

# A Novel Combination Treatment of Armed Oncolytic Adenovirus Expressing IL-12 and GM-CSF with Radiotherapy in Murine Hepatocarcinoma

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## Oncolytic adenovirus/Interleukin 12/Granulocyte-macrophage colony-stimulating factor/Radiation/Apoptosis.

In this study, a novel combination treatment of armed oncolytic adenovirus expressing interleukin 12 (IL-12) and granulocyte-macrophage colony-stimulating factor (GM-CSF) with radiation was investigated for antitumor and antimetastatic effect in a murine hepatic cancer (HCa-I) model. Tumor bearing syngeneic mice were treated with radiation, armed oncolytic virus Ad- $\Delta$ E1Bmt7 (dB7) expressing both IL-12 and GM-CSF (armed dB7), or a combination of both. The adenovirus was administered by intratumoral injection  $1 \times 10^8$  PFU per tumor in 50  $\mu$ l of PBS four times every other day. Tumor response to treatment was determined by a tumor growth delay assay. Metastatic potential was evaluated by a lung metastasis model. To understand the underlying mechanism, the level of apoptosis was examined as well as the change in microvessel density and expression of immunological markers: CD4+, CD8+ and Cd11c. The combination of armed dB7 and radiation resulted in significant growth delay of murine hepatic cancer, HCa-1, with an enhancement factor of 4.3. The combination treatment also resulted in significant suppression of lung metastasis. Increase of apoptosis level as well as decrease of microvessel density was shown in the combination treatment, suggesting an underlying mechanism for the enhancement of antitumor effect. Expression of immunological markers: CD4+, CD8+ and Cd11c also increased in the combination treatment. This study showed that a novel combination treatment of radiotherapy with armed oncolytic adenovirus expressing IL-12 and GM-CSF was effective in suppressing primary tumor growth.

## INTRODUCTION

Oncolytic adenovirus therapies have been designed to eliminate malignancies by direct infection.<sup>1,2)</sup> Armed oncolytic adenoviruses are currently being developed as novel antitumor therapeutics by creating oncolytic viral vectors armed with antitumor or immunomodulatory transgenes that may enhance viral-based therapeutic potential.<sup>3)</sup>

Recently, a novel concept of radiation enhanced viral oncolytic therapy (ReVOLT) has been proposed. In ReVOLT, ionizing radiation increases the recovery of infectious adenovirus compared to nonirradiated tumors.<sup>4)</sup> Qian *et al.* showed that ionizing radiation increases adenovirus uptake

and transgene expression in colon cancer xenografts.<sup>5)</sup> Also, in several studies, the combined use of radiation with a virus resulted in synergistic activity against the tumor cell lines.<sup>6-8)</sup> Although studies have shown that combined use of radiation with a virus can effectively inhibit tumor growth and metastasis in various tumor models, the additive effect of using radiation with an “armed” oncolytic virus expressing immunomodulatory transgenes: IL-12 and GM-CSF has not been tested so far.

In this study, a new treatment was developed which combine radiation, cytokines and an oncolytic adenovirus. Its antitumor and antimetastatic effects were tested in HCa-I. IL-12 induces interferon-gamma production, stimulates both T and natural killer cells, promotes T-cell responses, and inhibits neovascularization.<sup>9)</sup> GM-CSF is one of the most potent stimulators of a specific and long-lasting antitumor immunity and its important role in the maturation of antigen-presenting cells to induce T-cell activation.<sup>10)</sup> Combined IL-12 and GM-CSF gene therapy induces strong cytotoxic T lymphocytes reactions and antitumor effects from cellular immune responses.<sup>11)</sup> The strategy employs radiation treat-

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ment in combination with an armed oncolytic adenovirus expressing IL-12 and GM-CSF, which not only increases cytopathic effects by replicating adenovirus-induced apoptosis but also induces powerful antitumor activities by employing the cellular immune responses of IL-12 and GM-CSF.

## MATERIALS AND METHODS

### *Mice and tumor*

Male C3H/HeJ mice, 7–8 weeks old were used for this study. Experiments were in accordance with the Yonsei University Medical College guidelines and regulations approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The murine hepatocarcinoma syngeneic to C3H/HeJ, HCa-I is a highly radioresistant tumor with a TCD 50 (radiation dose yielding 50% tumor cure rate) of > 80 Gy. Tumor cell suspensions were prepared as previously described.<sup>12)</sup>

### *Viruses*

The oncolytic adenovirus Ad- $\Delta$ E1Bmt7 (dB7) and armed oncolytic adenovirus Ad- $\Delta$ E1Bmt7 expressing both IL-12 and GM-CSF (armed dB7) were provided by professor Chae-Ok Yun of the Yonsei Cancer Institute. The Ad- $\Delta$ E1Bmt7 adenovirus, generating an E1A and E1B double-mutant replication-competent adenovirus, was described previously.<sup>13,14)</sup>

### *Assay of tumor growth delay*

To determine the optimal sequence of armed dB7 and radiation treatment, 4 experimental groups were set: control, radiation alone, radiation followed by armed dB7 6 hr later, and armed dB7 followed by radiation 6 hr later.<sup>15)</sup>

