A novel combination treatment of armed oncolytic adenovirus expressing IL-12 and GM-CSF with radiotherapy in murine hepatocarcinoma

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Directed by Professor Jinsil Seong

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ABSTRACT

A novel combination treatment of armed oncolytic adenovirus expressing IL-12 and GM-CSF with radiotherapy in murine hepatocarcinoma

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Background: Hepatic cancer is a challenging disease due to poor response to anticancer treatment as well as frequent metastasis. 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 murine hepatic cancer (HCa-I) model.

Methods: Hepatic carcinoma HCa-I showing high resistance to antitumor treatment was used. Tumor bearing syngeneic mice were treated with radiation, armed oncolytic virus Ad-ΔE1Bmt7 (dB7) expressing both IL-12 and GM-CSF (armed dB7), or combination of both. The adenovirus was administered by intratumoral injection 1×10^8 PFU per tumor in 50 $\mu\ell$ of PBS four times every other day. Tumor response to treatment was determined by a tumor growth delay (TGD) assay. Metastatic potential was evaluated by assessing spontaneous pulmonary metastasis in a lung metastasis model. To understand underlying mechanism, the level of apoptosis was examined as well as change in microvessel density and expression of immunonlogical markers: CD4+, CD8+ and Cd11c.

Results: 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, suggesting additional mechanism underlying

suppression of metastasis.

Conclusion: Combination treatment of radiation and armed dB7 showed increased antitumor response as well as decreased lung metastasis. Underlying mechanism seems to involve increased apoptosis, decreased tumor microvessel as well as increased immunological factors. This study showed that a novel combination treatment of armed oncolytic adenovirus expressing IL-12 and GM-CSF with radiotherapy was effective in suppressing both primary tumor and distant metastasis.

Key words: oncolytic adenovirus, interleukin 12, granulocyte-macrophage colony-stimulating factor, radiation, apoptosis, microvessel density, tumor growth delay

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I. INTRODUCTION

Hepatic cancer is a challenging disease. Curative treatment is limited due to locally advanced disease as well as frequent metastasis. The most patients are subjected to the palliative treatment. Unfortunately, the tumor response to current antitumor treatment is not satisfactory.[1]

Tumor-targeted viral therapy is an emerging treatment of cancer, which is based on the development and application of viruses with tumor-restricted replication potential. In particular, oncolytic adenovirus therapies, which use a unique method of tumor destruction, have been designed to eliminate malignancies by direct infection and lysis of cancer cells.[2,3] Armed oncolytic adenoviruses are currently being developed as novel antitumor therapeutics by creating armed oncolytic viral vectors with antitumor or immunomodulatory transgenes that may enhance viral-based therapeutic potential.[4]

Recently, a novel concept of radiation enhanced viral oncolytic therapy (ReVOLT) has been proposed. In ReVOLT, ionizing radiation increases the recovery of infectious adenovirus or HSV mutants from infected tumor cells/xenografts compared to nonirradiated tumors. Then radiation therapy induced changes in the cellular environment can be exploited by the virus and can aid in its ability to replicate and spread within the tumor.[5] Qian *et al.* showed that ionizing radiation increases adenovirus uptake and transgene expression in cells and colon cancer xenografts.[6] Also, in several *in vitro* and *in vivo* studies, combined use of radiation therapy with oncolytic viral therapy had synergistic activity against the tumor cell lines glioblastoma multiforme U87, human non small-cell lung cancer cell line H1299 and

prostate cancer cell lines LNCaP.[7-9]

Another new strategy was developed by combining radiation, cytokines and an oncolytic adenovirus. This strategy displayed antitumor effect by using 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 cellular immune responses of IL-12 and GM-CSF.

In this study, a novel combination treatment of armed oncolytic adenovirus expressing IL-12 and GM-CSF with radiation was investigated for antitumor and antimetastatic effect in murine hepatic cancer model.

II. MATERIALS AND METHODS

1. 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 for the care and use of laboratory animals and the methods of this study were 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. The tumors were generated by inoculating viable tumor cells into the muscles of the right thighs of the mice. Tumor cell suspensions were prepared as previously described.[10]

2. Viruses

The oncolytic adenovirus Ad-ΔE1Bmt7 (dB7) and armed oncolytic adenovirus Ad-ΔE1Bmt7 expressing both interleukin 12 (IL-12) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (armed dB7)

were provided by professor Chae-Ok Yun of the Yonsei Cancer Institute. The Ad-ΔE1Bmt7 adenovirus, generating an E1A and E1B double-mutant replication-competent adenovirus, was described previously.[11,12]

3. 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.

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 + IL12 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 clinical linear accelerator (Varian Medical Systems Inc., Palo Alto, CA, USA). When tumors were in the range of 7.5-8 mm in mean diameter, PBS or adenoviruses mixed with LipofectAMINE^{TM} and LipofectAMINE^{TM} Plus solution (Invitrogen Co., Carlsbad, CA, USA) at 2:6 ratio were administered intratumorally [$1*10^8$ plaque forming units (PFU) per tumor in 50 $\mu\ell$ 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 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 treated group by drugs only.[13]

4. Assay of lung metastasis

HCa-I, grown in thighs, develops spontaneous lung metastasis in 10 – 20 days after tumor implantation.[14] The antimetastatic potential in the 6 experimental groups was tested with the spontaneous lung metastasis model. Briefly, 1×10^7 HCa-I cells per mouse were administered to the muscles of the right thighs of the mice of a 7-8 week-old male C3H/HeJ mouse. When the tumor reached a diameter of 7.5-8 mm, mice were randomly assigned to one of six groups: control + PBS, radiation + PBS, dB7 alone, dB7 + radiation group, armed dB7 alone, and the armed dB7 + radiation group (five mice per group). 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 (×4).

5. Analysis of apoptosis

The level of induced apoptosis was evaluated in tissue sections for 6 experimental groups of control + PBS alone, radiation + PBS, dB7 alone, dB7 + radiation group, armed dB7 alone, and the armed dB7 + radiation group, 5 mice in each group. 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-㎛ sections were cut and stained with the ApopTag staining kit (Chemicon, California, CA, USA).[14] Apoptotic cells were scored on coded slides at 400X magnification according to the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells as apoptotic only when accompanied by apoptotic morphology. Apoptotic index is a percent number of apoptotic bodies per 1000 nuclei. 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.

