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ARTICLE

Combination Treatment of Murine Tumors by Adenovirus-Mediated Local B7/IL12 Immunotherapy and Radiotherapy

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Failure of local tumor control still poses a problem for radiotherapy and translates into reduced survival. Combining radiation with chemotherapy or other newer modalities has shown promising results. Immunological approaches to tumor therapy have found renewed interest due to improved insight into mechanisms involved in the immune response to tumors. In this paper, we studied tumor growth delay after various combination regimens of locally injected adenovirus constitutively expressing IL12 and B7.1 (AdIL12/B7.1) and fractionated radiotherapy in two non-immunogenic murine tumor models, 4T1 and B16.F10. Effects of radiation and virus infection on surface antigen expression in these tumor lines were assessed. Mechanisms of action of AdIL12/B7.1 were studied by conducting additional experiments with and without depletion of NK-cells and/or T-cells, and by cytotoxic T-lymphocyte assays, and immunohistochemical evaluation of tumor blood vessels. Both B7.1 and IL12 were effectively expressed in both irradiated and unirradiated 4T1 and B16.F10 tumor cells but did not add significantly to radiation-induced cell killing *in vitro*. However, local tumor infection by AdIL12/B7.1 after irradiation significantly increases the effectiveness of radiotherapy when applied after completion of radiotherapy. The mechanism appears to be complicated, involving a host of factors that included the ability of IL12 to activate T-cells and NK-cells and to inhibit angiogenesis and the ability of radiation to induce apoptosis or necrosis among tumor cells. These data support the combination of radiotherapy with adenovirus-mediated immunotherapy and suggest that the concept of adding genetic immunotherapy after radiotherapy in a combined regimen merits further study.

Key Words: **interleukin 12; IL12; costimulatory molecules; radiotherapy; immunotherapy; multimodality therapy.**

INTRODUCTION

Radiotherapy has been proven to be very effective in the local control of a wide variety of tumors. However, it also has serious limitations. In a significant number of cases, radiation can not significantly control local tumor growth. In addition, radiation is usually not curative if distant metastases occur, which is the major source of mortality for many cancers. Due to the ubiquitous presence of the immune system, immunotherapy offers one of the most promising new strategies for cancer treatment, possessing the potential of fighting both the primary tumor and distant metastases. However, little success was achieved so far despite widespread enthusiasm. One reason is that most of the early methods involve systemic administering of immunostimulatory cytokines such as IL2, IL12, or TNF-α. Such systemic administering approaches turned out to be highly toxic to normal tissues (1–3). Therefore, therapeutically effective doses can not be achieved in most cases. In addition, it is now realized that sustained, high-level expression of cytokines is necessary in the tumor cells to promote an adequate antitumor immune response.

With the advent of gene therapy, novel approaches that may significantly reduce the systemic toxicity of cytokines while preserving their antitumor potency are emerging. Genetic immunotherapy is one such promising strategy. Generally, it involves the transduction of an immmunostimulatory gene or genes into tumor cells

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directly by plasmids or viruses. The most significant advantage of such approaches is that the immunostimulatory cytokines are produced locally in the tumor cells. Therefore, much higher levels of the cytokines in the tumor can be achieved. In addition, the levels of the immunostimulatory cytokines can be sustained for days or weeks as they are constantly being produced by transduced cells. Indeed, there are numerous reports demonstrating the success of this strategy (4–7). Interleukin 12 (IL12) is among the most promising cytokines used in local tumor therapy. IL12 alone or in combination with B7.1 (8) in different gene therapy approaches showed exquisite potency in various nonimmunogenic model tumor systems, resulting in tumor rejection (9), with the effect being mainly T-cell mediated (10–12). Using a locally injected adenovirus vector that constitutively expresses IL12 and B7.1 potentially reduces the toxicity observed with systemic injection of IL12 (13). Combining these approaches with conventional radiation treatment thus appears warranted. To address the efficacy of this novel approach, we studied the tumor response after various combination regimens of locally injected adenovirus constitutively expressing IL12 and B7.1 and fractionated radiotherapy in two nonimmunogenic murine tumor models. In addition, experiments were also carried out to elucidate the mechanisms of the antitumor efficacy of this approach.

METHODS

Tumor models/cell culture. 4T1 is a well-characterized 6-thioguanine-resistant subline derived without mutagen treatment from a spontaneously arising mammary tumor in a BALB/cfC_3H mouse (14). It is maintained in DMEM and was obtained from Dr. Fred Miller, Michigan Cancer Foundation (Detroit, MI). It metastasizes spontaneously, preferably to the lungs and the liver. B16.F10 (ATCC, Manassas, VA) is a spontaneously arising subline of the B16 melanoma syngeneic with the C57BL/6 mice that also metastasizes spontaneously. It is maintained in DMEM. Both tumors are nonimmunogenic in the sense that no protective immunity against viable wild-type cells can be elicited by injection of lethally irradiated wild-type cells. When transplanted at 10^6 cells/animal in the respective syngeneic animals, tumors were established in 100% of mice for both cell lines. The 293 cells (ATCC) for virus propagation were maintained in DMEM. All cell culture media were supplemented with 10% FBS (Hyclone, Logan, UT) and 1% Penicillin/Streptomycin (Gibco/Life Technologies, Grand Island, NY).

