tumors survived over 200 days (2/8 = 25%), whereas no significant prolongation of survival was seen in the group treated with irradiation alone. The combination therapy dramatically and significantly improved the survival of animals; six of eight animals (75%) survived over 200 days without any recurrence. The data were analyzed by the Kaplan–Meier method, and statistical relevance was determined using the log-rank test. $*P<0.001$. DC, dendritic cell; i.t., intratumoral; mIFN-β, murine interferon-β; rSeV, recombinant Sendai virus; s.c., subcutaneous injection. (See online version for color figure.)

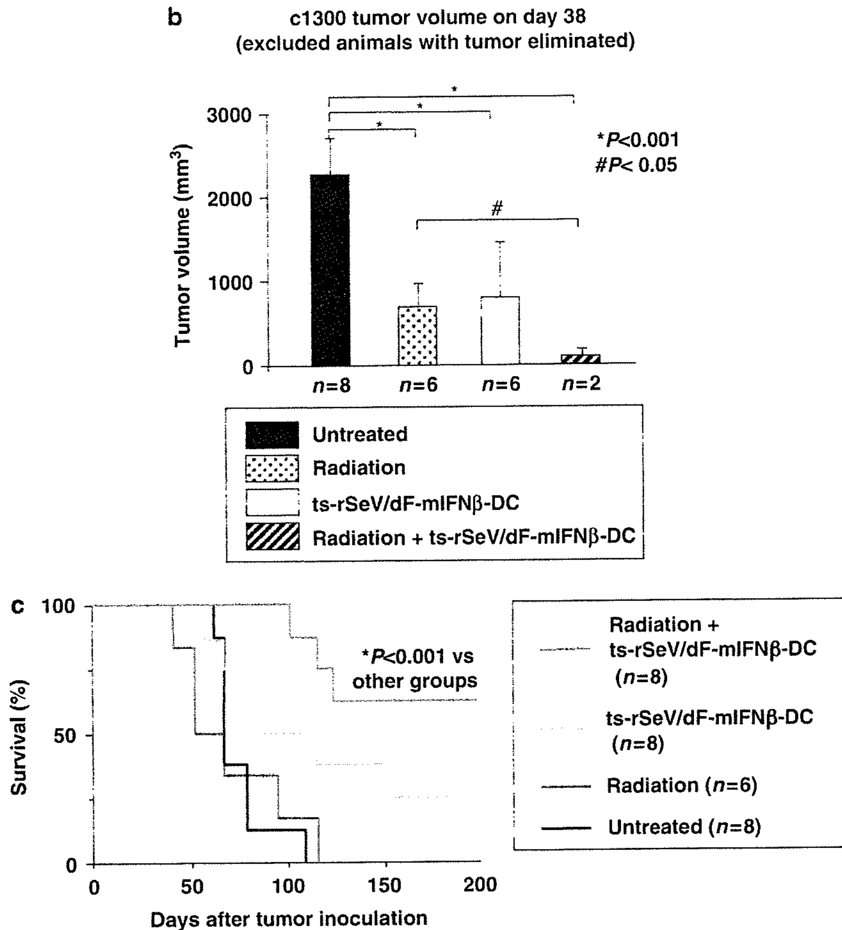


Figure 4 Continued.

death may together be responsible for the abscopal effect after radiation therapy.

It is of interest, however, that abscopal regression of distant tumors has been inferred in the use of certain tumors in experimental studies, but has rarely been seen in clinical settings.[33] In fact, we here demonstrated that tumor elimination through overdose irradiation did not protect against the second challenge of c1300 inoculation; rather, the addition of DC immunotherapy was required to establish a protective immunity. These findings could be supported by an important report describing that the induction of the T-lymphocyte-mediated abscopal antitumor response was tumor type specific.[25] Therefore, we concluded that DC-based immunotherapy would be required to induce protective immunity when tumor cells were destroyed by irradiation.