6. Immunohistochemical analysis for mechanism study

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 method 3. Assay of tumor growth delay. Immunohistochemical staining was performed with 4-㎛, formalin-fixed, paraffin-embedded tissue samples. After incubating the slide sections attached to a silane-coating slide overnight at 37℃, the tissue sections were deparaffinized in xylene (3×10 min) and

rehydrated through a series of graded alcohols (100%, 95%, 90%, 80%, and 70%) to diluted water. The deparaffinized sections were then heated and boiled (10 min) by microwaving in a 0.01 M citrate buffer (pH 6.0) to retrieve the antigens. The antibodies used were at 4, 8, 12, 24 hr: a mouse monoclonal antibody against CD31 (PECAM-1) protein (557355; 1/100 dilution; BD PharMingen, San Diego, CA, USA) at 4℃ for overnight; anti-hypoxia-inducible factor 1alpha (Hif-1α) monoclonal antibody (400080; 1/100 dilution; Calbiochem/EMD biosciences, Darmstadt, Germany) at 4℃ for overnight. The antibodies used were at day 2, day 7: a mouse anti-adenovirus (Ad-hexon) monoclonal antibody (MAB8052; 1/100 dilution; Chemicon, Billerica, MA, USA) at 4℃ for overnight; a mouse monoclonal antibody raised against full length recombinant IL-12 of human origin (sc-74147; 1/100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4℃ for overnight; a rat monoclonal antibody raised against GM-CSF of mouse origin (sc-71165; 1/100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4℃ for overnight; a purified rat anti-mouse CD4+ antibody (550278; 1/100 dilution; BD PharMingen, San Diego, CA, USA) at 4℃ for overnight; a monoclonal antibody to CD8+ (AM05640PU-T; 1/100 dilution; Acris, Herford, Germany) at 4℃ for overnight; a monoclonal antibody to CD11c (AM05514PU-T; 1/100 dilution; Acris,

Herford, Germany) at 4℃ for overnight. After washing three times with PBS, sections were incubated with biotinylated link (LSAB2; Dako A/S, Glostrup, Denmark) for 20 minutes. They were then washed three times with PBS, treated with streptavidin-hrp (LSAB2; Dako A/S, Glostrup, Denmark) for 20 minutes, and again washed three times with PBS. 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*.[15] The areas of highest neovascularisation were identified as regions of invasive carcinoma with the highest numbers of discrete microvessels stained for CD31.

The immunohistochemical results for Hif-1α protein were classified on coded slides at 400X magnification. Hif-1α index is a percent number of Hif-1α - nuclear staining - positive cells per 1000 nuclei. Ten fields in non-necrotic areas were selected randomly across each tumor section, and in each field Hif-1α - nuclear staining - positive cells were expressed as a percentage based on the scoring of 1000 nuclei.

7. Statistical analysis

Results are expressed as mean ± 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.

III. RESULTS

1. Determination of optimal sequence of radiation and armed dB7 + radiation

First of all, the optimal sequence of radiation and armed dB7 radiation followed by armed dB7 (Prior combination), or armed dB7 followed by radiation (Post combination) was investigated. There was no difference in tumor growth delay between the two groups (Figure 1). Therefore, the following experiments adopted the sequence of radiation followed by armed dB7.

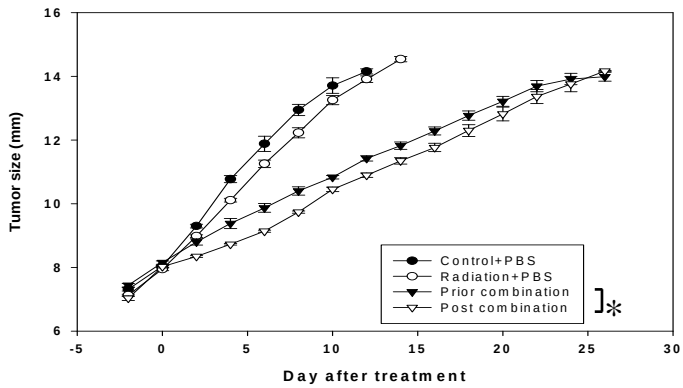

Figure 1. Tumor growth delay by different sequence of radiation and virus; radiation first group and virus first group.

* $p>0.05$ vs. Prior combination group.

2. Enhancement of tumor radioresponse by oncolytic adenovirus dB7 expressing IL-12 or GM-CSF

The time for tumor growth from 8 to 12 mm was 9.33 days and 11.33 days in radiation alone and in oncolytic adenovirus dB7 expressing IL-12 alone group (Figure 2. A.), respectively, which accords with 0.66 days and 2.66 days of the AGD, in oncolytic adenovirus dB7 expressing IL-12 alone and radiation alone group. When radiation was combined with oncolytic adenovirus dB7 expressing IL-12, the time for growth from 8 to 12 mm was 12.67 days and NGD was 2 days. Enhancement factor was 2.03 (Table 1). The time for tumor growth from 8 to 12 mm was 9.33 days and 10.67 days in radiation alone and in oncolytic adenovirus dB7 expressing GM-CSF alone group (Figure 2. B.), respectively, which accords with 0.66 days and 2 days of the AGD, in oncolytic adenovirus dB7 expressing GM-CSF alone and radiation alone group. When radiation was combined with oncolytic adenovirus dB7 expressing GM-CSF, the time for growth from 8 to 12 mm was 11.33 days and NGD was 0.66 days. Enhancement factor was 1 (Table 1).

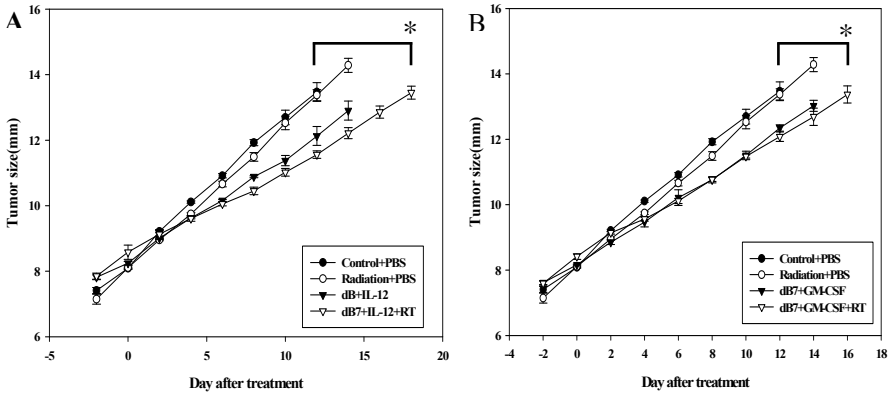

Figure 2. Effect of dB7+IL-12 or dB7+GM+CSF combined with radiation on the growth of HCa-I. dB7+IL-12 (A) or dB7+GM-CSF (B) increased the antitumor effect of radiation with an enhancement factor (E.F.) of 2.03 or 1.