For tumor growth delay analysis of oncolytic adenovirus dB7 expressing IL-12 or GM-CSF, six experimental groups were set; control + PBS (phosphate buffered saline) alone, radiation + PBS, dB7 + IL-12 alone, dB7 + IL-12 + radiation, dB7 + GM-CSF alone, and dB7 + GM-CSF + radiation group. For tumor growth delay analysis of armed dB7 expressing both IL-12 and GM-CSF, six experimental groups were set: control + PBS alone, radiation + PBS, dB7 alone, dB7 + radiation, armed dB7 alone, and armed dB7 + radiation group. The tumors were generated by inoculating viable tumor cells into the muscles of the right thighs of the mice. When tumors reached a mean diameter of 7.5–8 mm, mice were randomly assigned to one of the six groups receiving PBS, dB7, and armed dB7. Eight mice were allocated in each group. Radiation treatment was delivered when the tumors had grown to 7.5–8 mm in mean diameter (Day 0). The tumor-bearing legs were treated with a single dose of 25 Gy using a clinical linear accelerator (Varian Medical Systems Inc., Palo Alto, CA, USA).<sup>17)</sup> When tumors were in the range of 7.5–8 mm in mean diameter, PBS or adenovi-

ruses mixed with Lipofectamine<sup>TM</sup> and Lipofectamine<sup>TM</sup> Plus solution (Invitrogen Co., Carlsbad, CA, USA) at 2:6 ratio were administered intratumorally [ $1 \times 10^8$  plaque forming units (PFU) per tumor in 50  $\mu$ l of PBS] four every other day. In the radiation plus adenovirus group, radiation was administered followed by adenovirus injection 6 hrs later (Day 0). For the tumor growth delay assay, the tumors were regularly measured at three orthogonal tumor diameters until the tumors grew to at least 12 mm in diameter. The effect of the treatment on tumor growth delay was expressed as the absolute growth delay (AGD), which was defined as the time in days for the 8 mm tumors to grow to 12 mm in the treated group minus the mean time for the 8 mm tumors to reach 12 mm in the untreated control group. The enhancement factor (EF) was calculated by dividing the normalized tumor growth delay (NGD) by the AGD. The NGD was defined as the time in days for tumors to grow from 8 mm to 12 mm in mice treated with the combination treatment minus the time in days for tumors to reach 12 mm in the group treated by drugs only.<sup>16)</sup>

### *Assay of lung metastasis*

HCa-I, grown in the thigh, develops lung metastasis in 10–20 days after tumor implantation.<sup>17)</sup> The antimetastatic potential in the 6 experimental groups was tested with the spontaneous lung metastasis model. When the tumor reached a diameter of 8 mm, mice were randomly assigned to one of six groups as described in the assay of tumor growth delay. Mice lungs were taken at days 10, 15, and 20 after treatment and fixed with Bouin's solution for counting lung nodules under a polarizing microscope ( $\times 4$ ).

### *Analysis of apoptosis*

The level of induced apoptosis was evaluated in tissue sections for 6 experimental groups as described in assay of tumor growth delay. The tumors were immediately excised and placed in neutral buffered formalin at 4, 8, 12, and 24 hr after treatment as previously described. The tissues were embedded in paraffin blocks and 4- $\mu$ m sections were cut and stained with the ApopTag staining kit (Chemicon, California, CA, USA). Apoptotic cells were scored on coded slides at 400 $\times$  magnification according to the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells as apoptotic only when accompanied by apoptotic morphology. Ten fields in non-necrotic areas were selected randomly across each tumor section, and in each field apoptotic bodies were expressed as a percentage based on the scoring of 1000 nuclei.

### *Immunohistochemical analysis*

The tumors were immediately excised and placed in neutral buffered formalin at 4, 8, 12, 24 hr and at day 2, day 7 after treatment as described in the assay of tumor growth delay. After incubating the slide 2 hr at 57°C, the tissue sec-

tions were deparaffinized in xylene and rehydrated through a series of graded alcohols to diluted water. The deparaffinized sections were then boiled by microwaving in a 0.01 M citrate buffer (pH 6.0) to retrieve the antigens. The antibodies were used at 4, 8, 12, 24 hr: a mouse monoclonal antibody against CD31 (BD PharMingen, San Diego, CA, USA); anti-hypoxia-inducible factor 1 $\alpha$  (Hif-1 $\alpha$ ) monoclonal antibody (Calbiochem, Darmstadt, Germany) at 4°C for overnight. The antibodies were used at day 2, day 7: a mouse anti-adenovirus (Ad-hexon) monoclonal antibody (Chemicon, Billerica, MA, USA); a mouse monoclonal IL-12 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); a rat monoclonal GM-CSF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); a purified rat anti-mouse CD4+ antibody (BD PharMingen, San Diego, CA, USA); a monoclonal antibody to CD8+ (Acris, Herford, Germany); a monoclonal antibody to CD11c (Acris, Herford, Germany) at 4°C for overnight. After washing with PBS, slides were stained using EnVision-HRP Detection Kit (Dako A/S, Glostrup, Denmark). The peroxidase binding sites were detected by staining with diaminobenzidine (DAB; DAKO A/S, Glostrup, Denmark), and the sections were finally counterstained with Mayer's hematoxylin and observed under a light microscope.