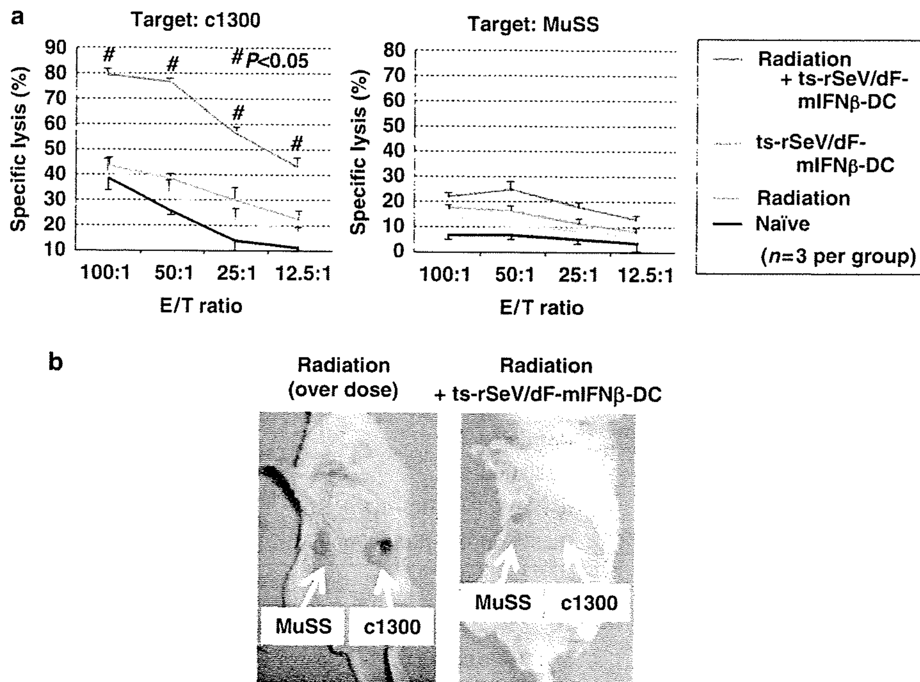
Related to this point, we have to discuss why c1300 tumor elimination through overdose irradiation (34 Gy × 3) did not induce protective immunity against the second challenge. In this case, a sustained dermal inflammation and burns were found (data not shown), possibly implying that the sustained dermal inflammation due to overdose irradiation might disturb the establishment of antitumor immunity. Therefore, clinically appropriate doses of irradiation, probably associated with tumor cell disruption proper for antigen uptake, processing and presentation by antigen-presenting cells, should be examined to obtain optimized antitumor immune responses following additional DC immunotherapy.

In summary, we here demonstrated that the i.t. injection of rSeV/DCs expressing mIFN-β to established c1300 tumors pretreated with a clinically reasonable dose of irradiation efficiently led tumors to a complete elimination *in vivo*. In addition, this regimen simultaneously induced a long-lasting protective immunity. Therefore, this results strongly suggest that the regimen warrants further investigation in research as well as in clinical trials.

Materials and methods

Mice and tumor cell lines

Female 6- to 8-week-old A/J mice (H-2[a]) were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and kept under specific pathogen-free and humane conditions. Murine neuroblastoma c1300 and MuSS murine malignant fibrous histiocytoma (under kind permission by Dr Itaru Watanabe, Department of Surgery, Tasuda Hospital)[26] were obtained from the RIKEN BioResearch Center (Tsukuba, Ibaraki, Japan). B16F10 murine melanoma, SK-N-SH human neuroblastoma without *MYCN* amplification and IMR32 human neuroblastoma



Summary of second challenge

	Total examined	Second challenge (c1300 and MuSS)			
		Alive (tumor free)	Used for second challenge	Rejection of c1300	Rejection of MuSS
Untreated	4	0	-	-	-
Radiation (4 Gy×3)	3	0	-	-	-
Radiation (over dose: 34 Gy×3)	4	3	3	0	0
ts-rSeV/dF-mIFNβ-DC	4	1	1	0	0
Radiation (4 Gy×3) + ts-rSeV/dF- mIFNβ- DC	4	3	3	3	0

Figure 5 Combination therapy of irradiation and rSeV/dF-mIFNβ-DC induces long-lasting and tumor-specific protective immunity. (**a**) Assessment of CTL activity for c1300. Induction of tumor-specific CTLs after i.t. administration of rSeV/dF-mIFNβ-DC, which was repeated twice according to the late treatment regimen. Control included tumor-bearing mice without any treatment and MuSS was also used as a target of a third party. Seven days after the last treatment, splenocytes were isolated and restimulated *in vitro* for 5 days with mitomycin C-treated MH134 cells, and cytolytic activity against ^{51}Cr-labeled targets was measured. Each group contains $n=3$. (**b**) Seven days after s.c. c1300 tumor inoculation into the left thigh, the tumors were irradiated three times daily at 4 Gy day^{-1}. At day 10 ('later' treatment regimen), 10^6 cells of ts-rSeV/dF-DCs with exogenous mIFN-β expression were injected weekly through the i.t. route. Four animals with completely eliminated tumors through overdose irradiation (34 Gy × 3 for 3 days) were also included. On day 184, live animals without primary tumor formation were subjected to a second challenge, simultaneous tumor inoculation with c1300 and MuSS (third party) on the abdominal wall. Fifteen days later, tumor formation was determined. DC, dendritic cell, i.t., intratumoral; mIFN-β, murine interferon-β; rSeV, recombinant Sendai virus; s.c., subcutaneous injection. (See online version for color figure.)