* $p<0.05$ vs. Control + PBS 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 †	AGD‡	NGD§	EF‖
control + PBS	**8.67**			
radiation + PBS	**9.33**	**0.66**		
dB7 + IL-12	**11.33**	**2.66**		
dB7 + IL-12 + RT	**12.67**	**4**	**2**	**2.03**
dB7 + GM-CSF	**10.67**	**2**		
dB7 + GM-CSF + RT	**11.33**	**2.66**	**0.66**	**1**

* Mice bearing 8-mm tumors in the right thighs were given i.t. 1×10^8 PFU adenoviruses or 25 Gy local tumor irradiation. Groups consisted of 3 mice each.

† Mean. ‡ 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.

§ 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.

‖ EF was calculated as the ratio of NGD in mice treated with combination group to AGD in mice treated by radiation alone.

3. Enhancement of tumor radioresponse by armed oncolytic adenovirus

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 (Figure 3). 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 the enhancement was much more potent with the armed virus.

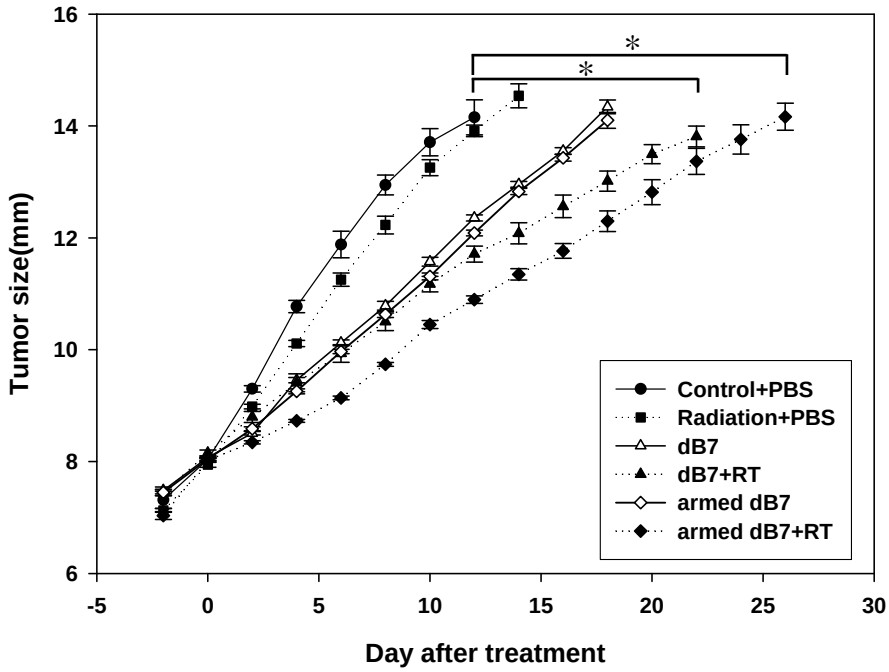

Figure 3. Effect of dB7 or armed dB7 combined with radiation on the growth of HCa-I. Treated with PBS (phosphate buffered saline) alone (●), radiation + PBS (■), dB7 alone (△), dB7 + radiation (▲), armed dB7 alone (◇), and armed dB7 + radiation group (◆). Significant growth delay is shown in armed dB7+ radiation group. 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.

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 †	AGD‡	NGD§	EF‖
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. 1×10^8 PFU‖ armed dB7 or 25 Gy local tumor irradiation. Groups consisted of 8 mice each.

† Mean ± SE. ‡ 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.

§ 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.

‖ EF was calculated as the ratio of NGD in mice treated with armed dB7 + RT to AGD in mice treated by radiation alone.

4. Suppression of tumor metastasis by armed oncolytic adenovirus

The murine HCa-I model presents early and frequent metastasis to the lung. Fifteen days after tumor transplantation, the average number of lung nodules was 8.4±0.6. The number of lung nodules significantly decreased to 1.4±0.4 in the dB7 + radiation group ($p<0.05$) and to 1±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±3.1 in the control group. The number of lung nodules was 2.1±0.7 ($p<0.05$) and 1.1±0.3 ($p<0.05$) in the dB7+ radiation and armed dB7 + radiation groups, respectively (Figure 4). These results suggest that both the oncolytic virus and armed oncolytic virus significantly suppressed tumor metastasis to the lung. A slightly better effect was observed with the armed virus, but the oncolytic virus seemed to play a major role in this setting.

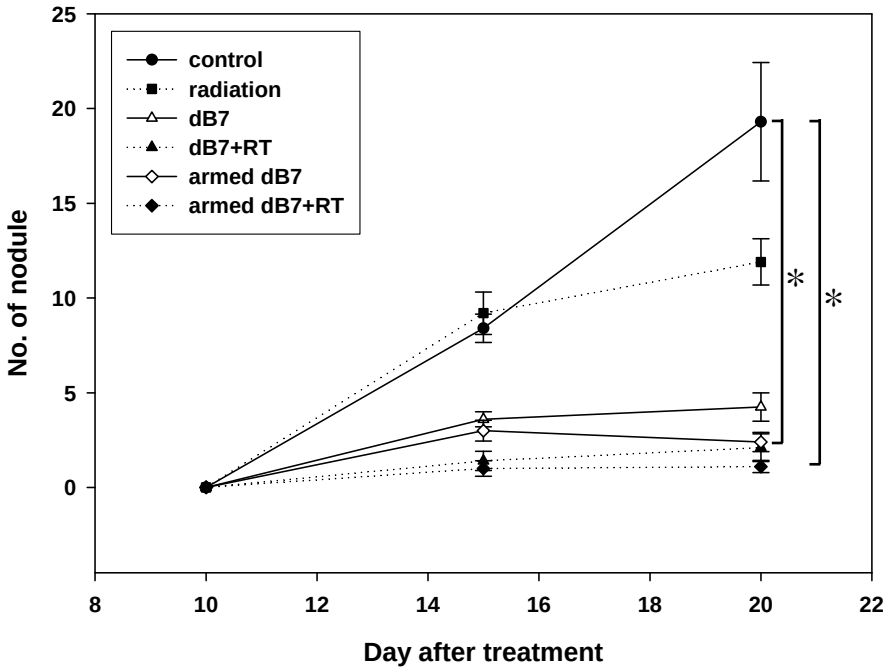

Figure 4. The number of metastatic lung nodules by the treatment in HCa-I. PBS (phosphate buffered saline) alone (●), radiation + PBS (■), dB7 alone (△), dB7 + radiation (▲), armed dB7 alone (◇), and armed dB7 + radiation group (◆). Significant decrease of lung nodules was shown in the dB7+radiation and the armed dB7 + radiation group.

