Anti-tumor effects of fusion cells of type 1 dendritic cells and Meth A tumor cells using hemagglutinating virus of Japan-envelope

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Abstract. It has been reported that the fusion cells of dendritic cells (DCs) and tumor cells have anti-tumor effects. In this experiment, we examined the anti-tumor effects of fusion cells of bone marrow-derived DC type 1 (DC1) and irradiated tumor cells using a newly commercially available hemagglutinating virus of Japan-envelope (HVJ-E) after cell fusion, in a mouse model. To induce DC1, bone marrow cells (BMCs) from BALB/c mice were cultured with GM-CSF, IL-12 and IFN-γ. BMC-derived DC1 were fused with 20-Gy-irradiated Meth A cells (BALB/c-derived fibrosarcoma) using HVJ-E. We subcutaneously injected: i) the BMC-derived DC1, or ii) the fusion cells of the DC1 and the irradiated Meth A cells, into Meth A-bearing BALB/c mice. The injection of only DC1 showed a moderate anti-tumor effect, as we previously described. However, the fusion cells were more effective in not only suppressing tumor growth but also prolonging survival. These results suggest that the fusion cells of DC1 and the irradiated tumor cells using HVJ-E were more effective in tumor suppression than DC1 alone.

Introduction

Dendritic cells (DCs) play a central role in the initiation and regulation of the immune system and can prime naive T cells (1). It has been reported that there is a positive correlation between the number of DCs in tumors and the suppression of the tumor growth, suggesting that DCs infiltrating tumors suppress the tumor growth (2-4). Based on these findings, anti-tumor therapies using DCs have been tried experimentally and clinically with some success in terms of the suppression of tumor growth. These therapies have included the administration of fusion cells of DCs and tumor cells (5), tumor-related peptide-pulsed DCs (6-8) and tumor-related gene-transfected DCs (9). In particular, the fusion cells showed significant effects on the suppression of tumor growth in the first presented report (5). However, subsequent data showed that the fusion cells of DCs and tumor cells have wide-ranging anti-tumor effects. The mechanisms underlying the anti-tumor effects of DCs are supposedly due to the activation of tumor-specific cytotoxic T lymphocytes (CTLs) (10). It has been reported that DCs induced by GM-CSF-based culture conditions could be temporarily designated as DC0, DC1 or DC2 (11). DC1 (not DC2) can polarize $CD4^+$ T cells to Th1 cells, resulting in the suppression of tumor growth (11-13). We have also reported that the inoculation of DC1 with or without formalin-fixed tumor cells has an anti-tumor effect (14,15).

In this study, we prepared fusion cells of DC1 and mouse fibrosarcoma Meth A cells using a new hemagglutinating virus of Japan-envelope (HVJ-E), and examined the anti-tumor effects of the fusion cells *in vivo*.

Materials and methods

Animals. Male BALB/c mice were purchased from Shimizu Laboratory Supplies (Shizuoka, Japan) and used at 8 weeks of age. The animals were handled in accordance with the Kansai Medical University Guide for the Care and Use of Laboratory Animals.

Culture of dendritic cells. DCs were induced from bone marrow cells (BMCs) of BALB/c mice, slightly modified as previously described. BMCs were obtained from femoral bones, tibiae, and humeral bones of BALB/c mice, and then filtered through a 70-μm nylon cell strainer (BD Bioscience-Labware, Franklin Lakes, NJ, USA). BMCs from BALB/c mice were cultured with GM-CSF (10 ng/ml) (PeproTech EC, London, UK) for 7 days to induce DC0, as previously described (13). Type 1 dendritic cells (DC1) were induced as described elsewhere (26). Briefly, BMCs from BALB/c mice

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were cultured in the presence of GM-CSF (10 ng/ml). At this point, >90% of cultured cells were $CD11c^+$, which is a specific marker of murine DCs. Next, IL-12 (5 ng/ml) (PeproTech EC) and IFN-γ (5 ng/ml) (PeproTech EC) were added to the culture. After another 3 days of culture, the DCs were used as DC1 for the experiments.

Establishment of Meth A expressing luciferase (luc Meth A). luc Meth A are Meth A cells expressing luciferase to examine tumor growth *in vivo*. Tax gene and bsr gene were cut off from pRTaxbsr (16), followed by insertion of luc2 gene and puro gene from pGL4.10[luc2] vector (Promega, Madison WI, USA), instead of tax and bsr genes, respectively. The thus-prepared pRluc2-pur was transfected into BOSC23 cells (packaging cells), and the retrovirus in the culture supernatant of the transfected BOSC23 cells was added into the Meth A cell-culture condition. Retrovirus-infected Meth A cells were cultured in DMEM containing puromycin. A single clone of the Meth A cells showing high luciferase activity was obtained. Thus, we prepared Meth A cells expressing high luciferase activity (luc Meth A cells).