Adenovirus vector. The adenoviral vector AdIL12/B7.1 (kindly provided by Dr. Frank L. Graham, McMaster University, Hamilton, Ontario, Canada) used in this study was described previously and is based on an Ad5 recombinant system (11). In short, both mIL12 subunit cDNAs were inserted in the E1 region and placed under control of the murine cytomegalovirus (MCMV) promoter. To achieve efficient expression of both IL12 subunits, an internal ribosome entry site was placed in between. An MCMV-B7.1 expression cassette was inserted in the E3 region. In the control virus (obtained from Dr. Peter Corry, William Beaumont Hospital, Detroit, MI), a CMV promoter driven green fluorescence protein (GFP) expression cassette was inserted in the E1 region. Viruses were propagated in 293 cells and purified by CsCl-banding according to a standard protocol (15) resulting in a particle/pfu ratio of 100.

Flow cytometry. Expression of B7.1 in irradiated or nonirradiated 4T1 and B16.F10 cells was examined by flow cytometry. Eighty percent confluent 4T1 cells were infected with AdIL12/B7.1 at an m.o.i. (multiplicity of infection) of 5 per cell for 5 h. Infection was performed alone, immediately preceding, or following a 6-Gy γ-irradiation (cesium irradiator, Sheperd and Associates, San Fernando, CA). Twenty-four hours after the beginning of infection, cells were detached with 0.05% trypsin (Gibco), resuspended in phosphate-buffered saline (PBS) with 3% normal rat serum and sequentially incubated with monoclonal biotin-labeled anti-mouse B7.1 (30 min) (Caltag, Burlingame, CA) and streptavidin–phycoerythrin (30 min) (Pharmingen) at 4°C with PBS washes in between. After final resuspension in PBS, samples were measured on a FACStar flow cytometer (Becton–Dickinson, Franklin Lakes, NJ). MHC I expression levels for both 4T1 and B16.F10 cells pre and post irradiation [1×18 Gy or 3×6 Gy (in 3 days)] were examined according to the same protocol, using monoclonal biotin-labeled anti-mouse MHC I (Pharmingen) as the primary antibody after the last irradiation.

Measurement of IL12 levels. IL12 levels in conditioned medium, serum samples and tumor extracts were detected with an mIL12 ELISA kit detecting the p70 heterodimer (R & D Systems, Minneapolis, MN) with a detection level of 2.5 pg/ml. Conditioned medium of uninfected and AdIL12/B7.1 infected 4T1 and B16 cells (m.o.i. 10/cell and 25/cell) was sampled 24, 48, and 72 h after infection with no medium changes in between. Serum was obtained from blood samples drawn from the tail vein before and 2 days after intratumoral AdIL12/B7.1 injection. For detection of intratumoral IL12, tumors were harvested without being infected and 2 days after local AdIL12/B7.1 infection (3×10^8 pfu in 50 μl). To assess the influence of irradiation on long term transgene expression, two 4T1-bearing Balb/c mice each were intratumorally injected with AdIL12/B7.1 and then irradiated (18 Gy single dose) or sham treated. These tumors were harvested after 9 days. Tumors were homogenized in PBS (1 ml/0.1 g tumor wet wt, with Complete protease inhibitor, Boehringer Mannheim), spun down and supernatant was collected for measurement.

Immunization studies. To study if lethally irradiated, AdIL12/B7.1 infected 4T1 cells can induce protective immunity, BALB/c mice were immunized subcutaneously with 10^6 lethally irradiated cells (70 Gy of γ-rays). The cells were treated with or without previous *in vitro* infection with AdIL12/B7.1 (m.o.i. of 20 per cell) prior to lethal irradiation. Animals were immunized once a week for 3 weeks. One week after the last immunization, 10^6 untreated 4T1 cells were transplanted in the right hind limb in both groups for observing tumor growth.

Tumor growth delay studies. Animal care and experimental procedures were in accord with institutional guidelines. About 10^6 cells in 50 μl PBS of either 4T1 or B16.F10 were transplanted in the right hind limbs of BALB/c or C57BL/6 mice respectively. Treatment was initiated when the tumors had reached a mean diameter of 5-7 mm. Each treatment group consisted of 6–10 animals and was treated with or without radiation and with or without a single intratumoral injection of AdGFP or AdIL12/B7.1. Adenoviral vectors were injected intratumorally either immediately after the first radiation fraction, immediately after the last fraction or—for 4T1 only—before the first fraction with the radiation treatment being postponed for 2 days. The virus dose injected was 3×10^8 pfu (in 50 μl PBS) except for the injection after the third radiation fraction in 4T1, when 3×10^7 pfu were used because of acute systemic toxicity, which, however, was only witnessed in Balb/c mice from one supplier. Tumors were irradiated locally with the mice being placed in plastic restrainers without anesthesia. The dosage used were 3×6 Gy for 4T1 and 3×11 Gy for B16.F10. Up to 12 mice were irradiated at a time, the legs placed in a 30 cm $\times$ 30 cm field with 1 cm water equivalent bolus on a 4MV linear accelerator (Varian, Palo Alto, CA) at a dose rate of 2 Gy/min. Growth curves are plotted as the mean relative treatment group tumor volume ± standard error (SE). Mean times to reaching four times initial tumor volumes (phase of exponential regrowth) for each group were calculated and compared using the Kruskal–Wallis and the two sided Mann–Whitney test (nonparametric).

Immunohistochemistry. C57BL/6 mice were transplanted with 10^6 B16.F10 cells in the right hind limb. After reaching a mean diameter of 5–7 mm, tumors were treated with injection of either 3×10^8 pfu AdGFP or AdIL12/B7.1. At 7 days after initiating treatment, tumors were harvested, deep frozen in liquid nitrogen with Tissue-Tek OCT compound

(Sakura, Torrance, CA) as embedding medium, sectioned and mounted. After blocking with serum (Jackson Immunoresearch, West Grove, PA), sections were stained according to a standard immunohistochemistry protocol (16) with rabbit-anti-mouse monoclonal anti-collagen type IV (Chemicon, Temecula, CA) and rat anti-mouse CD31 (Pharmingen, San Diego, CA) as primary antibodies and a biotinylated secondary antibody (Jackson Immunoresearch, West Grove, PA). Visualization of the immunoreaction was achieved by further incubation with a streptavidin–horseradish peroxidase conjugate and 3′-diaminobenzidine tetrahydrochloride Sigma (St. Louis, MO). After immunostaining, slides were counterstained with hematoxylin–eosin and mounted, and five vessel hot spots per tumor were assessed for vessel density at 400× magnification.