* $p<0.05$ vs. Control + PBS group.

5. Enhancement of radiation-induced apoptosis by armed oncolytic adenovirus

To further explore the mechanism of the increased cell killing of an armed oncolytic adenovirus and radiation treatment, the level of induced apoptosis was examined during the first 24 hr.

With radiation + PBS alone, the peak level of induced apoptosis was 11.3% at 8 hr ($p<0.05$) and decreased to 8.4% at 24 hr ($p<0.05$) (Figure 5). 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 dB7 ($p<0.05$) 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 group. 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 + radiation group ($p<0.05$) and 35.4% in the armed dB7+radiation group ($p<0.05$) at 24 hr. These results suggest that the armed oncolytic adenovirus renders HCa-I more susceptibility to radiation-induced apoptosis.

To examine the outcome of the early increase in induced apoptosis, TUNEL staining was also done at 20 days after tumor transplantation. In both the

armed dB7 group and armed dB7+radiation group, a wild area of necrosis was shown (Figure 6). These results suggest that armed dB7 increased the level of apoptosis as a key mechanism at early period, ultimately leading to a significant degree of tumor cell necrosis.

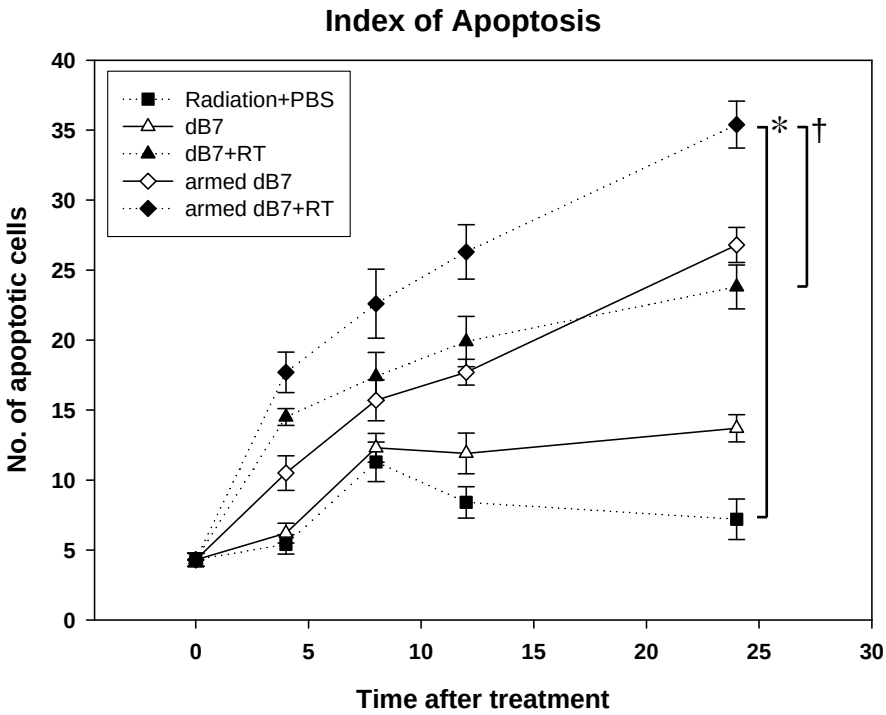

Figure 5. Change in the level of induced apoptosis in HCa-I treated with radiation + PBS (■), dB7 alone (△), dB7 + radiation (▲), armed dB7 alone (◇), and armed dB7 + radiation group (◆). Apoptotic index is a percent number of apoptotic bodies per 1000 nuclei. The maximum was 11.3% in radiation + PBS (■) at 8 hr and 35.4% in armed dB7 + radiation group (◆) at 24 hr. These data suggest that the armed oncolytic adenovirus renders HCa-I more susceptibility to radiation-induced apoptosis.

* $p<0.05$ vs. Radiation + PBS group.

† $p<0.05$ vs. dB7+RT group.