associated with *MYCN* amplification were purchased from ATCC (Manassas, VA, USA). These cell lines were maintained in complete medium (RPMI-1640 medium; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (BioWest, Nuaille, France), penicillin and streptomycin under a humidified atmosphere containing 5% CO_2 at 37 °C.

Temperature-sensitive mutant non-transmissible rSeVs (ts-rSeV/dF)

Temperature-sensitive mutant F-defective non-transmissible recombinant rSeVs (ts-rSeV/dF-null and ts-rSeV/dF-mIFNβ) were prepared and recovered as described earlier.[21,22] Briefly, vectors were prepared by using recombinant LLC-MK_2 cells carrying the F gene (LLC-MK_2/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used for the induction of F protein in LLC-MK_2/F7 cells (referred to as LLC-MK_2/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave ultraviolet irradiation and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units.[9] Murine IFN-β cDNA, which was

subcloned to the vector template ts-prSeV18+b(+)/dF, was cloned by reverse transcriptase PCR as described earlier.[14]

Generation of DCs and transfection with rSeVs

Murine bone-marrow-derived DCs were generated as described earlier,[14,15] and an endotoxin-free condition was maintained throughout the study by using endotoxin-free reagents. Briefly, bone marrow cells from A/J mice were collected and passed through a nylon mesh, and red blood cells and lineage-positive (B220, CD5, CD11b, Gr-1, TER119, 7/4) cells were depleted by using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Vancouver, British Columbia, Canada). These lineage-negative cells ($5–10 \times 10^4$ per 5 ml per well) were cultured in 50 ng ml^{-1} granulocyte-macrophage colony-stimulating factor (PeproTech, London, UK) and 25 ng ml^{-1} IL-4 (PeproTech) in endotoxin-free complete medium in six-well plates. On day 4, half of the culture medium was replaced by fresh medium supplemented with granulocyte-macrophage colony-stimulating factor and IL-4 at the same concentration. On day 7, DCs were collected and used for subsequent experiments. For ts-rSeV-mediated transduction, DCs (1×10^6 cells per ml) were simply incubated with rSeVs at a dose optimized earlier[14,15] and a multiplicity of infection of 100 (MOI = 100) without any supplementation. At this condition, gene transduction efficiency constantly showed over 95%. Note that the DCs used in this study were not loaded tumor antigens, as was also the case earlier.[15]

Major histocompatibility complex class I expression on tumor cells

c1300 (mouse), SK-N-SH (human) or IMR32 (human) neuroblastoma cells (1×10^5 per ml) were incubated in the presence or absence of mouse or human IFN-β (1000 U ml^{-1}) at 37 °C for 48 h. These cells were then stained with the corresponding fluorescein isothiocyanate-conjugated anti-major histocompatibility complex class I antibodies (mouse or human; BD Pharmingen, San Diego, CA, USA) and were analyzed using FACS Calibur (Becton Dickinson, San Jose, CA, USA) with CellQuest software (BD Biosciences Japan, Tokyo, Japan). Data analysis was performed using FlowJo 4.5 software (Tree Star, San Carlos, CA, USA). Dead cells were excluded by staining with propidium iodide.

DC-based immunotherapy to c1300 tumor

The DCs used in this study were not pulsed with any tumor antigen throughout the experiments.

'Early' treatment regimen. After the DCs were prepared, an immature DC phenotype appeared constantly. These immature DCs were incubated with ts-rSeV/dF-null or ts-rSeV/dF-mIFNβ for 8 h, as described earlier.[14,15] All of the DCs were added to 50 mg ml^{-1} of polymyxin B (Sigma-Aldrich) and were carefully washed twice before injection. Intradermal implantation (A/J for 1×10^6 of c1300 cells showing log-phase proliferation *in vitro*) was performed into the abdomen on day 0, and 1×10^6 DCs were injected i.t. on days 3, 10 and 17. For all injections, materials were suspended in a 100-μl volume of phosphate-buffered saline. Tumor size was assessed using microcalipers three times a week, and the volume was calculated by the following formula: tumor volume (mm^3) = 0.5236 × (long axis) × (short axis) × (height) (Figures 1 and 3).[14,15]

'Later' treatment regimen and radiation pretreatment. We further assessed the 'later treatment regimen' for tumors that were well established, measured 7–9 mm in diameter[14,15] and constantly showed a significant vascularization histologically (data not shown). γ-Irradiation pretreatment (^{60}Co source, 2 Gy day^{-1} for 3 days, daily) was performed if necessary.