CTL assay. Activity of cytotoxic T-lymphocytes (CTL) was assessed with a modified standard chromium-release assay (17, 18). Seven days after intratumoral injection of AdIL12/B7.1, AdGFP or normal saline, spleens of three animals each were harvested, pooled and disaggregated. Erythrocytes were lysed with ammonium chloride (3 min, 37°C) and T-cells were purified by two rounds of Nylonwool (Robbins Scientific, Sunnyvale, CA) separation. For restimulation, 10^7 T-cells/well were cocultivated with 10^6 target cells/well (4T1 or B16.F10, irradiated with 200 Gy to prevent overgrowth) in 6-well plates with c-RPMI lymphocyte medium (440 ml RPMI 1640, 100 u/100 mg penicillin/streptomycin, 2 mM l-glutamine, 55 mM 2-mercaptoethanol, 10% FCS, 10 mM Hepes) with or without 100 U/ml mIL2 for 5 days. Afterwards, the cell suspension was harvested and mononuclear cells were separated by centrifugation over a Histopaque 1083 gradient (Sigma). For the effector assay, 4×10^6 target cells (4T1, B16.F10 or Yac-1) were labeled with 150 mCi sodium ^{51}chromate for 70 min. Finally, 10^4 target cells were cocultivated with effector cells in varying effector/target ratios in triplicates in U-bottom 96-well plates for 4 h at 37°C. Minimum (no effectors) and maximum (1% Triton solution) release as well as the release in each effector/target well were determined by adding 100 ml of supernatant after centrifugation to 100 ml of scintillation fluid in a 96-well plate of a Wallac beta-counter. Specific lysis was determined as

$$\%\ \text{spec lysis} = (\text{release of sample} - \text{spont release})/(\text{max release} - \text{spont release}).$$

Spontaneous release was always <15% of the maximum release. Each experiment was repeated with similar results.

Studies in nude mice and NK-depletion. About 10^6 cells of either 4T1 or B16.F10 were transplanted in the right hind leg of Balb/c nu/nu mice (Taconic). Tumors in the range of 5–7 mm in diameters were established in 7–10 days. AdGFP or AdIL12/B7.1 (3×10^8 pfu) were then injected intratumorally to study the therapeutic effect of the virus in a T-cell-depleted host. The role of NK cells was studied by use of antibody-mediated NK depletion in nude mice. To asses NK-mediation of AdIL12/B7.1 effects, a group of animals was NK-depleted with the polyclonal antibody antiasiolo GM1 (Wako Chemicals, Richmond, VA). Fifty microliters of antibody stock solution (titer 1:1000) was injected intravenously in 150 µl H_2O on day –2 and 25 µl in 75 µl H_2O on day –1 and +1, +4, and +7 before and after the virus infection for the duration of the experiment. Success of depletion was assessed after two antibody injections with a splenocyte cytotoxicity assay as described above (17) after NK-activation by ip injection of 100 µg Poly I:C (Sigma) 18 h before spleen harvesting. However, in this case, no Nylonwool separation was performed and the NK-sensitive Yac-1 cell line (ATCC) was used as the target. Two spleens of antibody-treated and untreated animals each were pooled. NK activity was reduced to <2% as assessed by this assay.

RESULTS

In Vitro Gene Expression in 4T1 and B16.F10 Cells after Adenovirus Infection and/or Ionizing Radiation

The immunophenotype for the two tumor lines used is different. While both are negative for B7.1, 4T1 is positive for MHC-I while B16.F10 is negative for MHC I (Fig. 1A). Both B7.1 and IL12 were effectively expressed in AdIL12/B7.1 infected 4T1 cells grown *in vitro* with 40–60% of cells positive for B7.1 24 h after infection (Fig. 1A) and a maximal IL 12 concentration of 4.5 µg/ml in the supernatant 72 h after infection as seen in Fig. 1B (control: <8 pg/ml, the detection limit). The infection efficiency was lower for B16.F10 with 2–4% of cells being B7.1 positive 24 h after infection (Fig. 1A). The maximal IL12 concentration (Fig. 1B) was 1 ng/ml in the supernatant at 72 h after infection (control: <8 pg/ml). To assess whether radiation can enhance adenovirus medi-