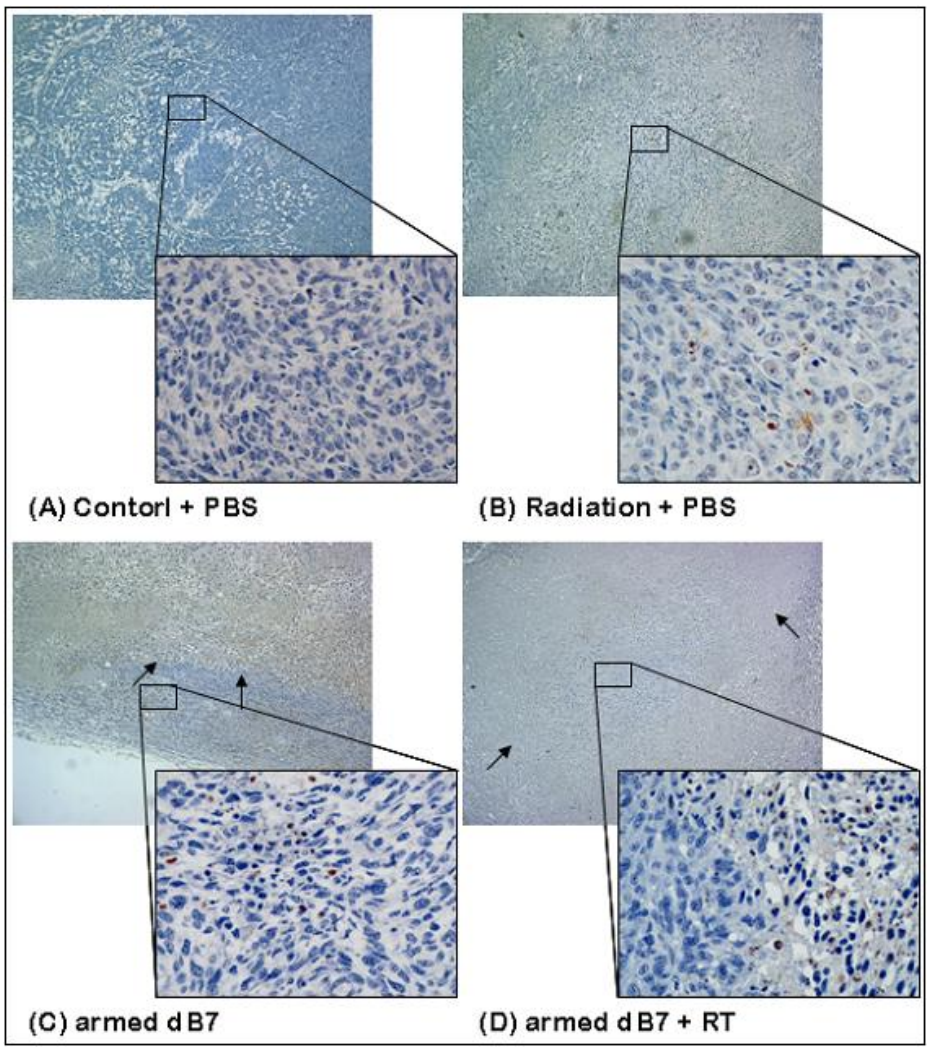

Figure 6. Induction of apoptosis in HCa-I tumors treated with PBS (A), radiation (B), armed dB7 (C) and armed dB7 + RT (D) at day 20. Wide area of necrosis is shown both in armed dB7 group and in armed dB7 +radiation group as indicated arrows.

6. Decreased microvessel density by armed oncolytic adenovirus and radiation

Although significant levels of apoptosis were induced in tumor cells by armed oncolytic adenoviruses and radiation, the microvessels can also be a target of the treatment. Therefore, the microvessel density was evaluated by an immunohistochemical technique with CD31 in each experimental group (Figure 7).

CD31 was overexpressed in the control group (23.3 ± 3.2; mean ± SE of microvessel density). In radiation group, CD31 expression remained at an almost similar level with only a slight decrease at 24 hr (18.7 ± 3.2 at 24 hr). However, in the armed dB7 group, CD31 expression showed a significant decrease in a time-dependent manner and ultimately reached 10% of the control level (1.7 ± 1.2 at 24 hr). CD31 expression in the armed dB7+radiation group appeared to be simply additive to the effects in the armed dB7 and radiation groups (Table 3). These data indicate that in armed dB7+radiation treatment, microvessel injury could be another mechanism of increased cell killing in addition to induced apoptosis of tumor cells.

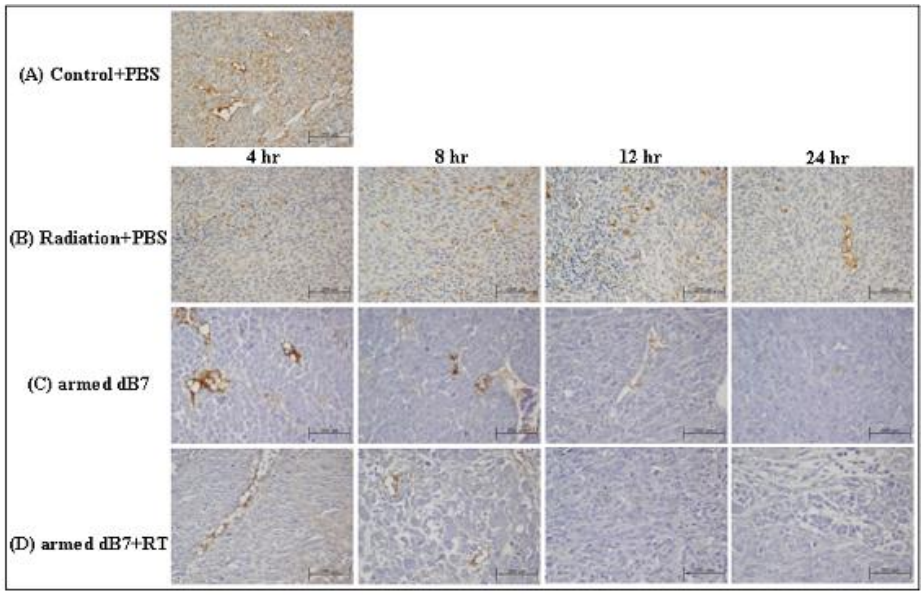

Figure 7. CD31 expression in the tumor tissues treated with control + PBS (A), Radiation + PBS (B), armed dB7 (C) and armed dB7 + RT (D). CD31 expression was decreased significantly in time dependent in armed dB7 group and in armed dB7 + radiation group comparing to radiation group.

Table 3. Change in microvessel density by time in CD31 immunohistochemical stain

Group	4 hr	8 hr	12 hr	24 hr
Control	23.3±3.2			
Radiation	21.7±2.5	21.3±2.1	20.7±2.1	18.7±3.2
armed dB7	11.7±2.1	7.3±3.2	4.7±2.08	2.7±0.6
Armed dB7+RT	10.7±2.1	6.3±2.7	3.7±2.08	1.7±1.2

7. Analysis of hypoxia condition by armed oncolytic adenovirus

In spite of the fact that significantly decrease of microvessel density was induced in tumor by armed oncolytic adenoviruses and radiation, the hypoxia condition can also be a target of the treatment. Therefore, hypoxia condition was examined by Hif-1α immunohistochemistry in each experimental group (Figure 8).

In radiation + PBS alone, the peak level of Hif-1α - nuclear staining - positive cells was 90.33% at 12 hr, which decreased to 55.67% at 24 hr. The expression of Hif-1α in armed oncolytic adenoviruses, significantly decreased compared to radiation + PBS. When radiation and armed oncolytic adenoviruses were combined, the expression of Hif-1α at 4 hr was increased in comparison to control + PBS alone or radiation + PBS alone. However, the expression of Hif-1α in armed oncolytic adenovirus and radiation was decreased time-dependently. These data represent that decrease of hypoxia condition might be involved in the mechanism of enhancement of antitumor effect in the combined group.

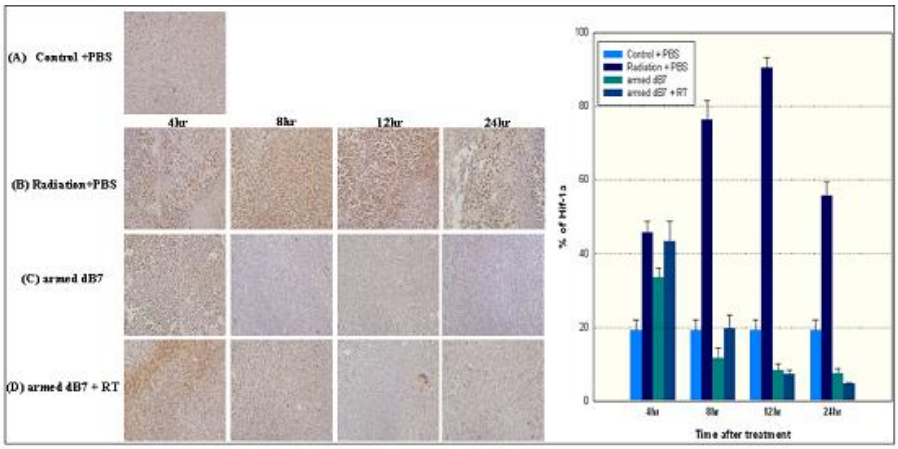

Figure 8. Positive nuclear immunostaing of Hif-1α of HCa-I treated with Control + PBS (A), Radiation + PBS (B), armed dB7 (C) and armed dB7 + RT (D). Hif-1α expression was decreased significantly in time dependent in armed dB7 group and in armed dB7 + radiation group comparing to radiation group.