Dendritic cells were collected as described above, and intradermal implantation (A/J for 5×10^5 c1300 cells) was carried out into the abdomen (Figure 1) or right thigh (Figures 3–5: to avoid irradiation-induced enterocolitis and so on) on day 0, and 1×10^6 DCs were injected i.t. on days 10, 17 and 24. Tumor size was assessed as described above.

^{51}Cr release assay for cytolytic activity of CTLs

Prepared DCs were i.t. administered twice into tumor-bearing mice (MH134) at 10^6 cells per 100 μl on days 10 and 17. One week after the last immunization, splenocytes were obtained and contaminated erythrocytes were depleted. For CTL assay, 4×10^6 splenocytes were cultured with 3×10^5 inactivated c1300 cells treated with 100 μg ml^{-1} mitomycin in a 24-well culture plate. Two days later, 30 IU ml^{-1} human rIL-2 was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells. Target cells (c1300 cells or MuSS for third party) were labeled with 100 μCi $Na_2^{51}CrO_4$ for 1.5 h, and Cr release assay was performed as described earlier.[14,15] The percentage of specific ^{51}Cr release of triplicates was calculated as follows: ((experimental c.p.m. × spontaneous c.p.m.)/(maximum c.p.m. × spontaneous c.p.m.)) × 100. Spontaneous release was always <10% of maximal Cr release (target cells in 1% Triton X-100).

Rechallenge of tumor cells

Nineteen animals bearing c1300 tumors were divided into five groups (Figure 6) (untreated: $n = 4$; 4 Gy × 3 days radiation: $n = 3$; 34 Gy × 3 days overdose radiation: $n = 4$; ts-rSeV/dF-mIFNβ-DC: $n = 4$; and 4 Gy × 3 days radiation+ts-rSeV/dF-mIFNβ-DC: $n = 4$) and treated as described in the schematic regimen in Figure 4. Seven animals had survived tumor-free on day 189 (34 Gy × 3 days overdose radiation: $n = 3$; ts-rSeV/dF-mIFNβ-DC: $n = 1$ and 4 Gy × 3 days radiation+ts-rSeV/dF-mIFNβ-DC: $n = 3$), and these were used for the second challenge. On day 189, 5×10^5 cells of c1300 (left) and MuSS (right) were inoculated into the bilateral dermis of the abdominal wall. Fifteen days later, tumor formation was assessed.

Statistical analysis

All data were expressed as means ± s.e.m. and were analyzed by one-way analysis of variance with Fisher's adjustment, except for animal survival. Survival was plotted using Kaplan–Meier curves, and statistical relevance was determined using log-rank

comparison. A probability value of $P<0.05$ was considered significant.

Abbreviations

ts-rSeV, temperature-sensitive mutant recombinant Sendai virus; ts-rSeV-DC, recombinant Sendai virus-modified DC; IFN-β, interferon-β; i.t., intratumoral injection; s.c., subcutaneous injection'

Acknowledgements

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Competing interest

Dr Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

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総　説

第24回日本小児がん学会教育セッション
神経芽腫に対する集学的治療法：化学療法を中心に

七野　浩之，陳　　基明，麦島　秀雄

I　概　　念[1)]

神経芽腫(neuroblastoma)の概念は，Pepper, Hutchinson, Wrightの3人の先駆者により確立した[2)]．1901年にPepperらは肝臓と副腎の先天性肉腫6例の報告を行った．続いて1907年にHutchinsonらが副腎腫瘍の眼窩転移例7例を報告した．そして1910年にWrightらが初めてneuroblastomaの用語を使用し神経芽腫の概念が確立している．

「神経芽腫」とは通常広義に用いられ，それは組織学的な分類として，狭義の神経芽腫(neuroblastoma)と神経節芽腫(ganglioneuroblastoma)及び神経節腫(ganglioneuroma)の3種類を総称するものである．最近では広義の神経芽腫を，脳腫瘍である中枢性神経芽腫と区別するために，末梢性神経芽腫群腫瘍(pNTs; peripheral Neuroblastic Tumors)と呼称するようになった[1,3)]．