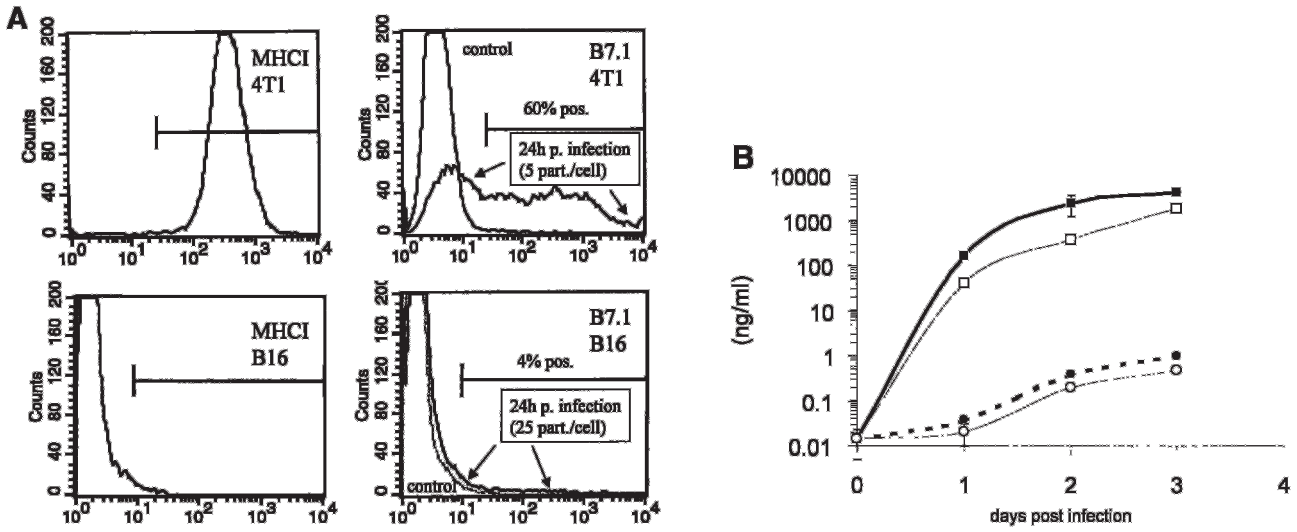

FIG. 1. (A) FACS analysis of the expression of MHCI (in noninfected and nonnirradiated cells) and B7.1 (in noninfected and AdIL12/B7.1 infected cells) on 4T1 and B16.F10 cells. The *x*-axis represents fluorescence intensity while the *y*-axis represents cell counts. B7.1 expression was measured before and 24 h after infection with AdIL12/B7.1 (m.o.i. 5/cell). (B) Concentration of IL12 in cell culture supernatant of 4T1 and B16 cells at 24, 48, and 72 h after infection with AdIL12/B7.1 (m.o.i. 10 or 25/cell): (□) 4T1, m.o.i. 10/cell; (■) 4T1, m.o.i. 25/cell; (○) B16, m.o.i. 10/cell, (●) B16, m.o.i. 25/cell.

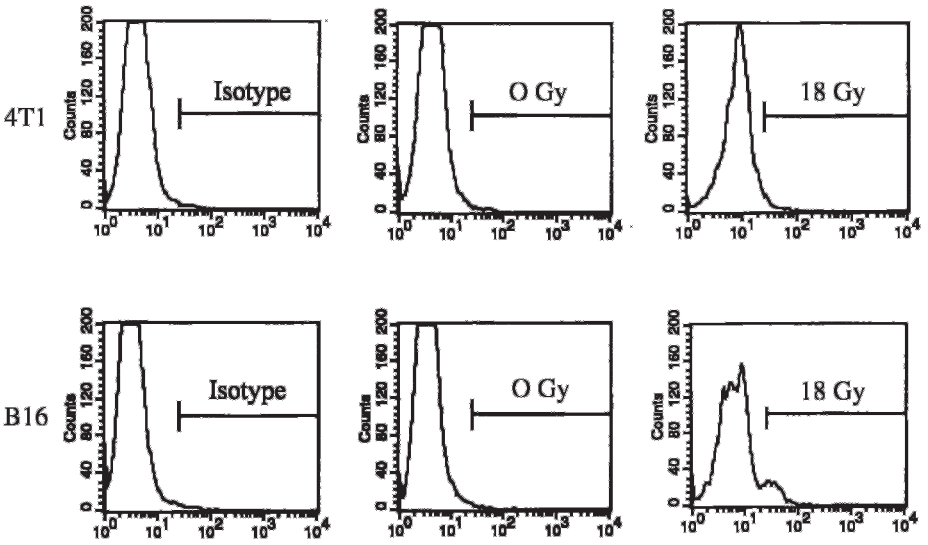

FIG. 2. FACS analysis of the expression of Fas Ligand on 4T1 and B16 cells 48 h after sham treatment or irradiation with 18 Gy. The two left panels represent controls with isotype antibody controls.

ated transgene expression. B16.F10 and 4T1 cells were irradiated at 0 and 18 Gy and immediately infected with AdIL12.B7. IL12 and B7 expression were examined at 48, 72, and 96 h after expression. Similar expression levels for both genes were obtained in both the nonirradiated and irradiated groups (data not shown). Another important observation is the lack of interaction between AdIL12.B7.1 and radiation in killing the 4T1 or B16.F10 cells *in vitro*. Adenovirus infection of either cell line did not cause any increased cell kill by radiation as evaluated by clonogenic assay performed as described in (19). Thus, it is unlikely that adenoviral toxicity will play any major role with respect to cell kill when combined with radiation at the dosage we adopted in this study.

Additional experiments were also conducted to examine whether radiation alone can alter expression of B7.1 and Fas-ligand genes. When irradiated with doses of 6–18 Gy after infection, no difference in B7.1 expression was observed when compared to unirradiated cells. Fas-ligand was upregulated several fold after a single dose of 18 Gy in the bulk of both B16.F10 and 4T1 cells (Fig. 2). In addition, a fraction of B16.F10 cells demonstrated more than 10-fold increase in Fas-L expression.