8. Expression of adenoviral hexon protein shown by immunohistochemical staining in tumor tissue

Viral distribution and persistence within the tumor mass was then confirmed by immunohistochemistry stain using an antibody specific to adenoviral hexon protein. Marked increase in hexon-immunoreactivity was detected in wide areas of armed oncolytic adenovirus and radiation group at day 7 and undetectable in control + PBS and Radiation + PBS (Figure 9).

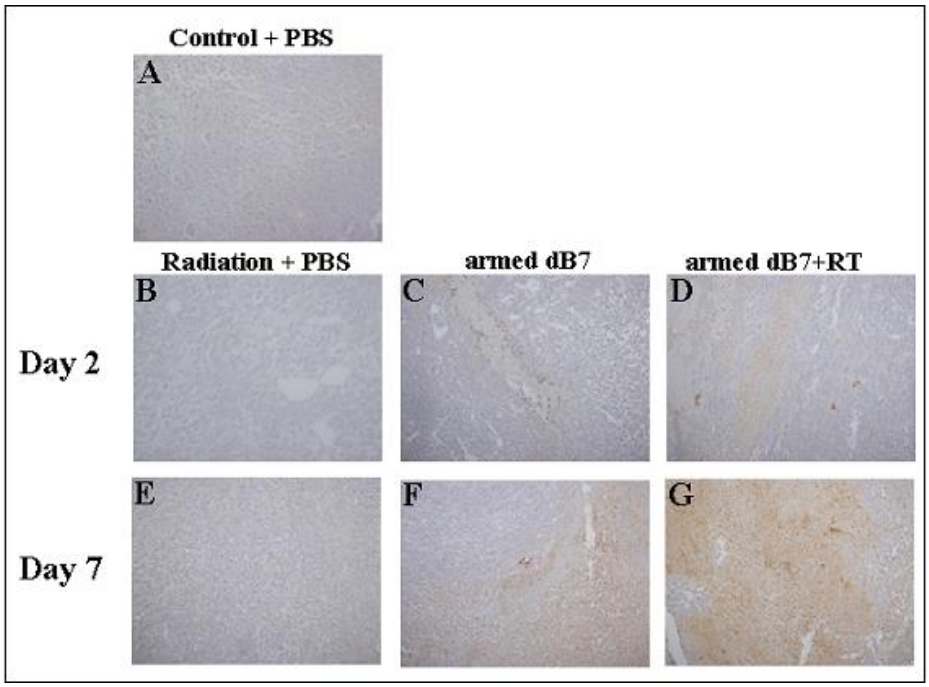

Figure 9. Ad-hexon immunostaining to assess the morphology of armed dB7 infected areas treated with control + PBS, radiation + PBS, armed dB7 and armed dB7 + RT at day 2, 7. Ad-hexon expression was increased significantly in day dependent in armed dB7 group and in armed dB7 + radiation group comparing to radiation + PBS group. (A; Control + PBS, B, C, D; Day2, E, F, G; Day7)

9. Expression of ad-hexon, IL-12 and GM-CSF in tumor tissue

To investigate the effects of IL-12 and GM-CSF on solid tumor, histological examination was carried out.

When radiation and armed oncolytic adenovirus were combined, ad hexon staining revealed in 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. These data show that the cytokine effect of IL-12 and GM-CSF in adenovirus increased the antitumor efficacy by armed oncolytic adenovirus (Figure 10).

In addition, with armed dB7 alone, the necrotic area was detected at day 7 (Figure 10. K, L, M). However, when radiation and armed oncolytic adenovirus were combined, the necrotic area was detected at day 2 (Figure 10. N, O, P). These data indicate that armed dB7 + radiation treatment was more efficient than armed dB7 alone treatment.

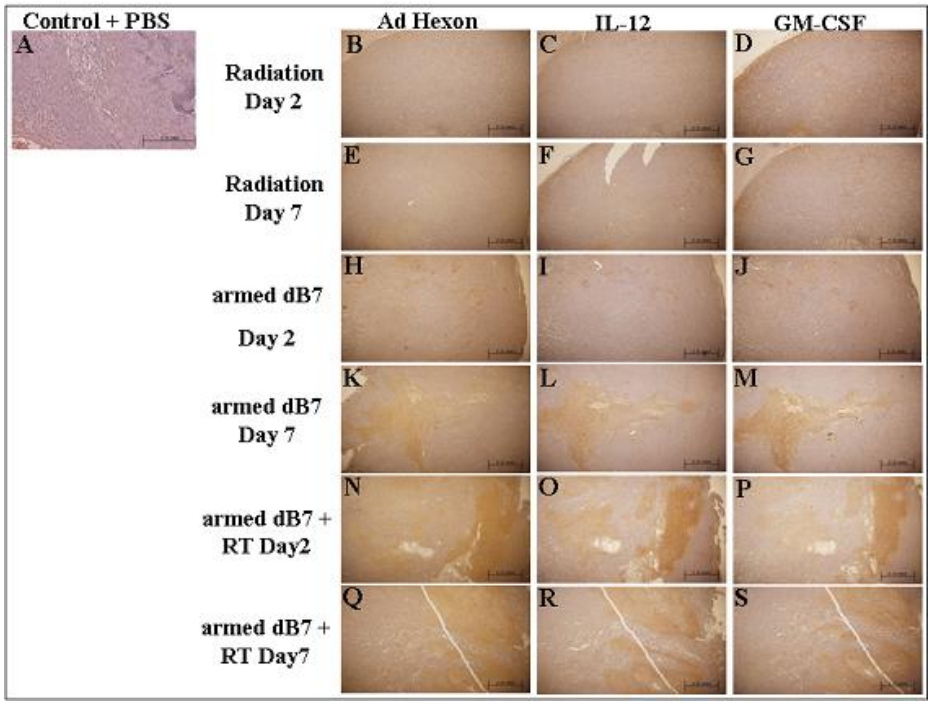

Figure 10. Expression of Ad-hexon, IL-12 and GM-CSF treated with Control + PBS, Radiation + PBS, armed dB7 and armed dB7 + RT at day 2, 7. The expression of IL-12 (R) and GM-CSF (S) was presented in same area of the expression of ad hexon (Q). (A; Control + PBS, B, C, D; Radiation Day 2, E, F, G; Radiation Day 7, H, I, J; armed dB7 Day 2, K, L, M; armed dB7 Day 7, N, O, P; armed dB7 + RT Day 2, Q, R, S; armed dB7 Day 7)