神経芽腫は，胎生期の神経堤（あるいは神経冠）(neural crest) を起源とする神経芽細胞が成熟分化せずに腫瘍化したものと考えられ，副腎髄質および交感神経系組織に発生する胎児性腫瘍である[1,3)]．

発生頻度は小児悪性固形腫瘍の中で脳腫瘍についで多く，日本の小児がんの中の10～20%を占める．アメリカでは7,000人出生に対し1人の割合で発生し，年間約600～650人の発生がある[1)]．日本では出生数あたりの発生頻度は把握されていないが，日本神経芽腫研究グループ（Japan Neuroblastoma Study Group/JNBSG）の種々のアンケート調査などからの推計では年間に90～130例，そのうち高リスク神経芽腫は年間50～70例程度と考えられる[4)]．アメリカChildren's Oncology Group (COG)の統計では年齢のピークは出生時であり，1歳未満は36%，5歳未満で89%，10歳未満で98%を占める[1)]．

II　病態生理

神経芽腫の好発部位は副腎で65%を占め，その他は頚部，後縦隔，後腹膜，骨盤腔などの交感神経節である．左右差は3：2の割合で左に多い．転移は骨，骨髄，肝，リンパ節，皮膚，眼窩等に認められる．硬膜，脳実質あるいは肺転移の報告もある[1)]．

ほとんどの神経芽腫は血清カテコラミン（ドパミン，アドレナリン，ノルアドレナリン）を産生し，その尿中代謝産物であるバニルマンデル酸(VMA) とホモバニリン酸 (HVA) などが上昇するため，これを腫瘍マーカーとして利用できる．また血中の神経特異エノラーゼ（neuron-specific enolase：NSE）が高値となることも多い[1)]．

組織学的に狭義の神経芽腫は，クロマチンに富む核と乏しい細胞質からなる小円形の神経芽細胞とわずかな神経線維からなり，その間に間質細胞が存在する．神経節芽腫は，未分化な神経芽細胞と分化した神経節細胞が混在するものである．神経節腫は，主として神経節細胞からなるものである．日本病理学会小児腫瘍組織分類委員会分類では，神経芽腫（花冠－細線維型・円形細胞型），

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神経節芽腫（分化型・混成型・低分化型），神経節腫に分類している[3]．

Beckwith らの研究から胎生期には多数の *in situ* neuroblastoma が存在し，その大多数は自然に成熟または消退し，残りのごく一部が神経芽腫になるものと推定されている．日本で行われたマススクリーニング症例の検討からも，マススクリーニングで発見された神経芽腫のうちあるものは自然退縮や分化成熟することが判明した[3,5]．しかし1歳半（あるいは1歳）以上で診断される症例はそのほとんどの病期は進行しており，また生物学的予後因子も不良なことが多く，治療抵抗性であることが多い．

1970年代に開始され連綿と継続されている研究から，現在では，予後と密接に関連している因子には，年齢，stage，病理組織学的分類，*MYCN*，DNA ploidy，染色体異常（1p－，11q－，17q＋）等が考えられている[1,3,6]．

Brodeur らの検討では，*MYCN* が増幅している頻度と3年生存率は，良性の神経節腫ではそれぞれ 0/64・100%，低リスク群病期1と2では 31/772・90%，4S では 15/190・80%，進行例では 612/1974・40% である．Shimada らの検討では Favorable Histology (FH) 329 例では *MYCN* 非増幅 325 例・増幅 4 例であるが，Unfavorable Histology (UH) では 243 例中 *MYCN* 非増幅 164 例・増幅 79 例であった．*MYCN* 増幅はすなわち予後不良であるが，非増幅は必ずしも予後良好ではない．UH の中では *MYCN* 増幅腫瘍は非増幅腫瘍より早期に発症する傾向にあり，分化傾向が見られず，high MKI を呈する特徴がある[1]．

DNA ploidy が3倍体の腫瘍は2倍体・4倍体に比し DNA が不安定であるため，細胞の生存及び分裂増殖に不利であり，治療に対する反応性が良いと言われている．Flow cytometry により DNA 量を検討する．DNA index (DI)＝1 は diploid を反映し予後不良であるが，FH では schwann 細胞が2倍体のため DI＝1 になる[1,3,6]．