Significant Enhancement of Radiotherapy by Adenovirus-Mediated IL12 and B7.1 Gene Expression

IL12 levels were below detection limit (2.5 pg/ml in lysate) in untreated tumors and reached a mean of 15.5 and 6.5 ng/g in 4T1 and B16.F10 tumors, respectively, 2 days after intratumoral injection of 3×10^8 pfu of AdIL12/B7.1. Therefore, significant levels of IL12 can be achieved *in vivo* by AdIL12/B7.1 injection. To see if ionizing radiation can enhance adenovirus mediated IL12 gene transduction, IL12 were measured up to day 9 after AdIL12/B7.1 injection with or without immediate irradiation (18 Gy). No significant difference in IL12 expression profiles was detected between the two groups of animals. In both groups, the level of IL12 in the tumor was negligible at day 9 postinjection, indicating that the combination of AdIL12/B7.1 injection and irradiation did not result in prolonged transgene expression due to potential radiation-aided integration of the virus into the host genome.

Figures 3 and 4 show the results of growth delay experiments after various combinations of radiation and intratumoral vector injection *in vivo*. AdGFP injection does not cause any growth delay in either tumor line. As expected, treatment with fractionated radiation and AdGFP injection resulted in a distinct, statistically significant ($P < 0.01$) growth delay of the tumors when compared with nonirradiated, AdGFP injected controls at the respective dose levels (3×6 Gy and 3×11 Gy) for both tumor models. AdIL12.B7.1 injection alone caused significant growth delay in both tumor lines. Most importantly, all combination treatments, with the exception of the 4T1 group in which radiotherapy was initiated 2 days after virus injection, were significantly ($P < 0.05$ for 4T1 and $P < 0.01$ for B16.F10) superior to radiation plus AdGFP. For both models, injecting AdIL12/B7.1 intratumorally immediately after the last radiation fraction was more effective than any other treatment schedule, especially when compared with the addition of AdIL12/B7.1 after the first fraction or postponing radiotherapy to 2 days after the virus injection. In the case of B16.F10 it was clearly superadditive in terms of growth delay and statistically significantly superior ($P < 0.05$). Even though the virus dose had to be reduced by a factor of 10 for this specific group in the

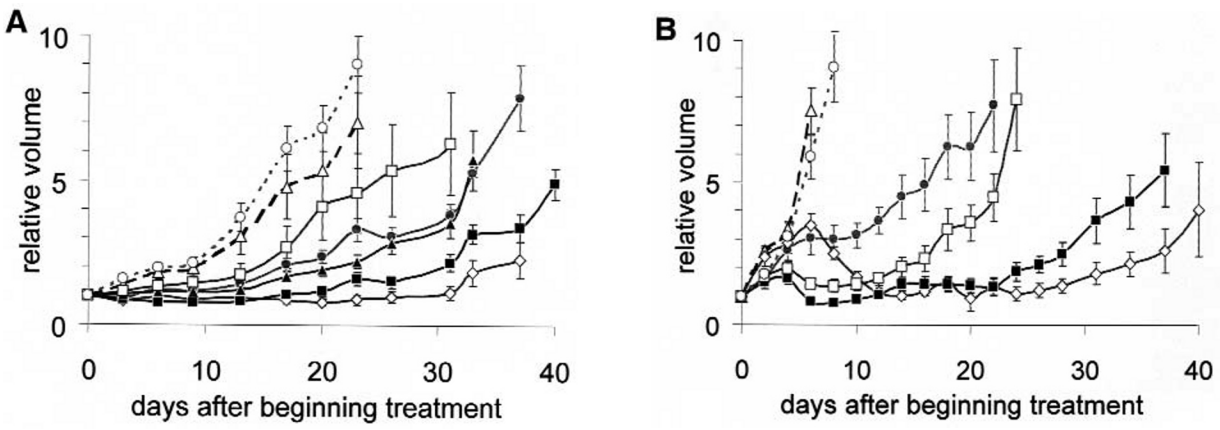

FIG. 3. Mean relative tumor volumes [±standard error (SE)] for different combinations of radiotherapy and adenovirus gene therapy in (A) 4T1 tumors in Balb/c and (B) B16.F10 tumors in C57BL/6 mice. (△) Untreated control, (○) injection of AdGFP on day 7 after transplantation (a. Tx.), no radiotherapy (RAD); (□) injection of AdIL12/B7.1 on day 7 a. Tx., no RAD; (●) initiation of RAD on day 7 a. Tx., injection of AdGFP after the last (third) RAD fraction; (■) initiation of RAD on day 7 a. Tx., injection of AdIL12/B7.1 after the first RAD fraction; (◇) initiation of RAD on day 7 a. Tx., injection of AdIL12/B7.1 after the last (third) RAD fraction; (▲) injection of AdIL12/B7.1 on day 7 a. Tx., initiation of RAD on day 9 a. Tx. The virus dose injected was 3×10^8 pfu (in 50 μl PBS) except for the injection after the third radiation fraction in 4T1, when 3×10^7 pfu were used.

4T1 bearing Balb/c mice, this sequence was still the most effective, also strongly suggesting a superadditive effect with respect to growth delay. Since initiating virus injection before radiotherapy appears to be inferior in 4T1 tumors, it was not studied in B16.F10. Although several complete responses were observed, all tumors finally regrew. Figure 3 shows the group means of relative tumor growth ± standard error (SE), while Fig. 4 shows the group means ± SE of the time it took the individual tumor to reach 4 times the initial treatment volume. As assessed by inspection, the combined treatments were tolerated without any increase in local toxicity (skin reaction) in the radiation + virus arms. However, in the radiation + AdIL12/B7.1 group, systemic toxicity was observed during the first week after virus injection, manifesting itself as weight loss and apathy. Over 90% of the symptomatic mice recovered after 1 week. In the animals injected with AdIL12/B7.1 we also observed splenomegaly (mean splenic weight of 0.7 g in AdIL12/B7.1 injected vs 0.07 g in uninjected animals). This is consistent with an earlier report (20).