10. Increased $CD4^+$, $CD8^+$ 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 $CD4^+$ and $CD8^+$ on tumor tissue was performed. Positive staining was observed for anti-CD4 and anti-CD8. The results from tumors analyzed at day 7 after treatments (Figure 11). Immunohistochemistry of the tumors of mice treated with armed dB7 demonstrated infiltration of $CD4^+$ and $CD8^+$ T cells in the center of the tumors compared with control + PBS and radiation + PBS. In particular, when radiation and armed dB7 were combined, the expression of $CD4^+$ and $CD8^+$ at day 7 (Figure 11.D, H) was increased in comparison to armed dB7 alone (Figure 11.C, G). 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. With armed dB7 alone, IHC analysis showed more heavy infiltration of $CD11c^+$ DCs into the tumor sites compared with radiation + PBS. When radiation and armed dB7 were combined, the expression of $CD11c^+$ at day 7 (Figure 11.L) was increased in comparison to armed dB7 alone (Figure 11.K). These data suggest that radiation treatment significantly enhanced the infiltration of armed dB7 into the tumor.

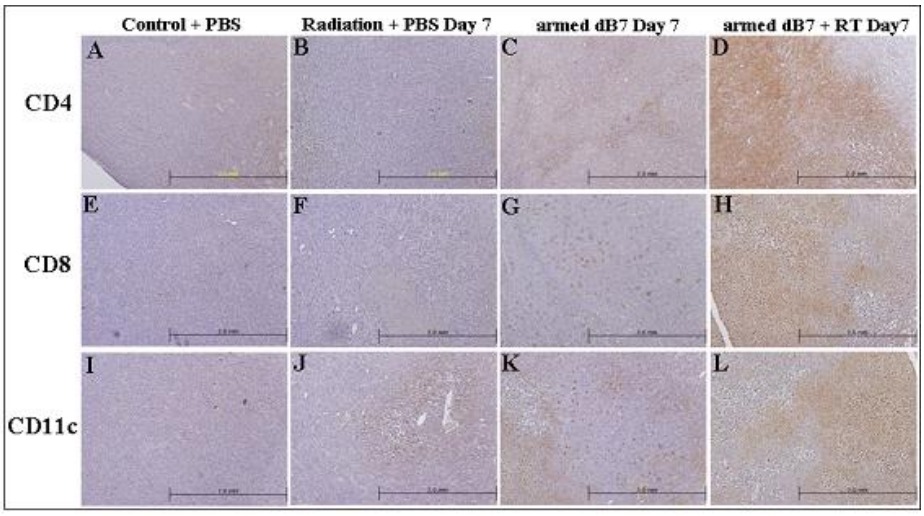

Figure 11. Tumor infiltration $CD4^+$ and $CD8^+$ lymphocytes and $CD11c^+$ mononuclear cells in Control + PBS, Radiation + PBS, armed dB7 and armed dB7 + RT at day 7. $CD4^+$, $CD8^+$ and $CD11c^+$ expression was increased significantly in armed dB7 + radiation group comparing to armed dB7 group and radiation + PBS group. (A, E, I; Control + PBS, B, F, J; Radiation + RT Day 7, C, G, K; armed dB7 Day 7, D, H, L; armed dB7 + RT Day 7)

IV. DISCUSSION

In this study, the present study showed that the armed oncolytic adenovirus Ad-ΔE1Bmt7 expressing both IL-12 and GM-CSF (armed dB7) greatly enhanced antitumor effect as well as suppressed lung metastasis compared to either treatment alone.

Oncolytic viral therapy has emerged as a new approach with potential utility in cancer treatment.[17] But the effectiveness of viral therapy in cancer treatment has been limited by its low infectivity and poor gene delivery to a solid tumor.[18,19] Recently, to overcome these limitations, Dilley et al. reported an oncolytic adenovirus CG7870 in combination with radiation therapy demonstrates synergistic enhancements of antitumor efficacy.[9] In addition, a few studies reported an armed therapeutic oncolytic adenovirus system is more efficacious than an oncolytic adenovirus therapy.[20,21] For these reasons, it is asked whether an armed dB7 could potentiate the antitumor efficacy of radiation *in vivo* and might be used as a treatment for cancer.

In previous studies, various attempts were tried to enhance radioresponse of HCa-I. Inhibition of ERK by PD98059 showed an EF of 1.87.[22] Farnesyltransferase inhibitor (FTI) and wortmannin showed an EF of 1.32 and 2.0.[23, 12] 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. On the other hand, the oncolytic adenovirus dB7 yielded an EF of 2.7. These data showed an armed dB7 greatly enhanced tumor radioresponse and suggested a possibly beneficial interaction between radiation and armed dB7.

Adenovirus-mediated therapy has been shown to suppress metastasis of primary tumors.[24] To investigate the effects of armed dB7 on tumor metastasis, a spontaneous lung metastasis model was used. Intratumoral injection of an armed dB7 inhibited pulmonary metastasis of HCa-I in mice. The number of nodules in radiation treatment combined with an armed dB7 decreased in comparison to radiation alone or an armed dB7 alone. However, there was no difference between dB7 alone and armed dB7 alone. It suggests that suppression of lung metastasis might not be by the immune response of IL-12 and GM-CSF but rather by maximum tumor cell kill of armed dB7.

It is considered a possible that an armed dB7 increased susceptibility of cells to radiation induced apoptosis. To elucidate how an armed dB7 increased enhancement of tumor radioresponse, the induction of apoptosis was examined by TUNEL analysis. In previous studies, it is reported that the

level of apoptosis in the combination group was no more than an additive effect, and that the level of apoptosis was not significantly increased by combination treatment.[12] However, in this study, the level of apoptosis in the combination group was more than an additive effect, and that the level of apoptosis was significantly increased by combination treatment. This result implies that an armed dB7 in combination with radiation has improved oncolytic activity caused by increased adenovirus infection.