Irradiation. Mice and Meth A cells were irradiated using the Gammacell 40 Exactor (Nordion International Inc., Ottawa, Ontario, Canada). Meth A cells irradiated with 20 Gy cannot proliferate.

Inoculation of tumor cells. To prepare the subcutaneous tumor models, the BALB/c mice were subcutaneously injected with murine fibrosarcoma cells, Meth A cells or luc Meth A cells ($2x10^5$/mouse) 1 day after radiation (3 Gy at 1.0 Gy/min). When the tumor reached ~1x1 cm in size, DCs or the fusion cells of DCs and irradiated or non-irradiated Meth A cells were injected.

To prepare the lung metastasis model, the BALB/c mice were intravenously injected with luc Meth A cells ($1x10^5$/mouse) 1 day after radiation (3 Gy at 1.0 Gy/min). Four days after the inoculation of luc Meth A cells, DC1 or a mixture of the fusion cells of DC1 and irradiated Meth A cells were injected subcutaneously.

Fusion of DCs and tumor cells. For fusion by hemagglutinating virus of Japan-envelope (HVJ-E) (GenomONE™-CF; Ishihara Sangyo Kaisha, Osaka, Japan), bone marrow-derived DCs and 20 Gy-irradiated or non-irradiated Meth A cells were mixed at a ratio of 10:1 [DC($1x10^7$/mouse): Meth A cells ($1x10^6$/mouse)] and centrifuged at 2500 rpm for 5 min. Buffer solution and diluted HVJ-E of GenomONE-CF were added to the cell pellet and incubated for 10 min, then centrifuged at 2000 rpm for 5 min and incubated at 37˚C, 5% CO_2 for 15 min. To confirm the fusion of the cells, BMC-derived DCs and Meth A cells were labeled with PKH-67 (Sigma-Aldrich Co. Ltd., St. Louis, MI) and with PKH-27 (Sigma-Aldrich Co. Ltd.), respectively. Next, they were fused using GenomONE-CF, followed by analyses using a flow cytometer (FACScan; Becton Dickinson, San Diego, CA), resulting in 20% of the Meth A cells being fused with DCs.

Experimental groups. When the first experiment using DC0 was carried out, Meth A cells were used as tumor cells in order to examine the anti-tumor effects. Several experimental groups were prepared, as follows: i) Control denotes Meth A-bearing BALB/c mice. ii) DC0 denotes Meth A-bearing BALB/c mice subcutaneously injected with only BM-derived DC0. iii) DC0+Meth A denotes Meth A-bearing BALB/c mice subcutaneously injected with the fusion cells of BM-DC0 and Meth A cells. iv) DC0+Meth A(R+) denotes Meth A-bearing BALB/c mice subcutaneously injected with the fusion cells of DC0 and irradiated Meth A cells. v) Meth A(R+) denotes Meth A-bearing BALB/c mice subcutaneously injected with irradiated Meth A cells.

For the following experiment using DC1, luc Meth A cells were injected subcutaneously or intravenously to prepare the subcutaneous tumor model or lung metastasis model in order to examine the anti-tumor effects. i) Control denotes tumor-bearing BALB/c mice. ii) DC1 denotes luc Meth A-bearing BALB/c mice subcutaneously injected with BMC-derived DC1. iii) DC1+Meth A(R+) denotes luc Meth A-bearing BALB/c subcutaneously injected with the fusion cells of DC1 and irradiated Meth A cells.

Measurement of tumor size. To evaluate the anti-tumor effect, tumor sizes were measured every 3-4 days. Tumor size was expressed as width (cm) x length (cm) x height (cm) = tumor size (cm^3).

Assessment of in vivo anti-tumor effects using IVIS® imaging system. In the lung metastasis model, the bioluminescent signal intensity (BLI) of tumor-bearing mice was examined every week using an IVIS imaging system (Xenogen, Alameda, CA, USA). The mice injected with luc Meth A were anesthetized 15 min after the intraperitoneal injection of D-luciferin (Xenogen) (3 mg per mouse). They were then placed in the light-tight chamber of the IVIS bioluminescent imaging system (Xenogen). We recorded the faint bioluminescent signal of the luc Meth A in the mice.