Microvessel density (MVD) was assessed using the criteria described by Weidner *et al.*<sup>18)</sup> The immunohistochemical results for Hif-1 $\alpha$  protein were classified on slides at 400 $\times$  magnification. Ten fields in non-necrotic areas were selected randomly across each tumor section, and in each field Hif-1 $\alpha$  positive cells were expressed as a percentage based on the scoring of 1000 nuclei.

#### Statistical analysis

Results are expressed as mean  $\pm$  SE. For statistical comparison, the t-test was used. All tests were two-sided, and a *p* value less than 0.05 indicated statistical significance.

## RESULTS

#### Enhancement of tumor radioresponse by armed oncolytic adenovirus

To determine the optimal sequence of radiation and armed dB7, 2 groups, radiation followed by armed dB7 (Prior combination) and armed dB7 followed by radiation (Post combination) were compared. There was no difference in tumor growth delay between the two groups (Fig. 1. a), thus the following experiments adopted the sequence of radiation followed by armed dB7. The time for tumor growth from 8 to 12 mm was 9.3 days and 11.3 days in the radiation alone group and the oncolytic adenovirus dB7 expressing IL-12 alone group (Fig. 1. b), respectively, which corresponded with 0.7 days and 2.7 days of the AGD, in the oncolytic adenovirus dB7 expressing IL-12 alone group and the radiation alone group, respectively. When radiation was combined

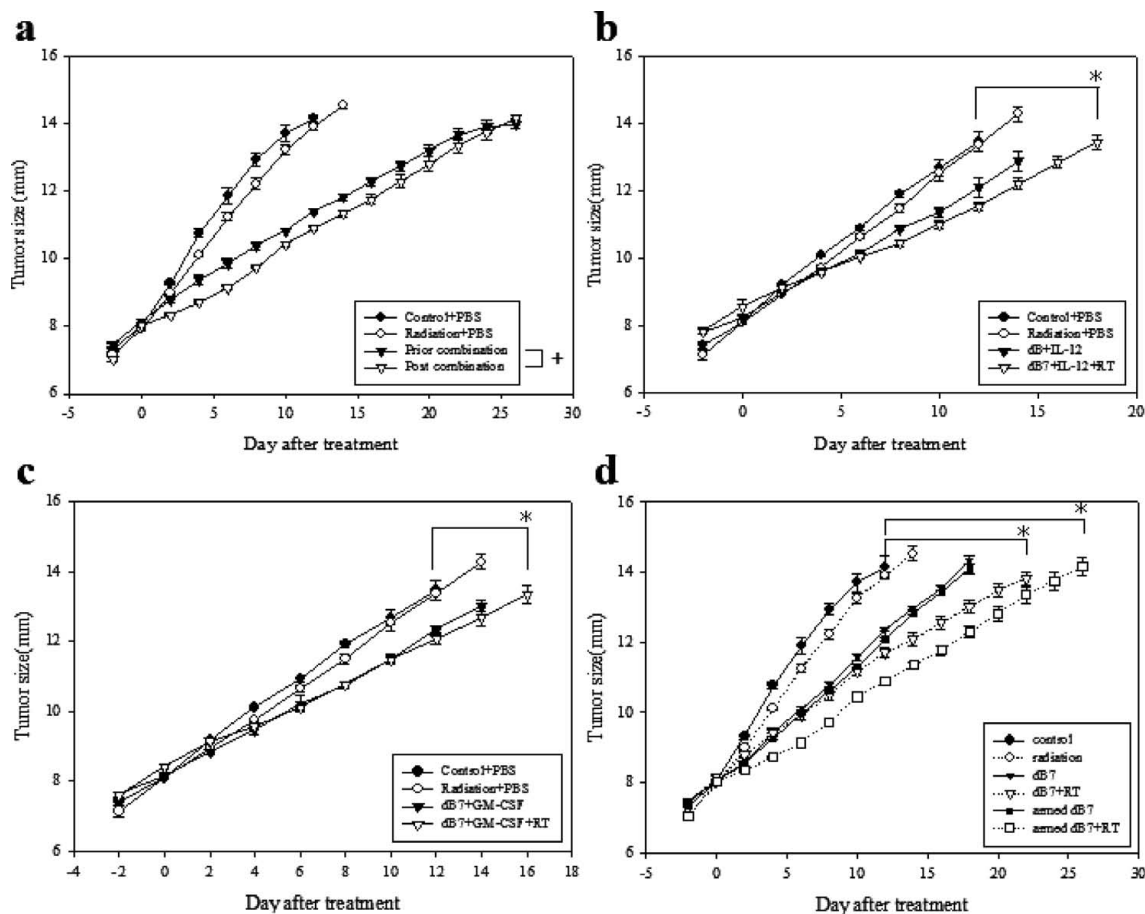
with oncolytic adenovirus dB7 expressing IL-12, the time for growth from 8 to 12 mm was 12.7 days and NGD was 1.3 days. The enhancement factor (EF) was 1.9 (Table 1). When radiation was combined with oncolytic adenovirus dB7 expressing GM-CSF (Fig. 1. c), EF was 1 (Table 1). The time for tumor growth from 8 to 12 mm was 8.3 days and 11.9 days in the radiation alone group and the armed dB7 alone group, respectively, which corresponded with 1.5 days and 5.1 days of the AGD in the radiation alone and armed dB7 alone groups, respectively (Fig. 1. d). When radiation was combined with armed dB7, the time for growth from 8 to 12 mm was 18.3 days and the NGD was 6.4 days with an enhancement factor of 4.3 (Table 2). In the case of dB7, the time for tumor growth from 8 to 12 mm was 11.2 days and 15.2 days in the dB7 alone, and dB7 + RT groups, respectively, which accorded with 4.4 days of the AGD and 4 days of NGD. The enhancement factor was 2.7. These data indicate that both the oncolytic and armed oncolytic viruses significantly enhanced the antitumor effect of radiation and that the enhancement was much more potent with the armed virus.