Thorne et al. have shown that virotherapy decreased microvessel density, which was consistent with enhanced antiangiogenic activity.[25] To examine the potential mechanism of interaction between radiation and armed dB7, the impact of treatment on tumor microvessels were investigated using CD31 as a marker for microvessel density and high expression was founded in the tumor control group. Immunohistochemical staining showed that the CD31 expression was significantly decreased in the combination group when compared to radiation alone. It is correlated with the anti-angiogenesis effects of IL-12 of an armed dB7. IL-12 can inhibit angiogenesis in vivo by inducing interferon-gamma (IFN-gamma) and other downstream mediators.[26] Furthermore, microvessel density concern with hypoxia condition. Benjamin et al. have shown that radiation increase Hif-1α levels and activity, and then radiation-induced Hif-1α activation induces the expression of angiogenic

growth factors.[27] In order to investigate the correlation between hypoxia condition and microvessel density, hypoxia condition was examined by Hif-1α immunohistochemistry in each experimental group and it is founded that Hif-1α expression showed a significant decrease time-dependently in the combination group when compared to radiation + PBS alone. Zagzag et al. demonstrated a correlation between HIF-1α overexpression and induction of angiogenesis in human brain tumors by assessment of the formation of blood vessels.[28] This finding revealed that angiogenesis was promoted by HIF-1α induced expression of VEGF. Therefore, overexpression of HIF-1α may contribute to increased MVD and tumor progression. On the contrary, in the present study, microvessel density and hypoxia condition were decreased in combination group. In other words, the anti-angiogenic/anti-hypoxic activity of combination treatment likely results from both the inhibition of the angiogenic function of tumor microvessels and the inhibition of the hypoxia condition. Taken together, these data suggest that tumor vascular injury and recovery of hypoxia condition might be involved in enhancing the antitumor 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.[29] Preclinical studies combining dendritic cells with radiation therapy have

shown increased tumor-specific CD8+ T cells and improved antitumor responses compared with radiation alone.[30,31] Other preclinical study has shown improved tumor control and/or survival by combining radiation with immune modulators such as IL-12.[32] To further investigate whether the generation of tumor-specific T-cell-mediated immune responses was responsible for the observed antitumor effect in present study, immunohistochemical stain was carried out. 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 oncolytic adenovirus Ad-ΔE1Bmt7 expressing both IL-12 and GM-CSF and radiation treatment comparing to each treatment alone. These observations demonstrate that local cytokine production could add to the already potent antitumor efficacy of oncolytic adenovirus and the combined armed dB7 and radiation treatment can induce stronger tumor specific cellular immunity.

A limitation of this study is that the mechanism by which cytokines of armed dB7 provoke greater apoptosis is not clearly understood. IL-12 plays a critical role in cellular immune responses such as the induction of Th1-mediated CD4+ T-cell differentiation and activation of natural killer cells.[33] GM-CSF plays an important role in the activation and maturation of professional antigen-presenting cells (APCs) by up-regulating MHC

molecules.[34] Combined IL-12 and GM-CSF gene therapy induces strong cytotoxic T lymphocytes (CTL) reactions and antitumor effects from cellular immune responses.[35] 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.

V. CONCLUSION

Combination treatment of radiation and armed dB7 showed increased antitumor response as well as decreased lung metastasis. Underlying mechanism seems to involve increased apoptosis, decreased tumor microvessel as well as increased immunological factors. This study showed that a novel combination treatment of armed oncolytic adenovirus expressing IL-12 and GM-CSF with radiotherapy was effective in suppressing both primary tumor and distant metastasis.

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ABSTRACT (IN KOREAN)

마우스 간암에서 IL-12와 GM-CSF가 동시 발현하는 종양 선택적 살상 아데노바이러스와 방사선치료의 새로운 복합치료

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김 원 우

간암은 빈번한 전이뿐만 아니라 항암 치료에 있어서 예후가 낮아, 이를 극복하고자 끊임없이 연구되는 암이다. 이 연구에서는 인터루킨-12(IL-12)와 마크로파지 콜로니 자극인자 (GM-CSF)가 동시 발현하는 종양 선택적 살상 아데노바이러스와 방사선 치료의 병합치료 효과를 통해 쥐 간암인 HCa-I 에서의 효과를 조사하였다.

C3H/HeJ 웅성 마우스에 방사선 치료에 강한 내성을 보이는 HCa-I 세포주를 단측 대퇴부 근육 내에 이식하여, 종양의 평균 직경 8

mm일 때부터 IL-12 와 GM-CSF가 동시 발현하는 종양 선택적 아데노바이러스를 $1*10^8$ PFU를 이틀 간격으로 4 번 종양 내 주사하였고, 방사선 치료는 종양 평균 직경 8 mm일 때 대퇴부만을 고정하여 25 Gy를 조사하여, 종양 성장 지연 양상을 관찰하였다. 폐 전이능은 폐 전이 모델에서 자연발생적으로 폐로 전이되는 것을 관찰하여 평가하였다. 종양 선택적 아데노바이러스와 방사선과의 상호 기작을 조사하기 위하여, apoptosis와 미세혈관 밀도를 계수하였고, 관련된 분자들을 면역화학염색법으로 Hif-1α, adhexon, IL-12, GM-CSF와 CD4+의 발현양상을 조사하였다. 종양 성장 지연 분석에서 증강지수가 4.3 으로 IL-12 와 GM-CSF가 동시 발현하는 종양선택적 아데노바이러스가 종양의 방사선 감수성을 증가시키는 것으로 나타났고, 각 치료군에 비해서 폐 전이가 억제되는 것을 관찰하였다. 복합 치료시의 Apoptosis 지수는 각 치료군에 비해서 부가적 효과보다 높은 효과를 보였다. 복합 치료시, 미세혈관 밀도의 감소뿐만 아니라 apoptosis의 높은 유도는 복합치료의 기작일 것이다. 마우스 간암에서 IL-12 와 GM-CSF가 동시 발현하는 종양 선택적 아데노바이러스의 사용으로 인하여 방사선 치료의 항암 효과를 증진 시켰이며, 폐 전이 역시 억제하였다. IL-12 와 GM-CSF가 동시 발현하는 종양 선택적

아데노바이러스과 방사선 치료의 새로운 복합치료는 항암 치료에 있어서 치료 효율의 상승을 유도할 수 있을 것으로 사료된다.

핵심되는 말: 종양 선택적 아데노바이러스, IL-12, GM-CSF, 방사선, apoptosis, 종양 성장 지연

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