Reverse transcriptase polymerase chain reaction (RT-PCR) for detection of mRNA expression for IL-4, IL-10, IL-12 p35, IL-12 p40, IFN-γ and TGF-β. For RNA preparation from the spleen of BALB/c mice, Meth A cells, induced DC1, the fusion cells and fusion cells cultured 1 h after fusion, cDNA synthesis, and PCR were carried out as described previously (17). Primers for the detection of mRNAs using this experiment were G3PDH (Toyobo, Tokyo, Japan), IL-4, IL-10, IL-12 p35, IL-12 p40 subunits, IFN-γ, TNF-α (Maxim Biotech, Inc., San Francisco, CA) and TGF-ß (Clontech Lab., Palo Alto, CA, USA).

Statistical analyses. The results are represented as mean ± SD. The significant difference of each group was analyzed using the Student's t-test. The significant differences of survival data were analyzed using the Kaplan-Meier method in the Stat Mate software. Differences were considered significant at $p<0.05$.

Results

Fusion cells of DC0 and tumor cells using HVJ-E can suppress tumor growth but cannot prolong the survival of tumor-bearing

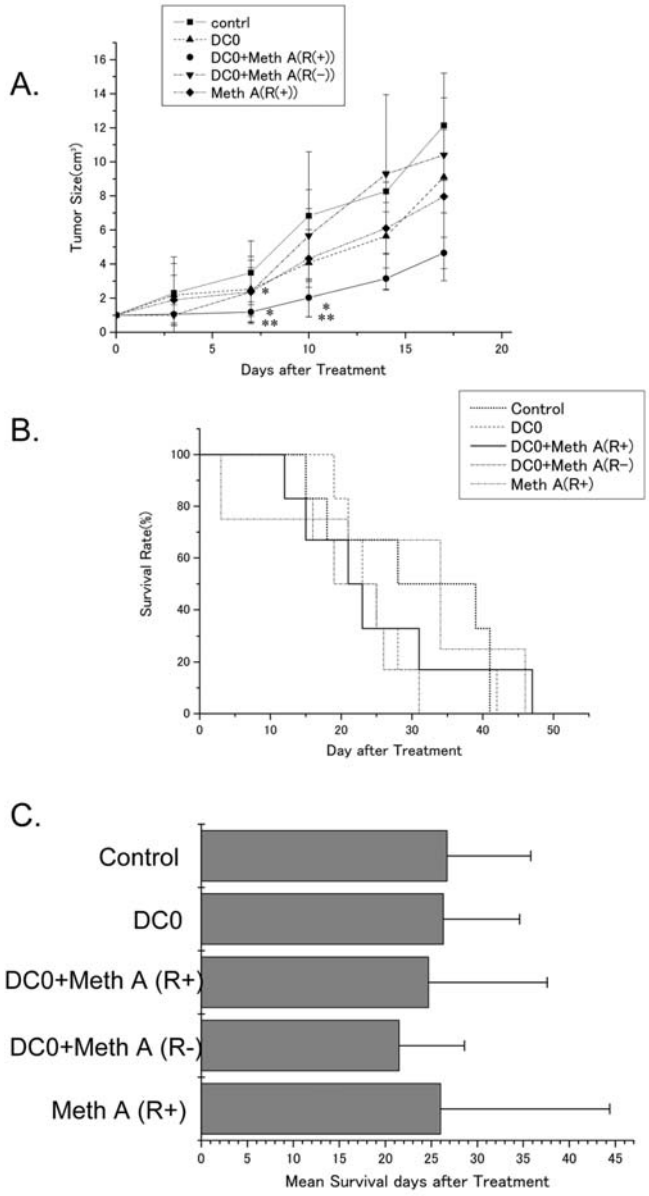

Figure 1. The fusion cells of DC0 and irradiated tumor cells have some effects on suppression of tumor growth but not on survival. Three-Gy irradiated mice were subcutaneously inoculated with Meth A. When the tumor reached 1x1 cm, DC0, irradiated Meth A [Meth A (R+)], the fusion cells of DC0 and non-irradiated Meth A[DC0+Meth A(R-)], the fusion cells of DC0 and irradiated Meth A[DC0+Meth A(R+)] were subcutaneously injected, thereby preparing 5 experimental groups; control, DC0, Meth A (R+), DC0+Meth A(R-) and DC0+Meth A(R+), as described in Materials and methods. As a control, only tumor injected mice were prepared. In the control mice, the experimental day was started when tumors reached 1x1 cm. Tumor size was measured every 3 or 4 days. (A) Mean and SD of the tumor sizes. (B) Survival rates. (C) Means and SDs of survival days of each group. There are no significant differences between these groups in A, B and C. $^{*}p<0.05$ as compared to control. $^{**}p<0.05$ as compared to DC1.

mice. First, we tried to fuse BMC-derived DCs and irradiated mouse fibrosarcoma cells (Meth A cells) using the HVJ-E, and then evaluated the anti-tumor effects. We labeled the DCs with PKH-67, and labeled the Meth A cells with PKH-26. We also fused the cells following the manufacturer's instructions and examined the percentages of fused tumor cells using a flow cytometer. When we fused the BMC-derived DCs and Meth A cells at the ratio of 10:1, 20% of the Meth A cells fused with the DCs.