#### Suppression of tumor metastasis by armed oncolytic adenovirus

Fifteen days after tumor transplantation, the average number of lung nodules was  $8.4 \pm 0.6$ . The number of lung nodules significantly decreased to  $1.4 \pm 0.4$  in the dB7 + radiation group (*p* < 0.05) and to  $1 \pm 0.4$  in the armed dB7 + radiation group (*p* < 0.05). The antimetastatic effect was more prominent 20 days after tumor transplantation; compared to  $19.3 \pm 3.1$  in the control group. The number of lung nodules was  $2.1 \pm 0.7$  (*p* < 0.05) and  $1.1 \pm 0.3$  (*p* < 0.05) in the dB7 + radiation and armed dB7 + radiation groups, respectively (Fig. 2). These results show that both the dB7 and armed dB7 significantly suppressed tumor metastasis to the lung even without radiation. Thus, radiation seems to play a minimal role in inhibiting metastasis to the lung in this model.

#### Enhancement of apoptosis by a combined treatment of radiation with armed oncolytic adenovirus

To study the mechanism, the levels of induced apoptosis were examined. With radiation + PBS, the peak level of induced apoptosis was 11.3% at 8 hr (*p* < 0.05) and decreased to 8.4% at 24 hr (*p* < 0.05) (Fig. 3. a). In contrast, the level of apoptosis continued to increase during the observation time (24 hr) both in the dB7 and armed dB7 groups. At 24 hr, the level of apoptosis was 13.7% in the dB7 group (*p* < 0.05) and 26.8% in the armed dB7 group (*p* < 0.05). In combination with radiation, induced apoptosis appeared to be simply additive at 8 hr in both groups. However at 24 hr, when the level of radiation-induced apoptosis decreased close to the basal level, apoptosis continued to increase more than additively, with 23.8% in the dB7 + radi-



**Fig. 1.** Tumor growth delay by different sequence of radiation and virus (a), effect of dB7 + IL-12 (b), dB7 + GM-CSF (c), dB7 or armed dB7 combined with radiation (d). An armed dB7 increased the antitumor effect of radiation with an enhancement factor (E.F.) of 4.3. \* $p < 0.05$  vs. Control + PBS group.

ation group ( $p < 0.05$ ) and 35.4% in the armed dB7 + radiation group ( $p < 0.05$ ) at 24 hr. These results suggest that combination treatment can synergistically increase apoptosis more than each group. To further examine the induced apoptosis of late time in experiment of lung metastasis model, TUNEL staining was also done at 20 days after treatment. In both the armed dB7 group and the armed dB7 + radiation group, a wide area of necrosis was shown (Fig. 3. b). These results suggest that armed dB7 increased the level of apoptosis as a key mechanism, ultimately leading to a significant degree of tumor cell necrosis.

#### *Analysis of microvessel density and hypoxia condition following the treatment with armed oncolytic adenovirus and radiation*

With the significant levels of apoptosis in tumor cells induced by combination treatment, the treatment could also induce a change in the microenvironment surrounding the tumor. In this regard, the microvessel density was evaluated via immunohistochemistry staining with CD31 (Fig. 4. a). CD31 was overexpressed in the control group ( $23.3 \pm 3.2$ ).

In the radiation group, CD31 expression remained at an almost similar level with only a slight decrease at 24 hr ( $18.7 \pm 3.2$ ). However, in the combination group, CD31 expression showed a significant decrease in a time-dependent manner and ultimately reached 7% of the control level ( $23.3 \pm 3.2$ ). CD31 expression in the armed dB7 + radiation group appeared to be simply additive to the effects in the radiation group (Fig. 4. b).

The combination treatment might exhibit antitumor effects via altering the expression levels of hypoxia-related genes. In the radiation group, the peak level of Hif-1 $\alpha$  positive cells was 90.3% at 12 hr, which decreased to 55.7% at 24 hr (Fig. 4. c). When radiation and armed dB7 were combined, Hif-1 $\alpha$  at 4 hr was increased in comparison to control or radiation. However, Hif-1 $\alpha$  in armed dB7 and radiation was decreased time-dependently (Fig. 4. d). These data indicate that decrease of Hif-1 $\alpha$  expression might be involved in the mechanism of enhancement of antitumor effect in the combined group.