A Complex Array of Mechanisms/Factors Contributed to the Observed Antitumor Effect

To decipher the mechanisms of the antitumor effects observed in the treatment regimens, a series of experiments were carried out. In the first experiment, immunization with AdIL12/B7.1 infected 4T1 cells resulted in a distinct growth delay over immunization with noninfected cells when animals were challenged with 10^6 untreated 4T1 cells 1 week after the last of three weekly immunizations (Fig. 5). This result indicates that

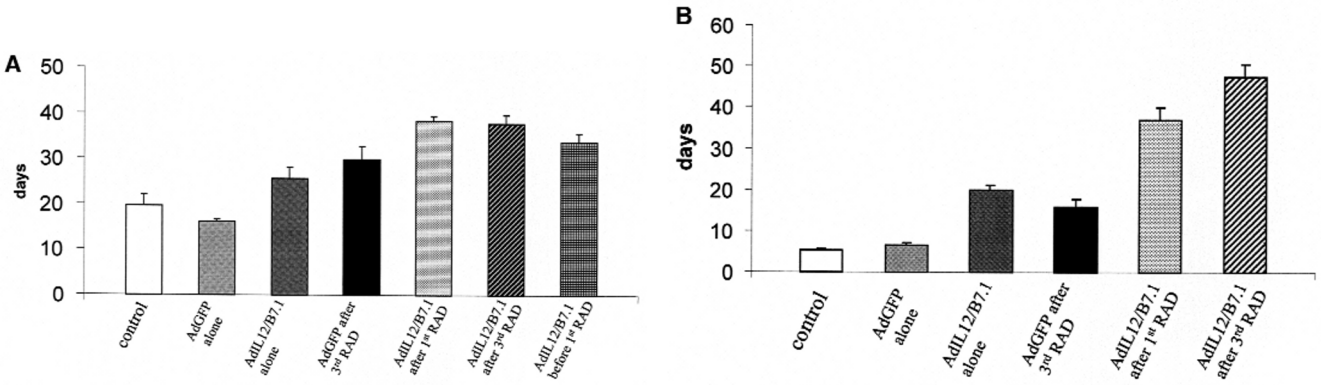

FIG. 4. Time to reach four times the initial treatment volume for different combinations of radiotherapy and adenovirus gene therapy in (A) 4T1 tumors and (B) B16.F10 tumors (group means ± SE).

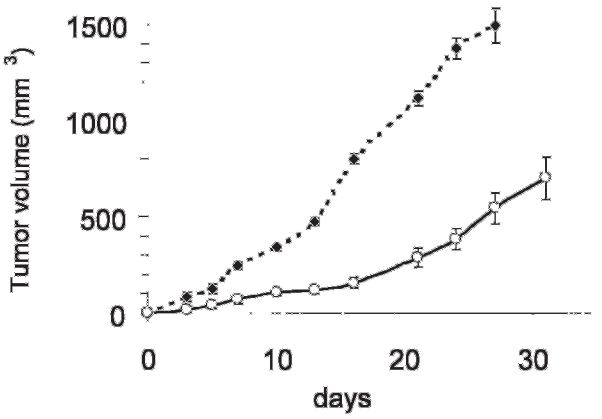

FIG. 5. Tumor growth after transplantation of 10^6 vital, wild-type 4T1 tumor cells with previous weekly subcutaneous immunization (three times) with lethally irradiated 4T1 cells that were either (♦) unmanipulated or (○) infected with AdIL12/B7.1 (group means ± SE).

immunological memory (possibly CTL activity although no CTL assay was performed in this case) was stimulated by the AdIL12.B7.1 infected 4T1 cells when compared with immunization with uninfected cells.

To further clarify the role of the T cells, experiments were carried out in nude mice bearing 4T1 and B16.F10 tumors. Both tumor lines grew faster than in their respective syngeneic hosts (Fig. 6), consistent with a role of T cells in delaying tumor growth even in those formally nonimmunogenic tumor models. In both B16.F10 and the 4T1 lines, the injection of AdIL12/B7.1 still resulted in significant growth delay ($P < 0.05$) when compared to AdGFP injection. Although not unequivocally assessable because of the difference in tumor growth rate between immunocompetent and nude animals, the effect, however, seemed to be diminished in nude mice when compared to immunocompetent mice (Fig. 6). This is especially pronounced in 4T1, where even the treatment group grew faster than the control in immunocompetent mice. Viral injection caused a more pronounced growth delay in B16.F10. As the absence of T cells did not completely abolish the effects of the adenovirus in either group, a further group was included in nude mice experiments with their NK cells depleted by an anti-NK antibody. Results indicate that NK-depletion in nude mice further reduced the therapeutic effect of AdIL12/B7.1 in B16.F10 significantly ($P < 0.05$). However, it is still not completely abolished. In 4T1, there also appears to be a further reduction in growth delay which, however, is statistically borderline ($P = 0.07$). Therefore, it appears that both T cells and NK cells played important roles in AdIL12/B7.1 mediated tumor growth delay for B16.F10 but neither is completely accountable for the total effect. While depletion of both T and NK abolished the therapeutic effects in the 4T1 model, it did not completely abolish the therapeutic effects in the B16.F10 model.