Previously, it was reported that the fusion cells of bone marrow cell (BMC)-derived DCs and tumor cells have significant anti-tumor effects (5). We therefore examined the anti-tumor effects of the subcutaneous injection of bone marrow-derived DCs induced by only GM-CSF (DC0) (11), and fusion cells of the DC0 and tumor cells. As shown in Fig. 1A, the DC0+Meth A showed no anti-tumor effects, and tumors developed at the injection site of the fusion cells, suggesting that unfused live Meth A cells proliferated at the injected site, and that the unfused Meth A cells suppressed the effects of fusion cells. DC0 and Meth A (R+) showed moderate suppression of tumor growth, while DC0+Meth A(R+) were more effective in the suppression of tumor growth. However, there were no significant differences in survival between these groups (Fig. 1B and C).

DC1 and fusion cells of DC1 and irradiated tumor cells can suppress tumor growth and also prolong the survival of tumor-bearing mice. Vaccination using fusion cells of DC0 and tumor cells did not significantly prolong survival in our system. Therefore, we prepared BM-derived DC1 induced by GM-CSF, IFN-γ and IL-12, as previously described (14), and we examined the anti-tumor effects of the fusion cells of the DC1 and Meth A, since it has been reported that DC1 are more effective than DC0 (11), and we have previously reported that the subcutaneous injection of DC1 has some anti-tumor effect on Meth A (14,15). In the following experiments, we used luc Meth A cells to prepare tumors: luc Meth A are Meth A cells expressing luciferase to examine tumor growth *in vivo*. As shown in our previous report, the tumors of mice treated with DC1 (DC1) were smaller than those of control mice (Fig. 2). We examined whether the fusion cells of DC1 and Meth A could enhance the anti-tumor effects of DC1. The tumors of the DC1+Meth A(R+) were significantly smaller than those of DC1 or control. These results suggest that the fusion cells can enhance the effects of DC1, thereby suppressing tumor growth.

We also examined the effects of the fusion cells of DC1 and tumor cells on survival (Fig. 2B and C). As shown in Fig. 2B and C, all the control mice died within 25 experimental days; the mean survival was 22.7±2.3 days. In contrast, the mean survival times of the DC1 and the DC1+ Meth A (R+) were 29.2±5.8 and 30.7±6.2 days, respectively. Thus, the DC1 and DC1+Meth A(R+) showed longer survival than the control, but the DC1+Meth A(R+) showed no significant prolongation of survival, over the DC1.

Fusion cells of DC1 and irradiated tumor cells have significant effects on suppression of tumor growth and survival of tumor-bearing mice in a lung metastatic model. We examined whether the fusion cells of DC1 and Meth A cells could enhance the anti-tumor effects of DC1 on the treatment of the established lung metastatic model, in which the mice had been injected with luc Meth A cells intravenously. When the tumor cells were injected into the vein, multiple metastatic lesions of the sarcoma grew rapidly in the lungs, and the mice died earlier (17.6±0.9 days), in contrast to the mice subcutaneously injected with the tumor cells (Fig. 3).