**Table 1.** Effect of armed oncolytic adenovirus expressing IL-12 or GM-CSF on radioresponse of HCa-I tumor in C3H/HeJ mice

Treatment*	Days required to form 8 to 12 mm sized tumor <sup>†</sup>	AGD <sup>‡</sup>	NGD <sup>§</sup>	EF <sup>¶</sup>
control + PBS	8.7 ± 0.5			
radiation + PBS	9.3 ± 0.6	0.7		
dB7 + IL-12	11.3 ± 0.7	2.7		
dB7 + IL-12 + RT	12.7 ± 0.7	4	1.3	1.9
dB7 + GM-CSF	10.7 ± 0.6	2		
dB7 + GM-CSF + RT	11.3 ± 0.8	2.7	0.7	1

\*Mice bearing 8-mm tumors in the right thighs were given i.t.  $1 \times 10^8$  PFU adenoviruses or 25 Gy local tumor irradiation. Groups consisted of 8 mice each. <sup>†</sup>Mean ± SE. <sup>‡</sup>AGD (Absolute growth delay) is defined as the time in days for tumors in the treated groups to grow from 8 to 12 mm minus the time in days for tumors in the untreated control group to reach the same size. <sup>§</sup>NGD (Normalized tumor growth delay) is defined as the time for tumors in groups treated with combination group to grow from 8 to 12 mm minus the time to reach the same size in mice treated with adenovirus alone. <sup>¶</sup>EF was calculated as the ratio of NGD in mice treated with combination group to AGD in mice treated by radiation alone.

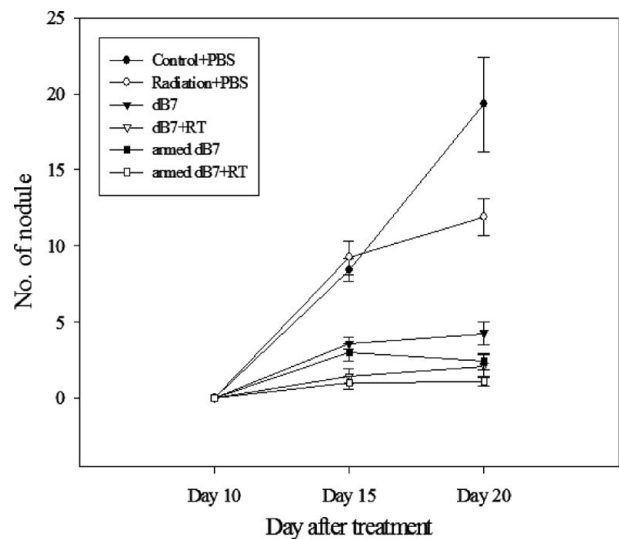
**Table 2.** Effect of armed dB7 on radioresponse of HCa-I tumor in C3H/HeJ mice

Treatment *	Days required to form 8 to 12 mm sized tumor <sup>†</sup>	AGD <sup>‡</sup>	NGD <sup>§</sup>	EF <sup>¶</sup>
control + PBS	6.8 ± 0.4			
radiation + PBS	8.3 ± 0.7	1.5		
dB7	11.2 ± 0.5	4.4		
dB7 + RT	15.2 ± 0.5	8.4	4	2.7
Armed dB7	11.9 ± 0.2	5.1		
Armed dB7 + RT	18.3 ± 0.7	11.5	6.4	4.3

\*Mice bearing 8-mm tumors in the right thighs were given i.t.  $1 \times 10^8$  PFU armed dB7 or 25 Gy local tumor irradiation. Groups consisted of 8 mice each. <sup>†</sup>Mean ± SE. <sup>‡</sup>AGD (Absolute growth delay) is defined as the time in days for tumors in the treated groups (armed dB7 or radiation or armed dB7 + RT) to grow from 8 to 12 mm minus the time in days for tumors in the untreated control group to reach the same size. <sup>§</sup>NGD (Normalized tumor growth delay) is defined as the time for tumors in groups treated with armed dB7 + RT to grow from 8 to 12 mm minus the time to reach the same size in mice treated with armed dB7 alone. <sup>¶</sup>EF was calculated as the ratio of NGD in mice treated with armed dB7 + RT to AGD in mice treated by radiation alone.

### Expression of adenoviral protein, IL-12, and GM-CSF in tumor

Viral distribution and persistence within the tumor mass was then confirmed using an antibody specific to adenoviral hexon protein. A marked increase in hexon-immunoreactivity was detected in wide areas of the combination group at day

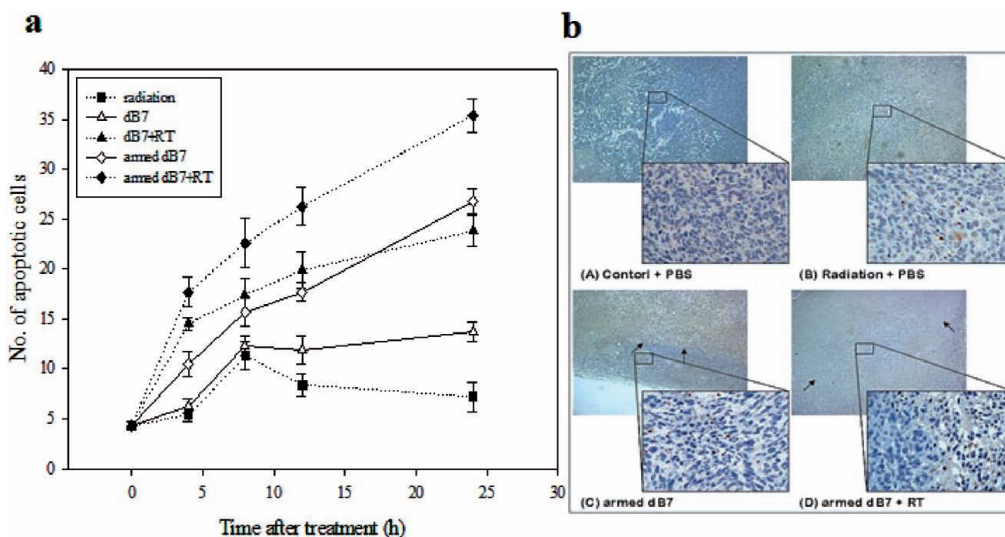
**Fig. 2.** The number of metastatic lung nodules by the treatment in HCa-I. Significant decrease of lung nodules was shown in the dB7 + radiation and the armed dB7 + radiation group.