To further elucidate the role of T and NK cells, CTL assays were conducted using splenocytes from AdIL12/B7.1 treated, syngeneic tumor bearing mice. The splenocytes were incubated with irradiated tumor cells for re-stimulation and subsequently tested for CTL activities against the tumor cells using the ^{51}Cr release assay. Although T-cells were necessary for maximum IL12 therapeutic effects, neither lymphocyte proliferation nor CTL response could be detected against naïve B16.F10 or 4T1 tumor cells in animals bearing the respective tumors 7 days after in control or AdIL12/B7.1 treated mice if IL2 is not added to the re-stimulation process. When IL2 was added during the restimulation (Fig. 7), splenocytes of B16.F10 tumor bearing C57BL/6 mice from control and AdGFP injected animals proliferated and showed activity against B16.F10 cells. However, the activity was also observed against Tramp-C cells (21), a prostate carcinoma line syngeneic with C57BL/6 and Yac-1 (an NK- and LAK sensitive cell line) in splenocytes from the C57BL/6 mice, thus implicating nonspecific LAK activity instead of cell specific cytotoxic T-cell response. Most intriguingly, splenocytes from AdIL12/B7.1 injected C57BL/6 mice

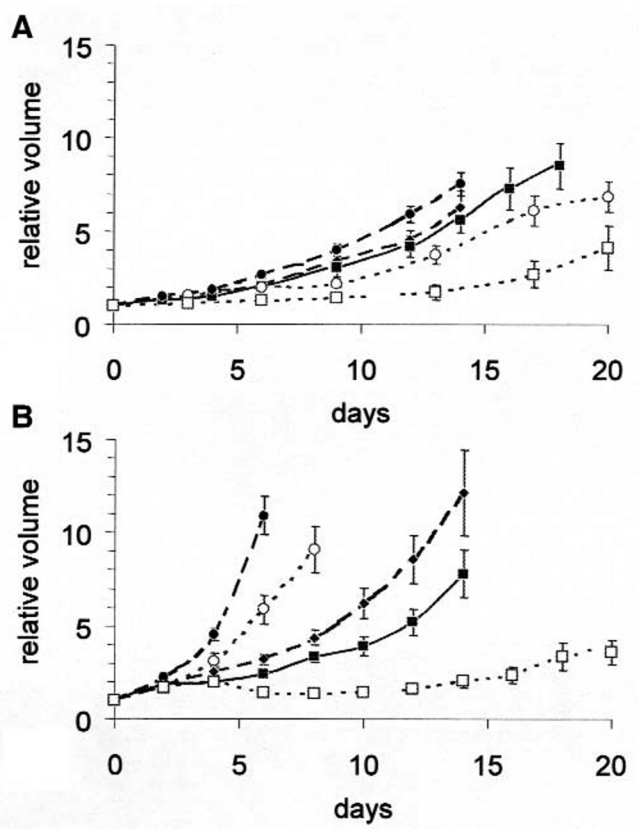

FIG. 6. Mean relative tumor volumes (±SE) for (A) 4T1 tumors or (B) B16.F10 tumors in Balb/c nu/nu mice after intratumoral injection of AdGFP or AdIL12/B7.1, with or without previous NK-cell depletion. (●) AdGFP, (■) AdIL12/B7.1, (♦) AdIL12/B7.1 in NK depleted animals. For comparison, the growth curves for tumors treated with AdGFP alone (○) and AdIL12/B7.1 alone (□) in the respective immunocompetent hosts (Balb/c or C57BL/6 mice) are also plotted.

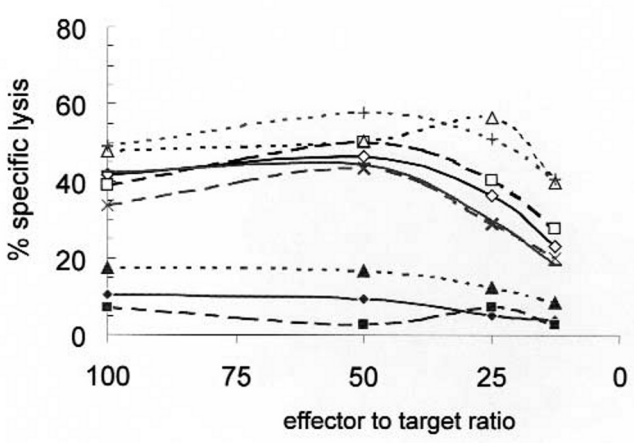

FIG. 7. CTL response of splenocytes from differently treated, B16 tumor-bearing C57 mice against B16.F10, Tramp or YAC1 cells. Splenocytes of untreated control animals against B16.F10 (no symbol), Tramp (X), or YAC1 (+). Splenocytes of animals injected intratumorally with AdGFP against B16.F10 (◇), Tramp (□), or YAC1 (△). Splenocytes of animals injected intratumorally with AdIL12/B7.1 against B16.F10 (◆), Tramp (■), or YAC1 (▲).

bearing B16.F10 still showed no response (neither proliferation, nor any CTL activity) with or without IL2 addition, indicating a suppressive rather than the expected stimulatory effect on T cells by the IL12/B7.1 treatment (Fig. 7).

As a sign of some immune activity against melanocyte antigens, depigmentation of AdIL12/B7.1 treated C57BL/6 mice was observed, mainly locally. In a few cases mild depigmentation was also noticed several weeks after treatment at distant sites.

As IL12 has also been indicated to possess potent antiangiogenesis effects (22, 23), vascular density was examined in B16 tumors that were treated with AdIL12/B7.1. Frozen sections of B16.F10 tumors 7 days after treatment with either AdGFP or AdIL12/B7.1 were stained with H & E or with an antibody against collagen IV or CD31 as a vessel marker (24). While AdGFP treated tumors showed even distribution of vessel density throughout the tumor, AdIL12/B7.1 treated tumors showed central necrosis with substantially reduced vessels centrally compared to AdGFP-treated tumors (Fig. 8, antibody shown is anti-collagen type IV, similar results with Anti CD31). This is typical of all the AdIL12/B7.1-treated B16 tumors. These results indicate that antiangiogenic activity may play some roles in the observed antitumor effects of the virus.