Ⅲ　臨床症状

臨床症状には原発部位の腫瘤による症状と転移による症状があり，年齢と原発部位・病期により様々である．

乳児期早期の症例は多くが限局例であり症状を認めない．乳児期のマススクリーニング発見例は一般的に無症状であった．4S 期では，瀰漫性肝転移による腹部膨満症状とそれによる胸部圧迫のための呼吸器症状を認めることがある．進行例には，腹部膨満，食欲不振，発熱などの他に，遠隔転移の症状としての顔面蒼白，貧血，眼球突出，眼瞼出血，骨痛，関節痛，跛行などが認められるが，発熱のみの場合や偶然の腹部腫瘤触知による発見まで無症状のこともある．特殊な症状として，Horner 症候群や opsomyoclonus，小脳性運動失調あるいは脊椎管内への腫瘍進展による神経麻痺，腫瘍から産生されるカテコラミンによる異常な発汗や高血圧，血管作動性腸ペプチド産生による水様性下痢などが見られることがある[1]．

Ⅳ　診　　断

診断は原発腫瘍または転移巣の開創生検を行い光学顕微鏡検査により病理組織学的に確定診断する．あるいは，骨髄検査で腫瘍細胞の転移が確認され，かつ尿中 VMA や HVA が明らかに高値である場合は，原発腫瘍の組織学的検討を行わずに神経芽腫と診断してよい．しかしながら原則としては，診断を確定し，治療方針を決定するために必要な腫瘍の生物学的予後因子を検討するために，原発腫瘍（あるいは転移巣）の開創生検を行うべきである．針生検による病理診断は正確な診断に至ることが困難な場合があり，また生物学的予後因子の検索ができないことが多く，神経芽腫以外の固形腫瘍の場合も含め薦められない[1,3]．

MYCN コピー数や DNA ploidy などの分子生物学的予後因子の検索を行うことが，リスク分類による治療方針の決定には必要である[1,3]．

病理組織学的分類は International Neuroblastoma Pathology Classification (INPC) が国際的に標準的である．これは，神経芽腫細胞の形態に，診断時年齢，Schwann 細胞の発達 (stroma)，神経細胞の分化程度（differentiation），核崩壊程度（Mitosis-karyorrhexis index）（MKI）を加味し，

組織型としては Neuroblastoma/ Ganglioneuroblastoma, intermixed/ Ganglioneuroma/ Ganglioneuroblastoma, nodular の4型に分類し，さらに予後のグループとして Favorable Histology Group (FH) と Unfavorable Histology Group (UH) に分類するものである[1,3]．

V 病期分類

これまで病期分類は日本小児外科学会悪性腫瘍分類，Evans system, St. Jude Children's Research Hospital and POG classification などが使用されてきたが，現在は神経芽細胞腫国際病期分類 (International Neuroblastoma Staging System/INSS) (表1) が使用される[7]．病期分類には初診時での原発腫瘍の広がり，リンパ節転移，肝転移，あるいは神経芽腫の好発部位である交感神経の経路に沿った部位への転移の把握が必要であり，これには全身のX線CTやMRIが必要である．さらに骨及び骨髄転移の検索が必須で，I-123 metaiodo-benzylguanidine (MIBG) シンチグラフィー及び Tc-99 m 骨シンチグラフィーが必要である．骨髄転移の検索には，治療効果の判定として International Neuroblastoma Response Criteria (INRC) (表2) を用いる場合には左右2箇所の腸骨での骨髄穿刺吸引検査と左右2箇所の骨髄生検が必要とされている[7]．

VI リスク分類

神経芽腫は，年齢，病期，病理学的特徴，分子生物学的特徴などにより著しく予後が異なる．このため治療選択の基準として，病期分類にさらにいくつかの予後因子を組み合わせたリスク分類の必要性が提唱されている．リスクは予後との関連により低リスク群，中間リスク群，高リスク群に分類することが一般的である．これまでは日米欧で独自のリスク分類が提案されてきたが，現在は国際的な統一分類の開発 (The International Neuroblastoma Risk Classification (INRG)) が企画され討議されている[8,9]．さらに今後は分子生物学的予後因子を組み込んだリスク分類が創案されることと考えられる．代表的なリスク分類であるCOGのリスク分類では，年齢とINSS病期分類，INPC組織分類，*MYCN* 増幅の有無及び DNA index により表3のように分類している[1,10]．