7 and was undetectable in control and radiation groups (Fig. 5. a).

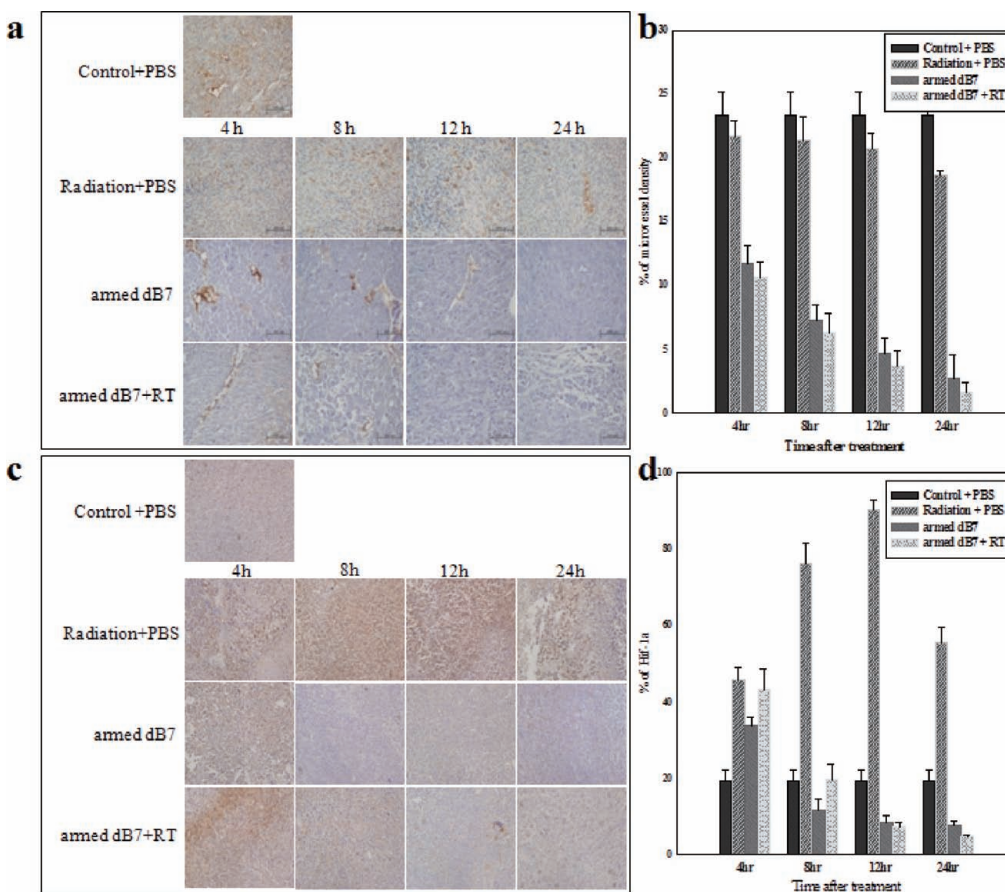
To investigate the distribution and persistence of IL-12 and GM-CSF on solid tumor, histological examination was carried out. In the combination group, ad hexon staining revealed wide areas of tumor tissue. Also, the expression of IL-12 and GM-CSF was presented in the same area of the expression of ad hexon (Fig. 5. b). In addition, with armed dB7 alone, the necrotic area was detected at day 7. However, in the combination group, the necrotic area was detected at day 2. These data indicate that combination treatment was more efficient than armed dB7 treatment in inhibiting hepatic tumor growth.

### Increased CD4<sup>+</sup>, CD8<sup>+</sup> T-cell and dendritic cell infiltration in armed dB7 + radiation-treated tumor

To test the possibility that lymphocytes infiltrate to the armed dB7-treated tumor tissue, immunohistochemical staining for CD4<sup>+</sup> and CD8<sup>+</sup> on tumor tissue was performed. Combination treatment increased infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the center of the tumors compared with control or radiation. In particular, when radiation and armed dB7 were combined, increased infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells at day 7 was observed compared to the armed dB7 group (Fig. 6). To further examine whether expression of GM-CSF stimulates the recruitment of dendritic cells (DCs), the presence of DCs in the tumor tissues was analyzed. When radiation and armed dB7 were combined, IHC analysis showed heavier infiltration of CD11c<sup>+</sup> DCs at day 7 into the tumor sites compared with the armed dB7 group. These data suggest that radiation treatment enhanced the infiltration of armed dB7 into the tumor.