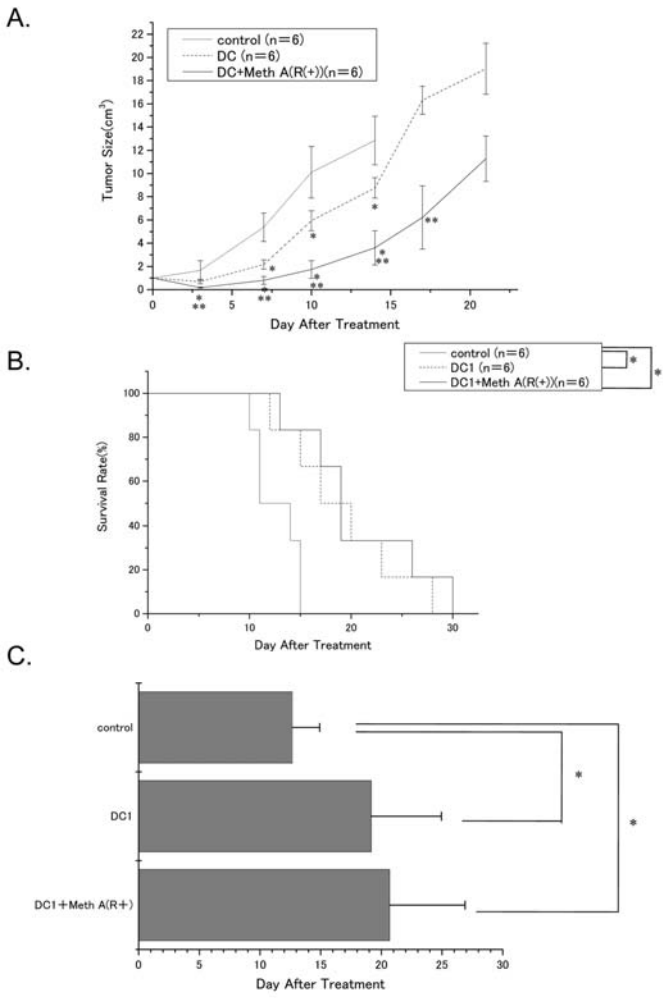

Figure 2. Fusion cells of DC1 and tumor cells are more effective than fusion cells of DC0 and tumor cells in suppression of tumor growth. Three groups were prepared in order to evaluate the anti-tumor effects of the fusion of DC1 and tumor cells; control, DC1 and DC1+Meth A(R+), as described in Materials and methods. (A) Means and SDs of the tumor sizes. (B) Survival rates. (C) Means and SDs of survival days. $^{*}p<0.05$ as compared to control. $^{**}p<0.05$ as compared to DC1.

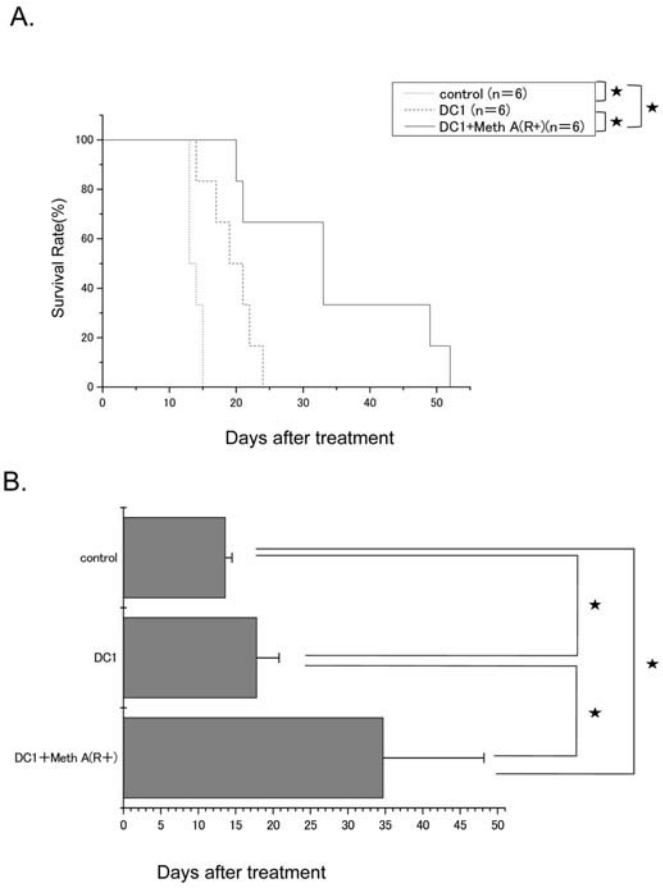

Figure 3. Survival was prolonged in DC1+Meth A(R+) even in lung metastatic model. Three-Gy irradiated mice were intravenously inoculated with Meth A. Four days after the inoculation of Meth A, the mice were injected with: i) DCs, or ii) the fusion cells. Survival rate (A) and mean and SD of survival days are shown (B). $^{*}p<0.05$.

Therefore, in this model, we can easily compare the anti-tumor effects of the treatment on survival. As shown in Fig. 3, DC1 showed a slightly longer survival (21.8±3.0 days) than control (17.6±0.9 days), and DC1+Meth A(R+) showed significantly longer survival (38.7±13.5 days) than control or DC1.