VII 鑑別診断

HE染色による形態判断では鑑別が困難な腫瘍群を小円形細胞腫瘍と呼び，悪性リンパ腫，ユーイング肉腫ファミリー腫瘍，横紋筋肉腫と神経芽腫が含まれる．これらの鑑別には生検あるいは摘出組織を利用して免疫染色や電子顕微鏡検査ある

表1 神経芽細胞腫国際病期分類 (International Neuroblastoma Staging System/INSS)

病期	定義
1	限局性腫瘍で，肉眼的に完全切除．組織学的な腫瘍残存は不問．同側のリンパ節に組織学的な転移を認めない．(原発腫瘍に接し，一緒に切除されたリンパ節転移はあってもよい)
2A	限局性腫瘍で，肉眼的に不完全切除．原発腫瘍に接しない同側リンパ節に組織学的に転移を認めない．
2B	限局性腫瘍で，肉眼的に完全または不完全切除．原発腫瘍に接しない同側リンパ節に組織学的に転移を認める．対側のリンパ節に転移を認めない．
3	切除不能の片側性腫瘍で，正中線（対側椎体縁）を越えて浸潤．同側の局所リンパ節の転移は不問．または，片側発生の限局性腫瘍で対側リンパ節転移を認める．または，正中発生の腫瘍で椎体縁を越えた両側浸潤（切除不能）か，両側リンパ節転移を認める．
4	いかなる原発腫瘍であるかに関わらず，遠隔リンパ節，及び／または，骨，骨髄，肝，皮膚，他の臓器に播種している．(4Sは除く)
4S	限局性腫瘍（病期1，2A，2B）で，播種は皮膚，及び／または，肝，骨髄に限られる（1歳未満の患者のみ）．骨髄中の腫瘍細胞は有核細胞の10%未満で，それ以上は病期4である．MIBGシンチが行われるならば骨髄への集積は陰性．

Brodeur GM, et al: J Clin Oncol 11(8) : 1466-77, 1993. より引用

表2　効果判定規準（International Neuroblastoma Response Criteria/INRC）

評価	原発巣	転移巣
CR (complete response)	腫瘍無し	腫瘍無し カテコールアミン代謝産物正常化
VGPR (very good partial response)	90%—99%縮小	腫瘍無し カテコールアミン代謝産物正常化，骨シンチでの集積は残存していてもよい（MIBG シンチは陰性化していなければならない）
PR (partial response)	50%以上縮小	測定可能病変が50%以上縮小 骨転移の病変数が50%以上減少 骨髄転移の病変数は0—1か所 （MIBG シンチでの集積は残存していてもよい）
MR (mixed response)	新病変の出現なし 原発巣および転移巣の測定可能病変において50%以上縮小する病変を認める 同時に他の病変は50%未満の縮小や25%未満の増大を示す	
NR (no response)	新病変の出現なし 原発巣および転移巣の測定可能病変は，50%未満の縮小や25%未満の増大を示す	
PD (progressive disease)	新病変の出現 あるいは原発巣および転移巣の測定可能病変において25%以上の増大を示す病変を認める もしくは骨髄の転移病変の新たな出現	

CR, VGPR, PR, MR, NR につては定義に述べられた全ての用件を満たしていることが必要である．PD に関しては定義に述べられたいずれかの要件を満たした状態である．

Brodeur GM, et al.: J Clin Oncol 11(8)：1466-7, 1993 より引用

表3　COG リスク分類

低リスク 1．患者の年齢を問わず INSS 1 期 2．1歳未満の INSS 2A 期及び 2B 期 3．1歳以上で，FHG の INSS 2A 期及び 2B 期 4．1歳以上で，*N-MYC* 増幅なしの INSS 2A 期及び 2B 期 5．1歳未満で，*N-MYC* 増幅なし，かつ FHG，かつ高二倍体 DNA である INSS4S 期
中間リスク 1．1歳未満で，*N-MYC* 増幅なしの INSS 3 期 2．1歳以上で，*N-MYC* 増幅なし，かつ FHG の INSS 3 期 3．1歳未満で，*N-MYC* 増幅なしの INSS 4 期 4．1歳未満で，*N-MYC* 増幅なし，かつ二倍体に近い DNA の INSS 4S 期 5．1歳未満で，*N-MYC* 増幅なし，かつ UFHG の INSS 4S 期
高リスク 1．1歳以上で，*N-MYC* 増幅あり，かつ UFHG の INSS 2A 期及び 2B 期 2．患者の年齢を問わず，*N-MYC* 増幅ありの INSS 3 期 3．1歳以上で，UFHG の INSS 3 期 4．1歳未満で，*N-MYC* 増幅ありの INSS 4 期 5．1歳以上の INSS 4 期 6．1歳未満で，*N-MYC* 増幅ありの INSS 4S 期