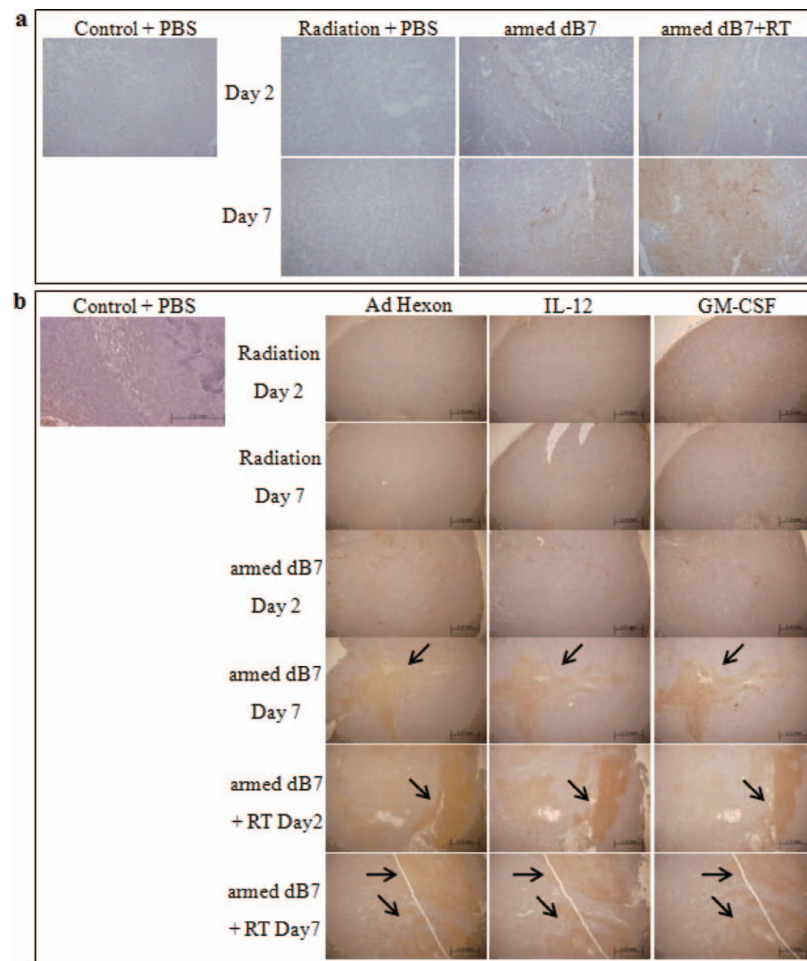


**Fig. 3.** Change in the level of induced apoptosis in HCa-I treated with radiation (■), dB7 alone (△), dB7 + radiation (▲), armed dB7 alone (◇), and armed dB7 + radiation group (◆) (a). Induction of apoptosis in HCa-I tumors treated with control + PBS, radiation + PBS, armed dB7 and armed dB7 + RT at day 20 in experiment of lung metastasis model (b). The maximum was 11.3% in radiation + PBS (■) at 8 h, and 35.4% in armed dB7 + radiation group (◆) at 24 h.

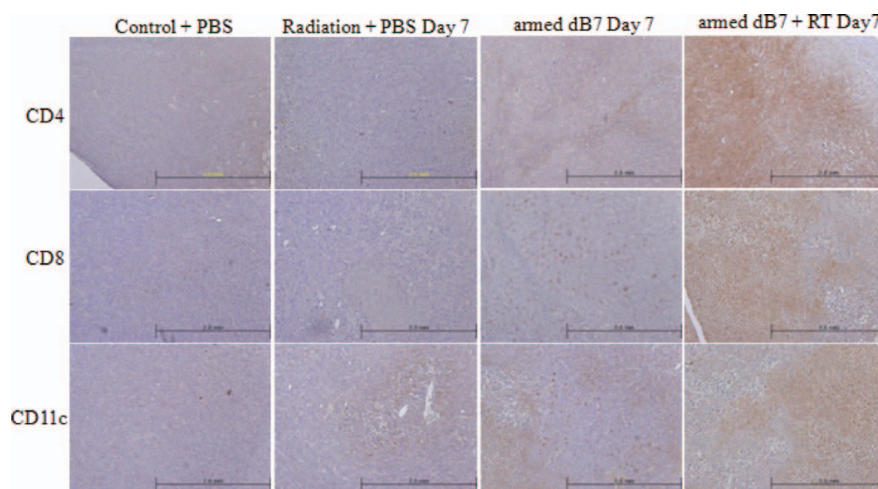


**Fig. 4.** CD31 expression in the tumor tissues treated with control + PBS, radiation + PBS, armed dB7 and armed dB7 + RT (a), change in microvessel density by time in CD31 stain (b), Positive nuclear immunostaining of Hif-1 $\alpha$  of HCa-I (c), the level of Hif-1 $\alpha$  positive cells of HCa-I (d). Hif-1 $\alpha$  expression was decreased significantly in a time-dependent in armed dB7 group and in armed dB7 + radiation group compared to radiation group.





**Fig. 5.** Ad-hexon immunostaining to assess the morphology of armed dB7 infected areas treated with control + PBS, radiation + PBS, armed dB7 and armed dB7 + RT at day 2, 7 (a). Expression of Ad-hexon, IL-12 and GM-CSF treated with control + PBS, radiation + PBS, armed dB7 and armed dB7 + RT at day 2, 7 (b). The expression of IL-12 and GM-CSF was presented in the same area of the expression of ad-hexon (arrows indicate; necrotic area).