To assess the effects of the fusion cells, we examined the development of the lung metastatic tumors by the *in vivo* bioluminescent signal intensity (BLI). We found that anti-tumor activity in DC1+Meth A(R+) resulted in delayed tumor growth, compared with the control or DC1 (Fig. 4). Moreover, we examined the lungs macroscopically and microscopically 14 days after the inoculation of the luc Meth A cells (Fig. 5). In the macroscopical examination, many micronodules were observed in the lungs of the control. Fewer and smaller nodules were detected in the lungs of the mice treated with DC1 than the control, while there were no visible nodules in the lungs of the DC1+Meth A(R+), the lung morphology being similar to normal lung. In the microscopical examination, many large tumor masses were seen in the lungs of the control, while fewer and smaller tumor masses were seen in the lungs of the DC1. Very few tumor cells were seen in the lungs of the DC1+Meth A(R+). These results suggest that there is significant suppression of tumor growth in the lungs of mice treated with the fusion cells by HVJ-E, resulting in longer survival.

Analyses of cytokine production of fusion cells. We have shown that DC1 induced by GM-CSF, IL-12 and IFN-γ express more mRNA of IL-4, IL-10 IL-12 p40 and IFN-γ than DC0 (17). Therefore, we examined mRNA expression of IL-4, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TNF-α and TGF-ß in the fusion cells using the RT-PCR method. Meth A cells express a very low level of TNF-α and do not express any other cytokines, as far as we examined. The expression of the cytokines in fusion cells became weaker than only DC1, possibly due to dilution of mRNA induced by the fusion. One hour after culture, the expression of mRNA of the cytokines except for IL-4 became weaker than those before the culture. These results suggest that induced DC1 express not only Th1 cytokines but also Th2 cytokines, and the expression of IL-4 in fusion cells increased after inoculation into tumor-bearing individuals.

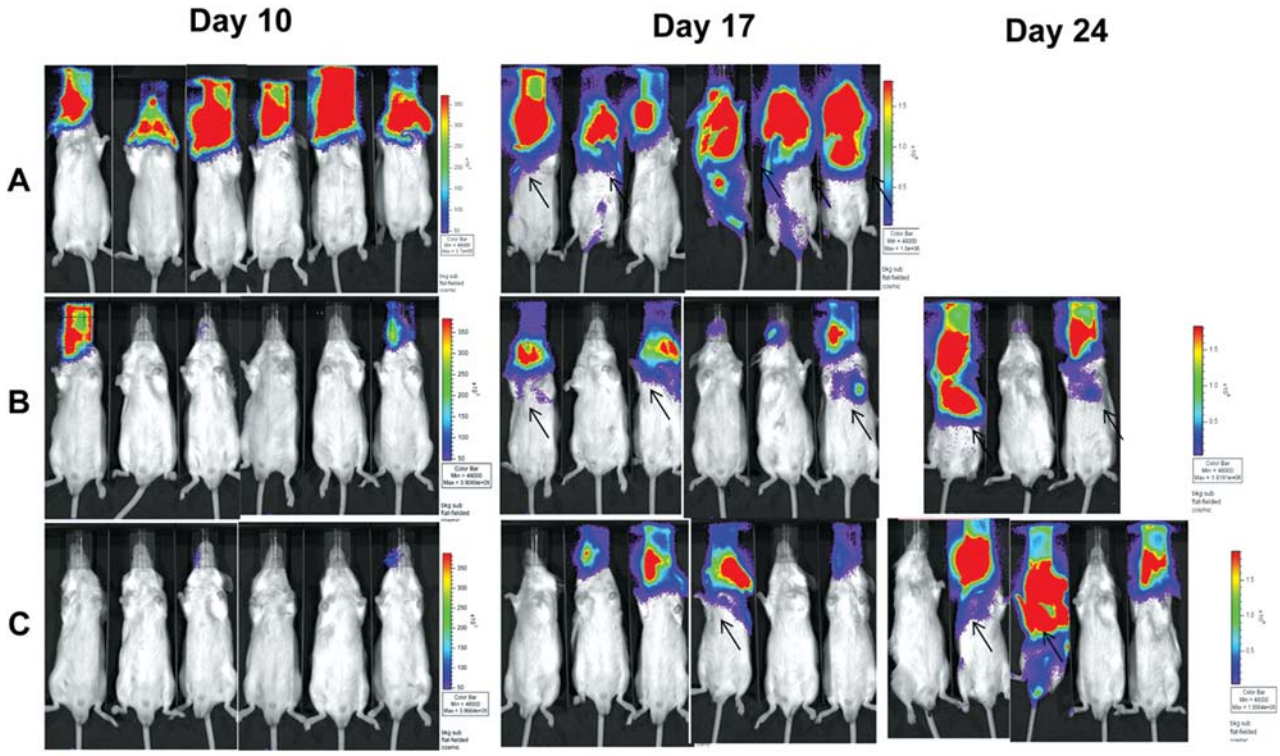

Figure 4. Whole body distribution of Meth A cells in *in vivo* BLI. Three-Gy irradiated mice in each group were intravenously injected with $1x10^5$ luciferase-expressing Meth A cells on day 0. The whole body distribution of transduced tumor cells was monitored using *in vivo* BLI every week from day 10 as indicated by the black arrows.