FHG;INPC で favorable histology group，UFHG;INPC で unfavorable histology group

記載のない項目は不問である

Castleberry RP. Eur J Cancer. 33：1430-1437, 1997 より引用

いは分子生物学的検査を施行する必要がある．また腫瘍マーカーとしての血清カテコールアミンやVMA，HVAなどを確認することが有効である．NSEは小円形細胞腫瘍ではいずれの腫瘍でも上昇することがある．また原発部位が腎近傍の場合には腎芽腫などの腎原発腫瘍との鑑別も必要である[1),3)]．

Ⅷ 予　後

予後を規定する最も重要な因子は適切な治療法である．このためリスク分類を設定し，リスクごとに治療強度を規定し，より適切な治療法の開発が検討されている．日本ではリスク分類は未だ議論の途上ではあるが，今後治療成績の集積により改善していくものと考えられる．今のところ1歳半以上あるいはstage 4あるいは*MYCN*が増幅している神経芽腫の予後は，骨髄破壊的大量化学療法を行っても3年無増悪生存割合として20～40%台に過ぎない．大量化学療法後の主な再発形式は骨あるいは骨髄再発である．現在アメリカではCOGのリスク分類に基づき3年全生存割合を低リスク群で90%超，中間リスク群で70～90%，高リスク群で30%超と推測している[1)]．日本全体としての治療成績は明らかではない．

Ⅸ 治療方針

神経芽腫の治療は以前よりリスクに基づいた治療戦略が行われてきた．現在のCOGの治療方針は低リスク群では外科切除後経過観察，中間リスク群では外科切除と通常の化学療法，高リスク群では集学的治療が必要となり，外科切除に加え積極的な化学療法と大量化学療法 ＋ 自家造血細胞移植及び放射線療法である．日本においても以前よりほぼ同様の治療方針が採られている．

低リスクあるいは中間リスク例の治療は第1選択としては外科切除を行う．外科療法単独で可能な場合や，あるいは6～12～24週間の化学療法を併用する場合がある．化学療法による障害を抑えるために薬剤使用量を少なくする努力が行われている．

ダンベル症候群をきたし脊髄圧迫症状を起こしている例では，神経学的な症状を一刻も早く改善し神経症状が永続するのを回避するために緊急化学療法を行うべきである．神経圧迫症状の期間が短いほど神経学的回復が期待される．椎弓切除術あるいは放射線療法も同等の治療効果が期待できるが，いずれもその後の化学療法が必要になるため，速やかな化学療法の実施が望ましいと考えられている[1)]．

Ⅹ 進行例に対する治療方針

種々の研究により明らかにされた予後不良因子（遠隔転移・*MYCN*増幅・UH・DNA index≦1など）を持つ高リスク神経芽腫は，現在なおその3年無増悪生存割合で20～40%であり，新たな有効な治療法の開発が切に待ち望まれている．

高リスク神経芽腫の進行は早く，発見後あるいは化学療法中にも急速に進展する例が多く見られ，原発部位の増大だけでなく骨・骨髄・リンパ節・肝・後腹膜・後縦隔などに急速に転移・浸潤する特徴がある．また，集学的治療が奏効し治療を終了できたとしても，治療後すぐから再発を認めることが多い．近年では原発巣局所に対する外科及び放射線療法が進歩してきており，以前よりも原発巣局所からの再発は減少して来たが，その代わりに骨や骨髄再発が主体となってきている．再発時期は骨髄破壊的大量化学療法施行直後から2年以内にほとんどの再発がみられ，その後も5年以上にわたり再発がみられる[1)]．

現在日米欧では，高リスク神経芽腫に対しては，診断時に原発巣を安全に全摘出できる症例がほとんどなく，骨・骨髄転移例が多く，速やかに全身化学療法を行わないと生命の危険性が高いと判断されることから，生検後に速やかに寛解導入化学療法(induction)が開始される．化学療法を数コース行った後，局所療法として外科切除術および局所放射線療法を組み合わせた治療を行い，その後強化した化学療法かあるいは骨髄破壊的大量化学療法による地固め療法(consolidation)を行うものが多い．化学療法としてはシスプラチン・カルボプラチン・シクロフォスファミド・イホスファミド・エトポシド・ビンクリスチン・ピラルビシ