**Fig. 6.** Tumor infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes and CD11c<sup>+</sup> mononuclear cells in control + PBS, radiation + PBS, armed dB7 and armed dB7 + RT groups at day 7. CD4<sup>+</sup>, CD8<sup>+</sup> and CD11c<sup>+</sup> expression was increased significantly in armed dB7 + radiation group compared to armed dB7 group and radiation + PBS group.

## DISCUSSION

Various attempts have been made to enhance the radioreponse of HCa-I. Inhibition of ERK by PD98059 showed an EF of 1.87.<sup>19)</sup> Farnesyltransferase inhibitor (FTI) and wortmannin showed an EF of 1.32 and 2.0.<sup>16,20)</sup> These results suggested that achieving enhancement by molecular targeting agents with this tumor is particularly difficult. However, in the present study, the increment of tumor radioresponse was the highest ever achieved, reaching an EF of 4.3, when an armed dB7 was combined with radiation treatment. These data showed that an armed dB7 greatly enhanced antitumor effect when combined with radiation treatment, which suggests a additive interaction between radiation and armed dB7.

Adenovirus-mediated therapy has been shown to suppress metastasis of primary tumors.<sup>21)</sup> The number of nodules in radiation treatment combined with an armed dB7 decreased in comparison to radiation. In the case of lung metastasis, the oncolytic virus alone was enough to inhibit metastasis to the lung. It suggests that IL-12 and GM-CSF play a minimal role in inhibiting lung metastasis in our model.

It might be possible that an armed dB7 increased the susceptibility of cells to radiation induced apoptosis. In previous studies, it has been reported that the level of apoptosis in the combination group was either not significantly increased or increased in an additive manner.<sup>16)</sup> However, in this study, the level of apoptosis in the combination group went beyond an additive effect. This result implies that an armed dB7 in combination with radiation has highly improved antitumor activity, possibly caused by increased adenovirus infection.

Thorne *et al.* have shown that virotherapy decreased microvessel density, which was consistent with enhanced antiangiogenic activity.<sup>22)</sup> Immunohistochemical staining showed that the CD31 expression was significantly decreased in the combination group when compared to the radiation alone group. This decrease might be due to the antiangiogenesis effects of IL-12 of an armed dB7. IL-12, which can inhibit angiogenesis *in vivo* by inducing interferon-gamma (IFN-gamma) and other downstream mediators.<sup>23)</sup>

Microvessel density is closely related to hypoxia condition. Hif-1 $\alpha$  has been regarded as a key factor in regulation of VEGF and other angiogenic factors. Immunohistochemical analysis of human tumor biopsies revealed that dramatic overexpression of Hif-1 $\alpha$  was seen in common cancers.<sup>24)</sup> and associated with tumor VEGF expression and vascularization.<sup>25,26)</sup> In addition, Benjamin *et al.* have shown that radiation increases Hif-1 $\alpha$  levels and activity, and that radiation-induced Hif-1 $\alpha$  activation induces the expression of angiogenic growth factors.<sup>27)</sup> In the present study, the level of Hif-1 $\alpha$  expression significantly decreased in the combination group when compared to the radiation alone

group. Furthermore, microvessel density was decreased time-dependently in the combination group, while the level of apoptosis in the combination group increased time-dependently. The anti-angiogenic activity of combination treatment likely results from both the suppression of tumor microvessels and the inhibition of Hif-1 $\alpha$ . Taken together, these data suggest that tumor vascular injury and inhibition of Hif-1 $\alpha$  activity might be involved in enhancing the anti-tumor effect observed in the combination group.

Radiation-induced cell death is also an immunogenic process that can potentially be exploited to stimulate tumor-specific immune responses.<sup>28)</sup> Preclinical studies combining dendritic cells with radiation therapy have shown increased tumor-specific CD8+ T cells and improved antitumor responses compared with radiation alone.<sup>29,30)</sup> Other preclinical study has shown improved tumor control and/or survival as a result of combining radiation with immune modulators such as IL-12.<sup>31)</sup> Immunohistochemical studies also showed a massive infiltration of CD4+/CD8+ T cells and CD11c+ into the tissues surrounding the necrotic tumor area after *in situ* delivery of armed dB7 and radiation treatment compared to each treatment alone. These observations demonstrate that local cytokine production could add to the already potent antitumor efficacy of oncolytic adenovirus and that the combined armed dB7 and radiation treatment can induce stronger tumor specific cellular immunity.

The mechanism by which cytokines of armed dB7 provoke greater apoptosis is not clear. IL-12 plays a critical role in cellular immune responses such as the induction of Th1-mediated CD4+ T-cell differentiation and the activation of natural killer cells.<sup>32)</sup> GM-CSF plays an important role in the activation and maturation of professional antigen-presenting cells (APCs) by up-regulating MHC molecules.<sup>33)</sup> In this study, cytokines might have increased induction of cellular immunity and therefore enhanced the antitumor effect. This, however, requires further investigation.

Overall, the present study showed that combination of an armed dB7 and radiation produce greater antitumor effect in terms of increased tumor response as well as decreased lung metastasis. The results also showed that induction of apoptosis and vascular damage might be involved as possible mechanisms. This novel combination treatment may have potential benefits in cancer treatment.

## ACKNOWLEDGEMENTS

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