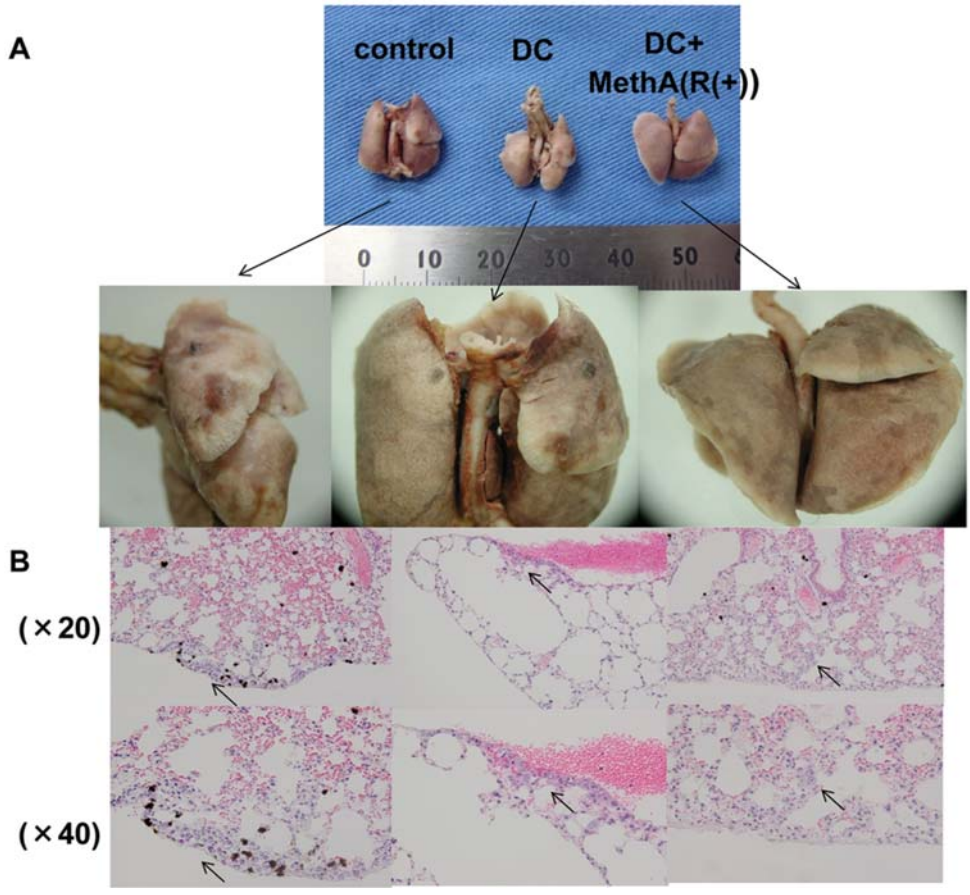

Figure 5. Macroscopical and histological examination of the lungs in each group. Three-Gy irradiated mice in each group were injected with $2x10^5$ luc Meth A cells intravenously through the orbital venous plexus on day 0. Four days after the injection of tumor cells, the mice were injected with DC1 (DC1) or the fusion cells of DC1 and irradiated Meth A cells [DC1+ Meth A(R+)]. The mice were sacrificed 14 days after the injection of luc Meth A cells. The lungs of each group were fixed with formalin. Representative data of the macroscopical (A) and microscopical (B) photographs. Arrows show tumor masses.

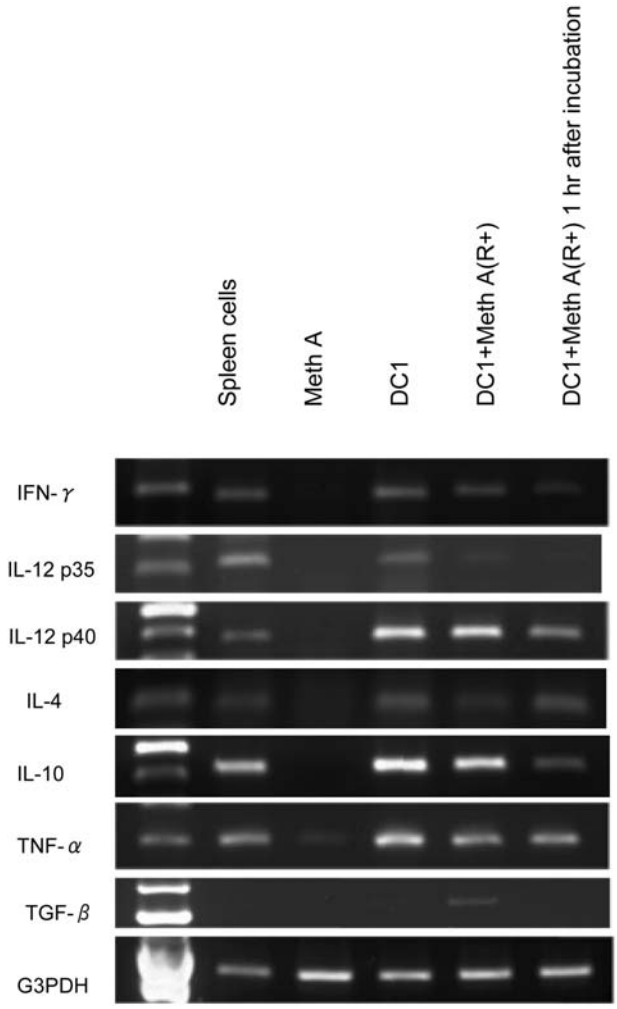

Figure 6. mRNA expression of DC1, Meth A and fusion cells. RT-PCR was carried out to examine the expression of mRNA of IFN-γ, IL-12 p35, IL-12 p40, IL-4, IL-10, TNF-α and TGF-ß. cDNA were prepared from spleen cells, Meth A, DC1, DC1+Meth A(R+) and DC1+Meth A (R+) after 1-h incubation. PCR was then carried out.

Discussion

It has been reported that fusion cells of DCs and tumor cells have anti-tumor effects. For cell fusion, PEG, electroporation or HSV have been used with a preference for PEG or electroporation, since HSV poses the risk of infection. Recently, a newly established HSV-E has become commercially available. This reagent obviates the risk of infection and its effect on cell fusion is stable. Therefore, we tried HSV-E to use cell fusion of DCs and tumor cells.

Fusion cells of DCs and tumor cells have shown various effects. Generally, fusion cells suppress tumor growth moderately but cannot totally inhibit it. Therefore, several additional methods have been carried out to enhance the effects, including allogeneic DCs (18), and induced DC1 plus Th1 cells (12). We have shown that BM-derived DC1 is useful for the suppression of tumor growth (12,13). In the experiments, we used BMC-derived DC1 or formalin-fixed tumor cell-pulsed BMC-derived DC1.

It has been reported that immature DCs have not only a minimal effect on the suppression of tumor growth but may also accelerate tumor growth in some cases. This is because immature DCs express no or low levels of the costimulatory molecules CD80 or CD86. It has been reported that immature DCs rather than mature DCs exist in several kinds of cancers (19,20) and that BMCs cultured with only GM-CSF differentiate into immature DCs, which induce T cell anergy (21). It has also been reported that malignant tumors release humoral factor(s) to suppress the maturation of DCs (22-24). Therefore, it is reasonable to accelerate the maturation of DCs *in vitro* in order to suppress tumor growth. In our experiment, GM-CSF-induced DCs showed no effects on the survival rate of tumor-bearing mice, even if they are fused with irradiated tumor cells. Therefore, in our system, the induction of DC1 is crucial for inducing the anti-tumor effects of DC vaccination.

Previously, it has been reported that TGF-ß released from tumor cells can suppress anti-tumor effects of fusion cells of DCs and tumor cells (25). Therefore, we examined the mRNA expression of TGF-ß of the luc Meth A. However, we did not detect the mRNA of TGF-ß. It has been reported that the fusion cells of DCs and tumor cells plus IL-12 show more anti-tumor effects than the fusion cells only (26), suggesting that the shift to Th1 rather than Th2 is crucial to achieve the anti-tumor effects. In our experiments, induced DC1 express not only Th1 cytokines but also Th2 cytokines, and the fusion cells also express Th2 cytokines. Therefore, it is suggested that the suppression of Th2 cytokines and/or the augmentation of Th1 cytokines induces more anti-tumor effects, even in our system.

In this experiment, we have tried a new commercially available HSV-E reagent to fuse BMC-derived DC1 and irradiated tumor cells, and have shown the anti-tumor effects of the fusion cells on murine fibrosarcoma, Meth A. These results suggest that the fusion cells of DC1 and irradiated tumor cells by GenomONE-CF are effective in the suppression of tumor growth, and that this method is a candidate for a new anti-tumor therapy.

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