DISCUSSION

Results from this study support the idea that a combined regimen of radiation and genetic immunotherapy may achieve better results than either alone. Specifically, intratumoral injection of an adenovirus AdIL12/B7.1 after radiation resulted in significantly more growth delay than radiation or immunotherapy alone in two murine tumor models. IL12 and B7.1 were chosen as immunostimulatory agents in this study because local application of either IL12 or B7.1 encoding virus alone as well as their combination has been used successfully in various tumor models. Both agents appear to improve immune responses against tumors (11).

It is somewhat puzzling that there is a huge difference in IL12 expression *in vitro* between the two cell lines that is not all that noticeable *in vivo*. While the *in vitro* results are not surprising given the fact that B16 is reported to be hard to infect with adenovirus (25), there are several reasons for the smaller difference in IL12 expression *in vivo*. It is unlikely to result from the differential promoter activities of CMV since plasmid transfection of 4T1 and B16 cells with the same expression cassette resulted in similar expression levels and retroviral infection actually resulted in higher efficiency for B16. A likely reason is the very limited volume that can be reached by intratumorally injected adenovirus vectors. We found that adenovirus encoded GFP is only expressed in a very small volume around the needletrack after injection in both tumors (data not shown). In this limited space, the virus concentration is probably extremely high. Therefore it is likely that infection in 4T1 tumors is saturated while infection in B16 benefits from the local high

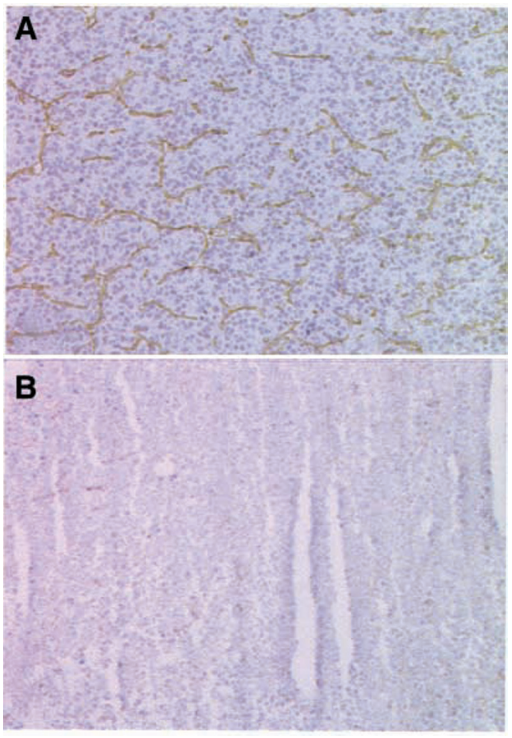

FIG. 8. Immunochemical staining for vessels of tumors (anti-collagen type IV) in B16 melanoma. (A) A 25× magnification photomicrograph of the center of a tumor treated with AdGFP. Notice the extensive network of stained tumor vessels (brown stain). (B) A 25× magnification photomicrograph of the center of a tumor treated with AdIL12/B7.1. Notice the almost complete absence of tumor vessels and the necrosis. (C) Vessel density in hot spots in tumors treated with AdGFP vs AdIL12/B7.1 (means ± SE).

virus doses. Another possible reason is the more equitable infection of nontumor host cells that are present in tumors but not vitro.

Another issue that was addressed in this study is the timing for administering immunostimulatory agents such as IL12 in a combined regimen with radiation. This is very important since radiation can potentially kill effector cells. Indeed, it has been reported that the number of T cells and dendritic cells in an irradiated volume (and if a large volume is irradiated, systemically) decreases immediately after irradiation. However, it has also been reported that T-cell levels rebound soon after completion of irradiation with CD4+ cell level climbing to even higher than baseline levels (26–28). This and the fact that a reduced tumor load after radiotherapy might leave an improved situation for immunomodulatory substances suggest that adding locally immunologically active agents after fractionated radiotherapy might be the appropriate strategy. Our results in two murine tumor models confirmed this idea. We found that adding AdIL12/B7.1-mediated gene therapy after the last radiation fraction proved superior to adding it after the first fraction, especially for B16.F10 but also for 4T1 given the fact, that the virus dose after the last fraction had to be reduced in the specific strain of Balb/c mice used in this experiment. The fact that even with the lower virus dose the post rad application was most effective emphasizes the advantage of this sequence. Our results are also consistent with an earlier study in which a combination of systemic IL12 injections with local radiotherapy seems to yield best results when IL12 is applied during and after radiotherapy rather than before (29). However, our results are slightly different from a recent study where a locally injected adenovector coding for IL12 was used (30). The authors reported improvement of outcome after radiotherapy with virus application even though the virus was injected before the first radiation fraction. The precise mechanisms for the enhanced efficacy in that study are still unresolved. Seetharam *et al.* speculated that the antiangiogenic properties of IL12 mainly constitute its antitumoral effect, based on vessel count data in treated and untreated tumors. The exact nature of the discrepancy is unclear at this point. It is likely that different tumor models behave differently. For example, it is possible that their tumor model is more susceptible to an antiangiogenic attack from IL12 when compared to the two tumor models we use.

As to the mechanism for the observed improved antitumoral effect through combination of AdIL12/B7.1 injection and radiation, the results from this study do not point to a definitive answer. There are several possibilities. The first is the potential interaction between radiation and the adenoviral vectors. While integration of SV40– and adenoviral genes is generally improved in irradiated cells (31–33), the infection efficiency seems to be only affected at high doses ranging between 60 and 120 Gy (25). Our data showed no effect of radiation doses up to 18 Gy on expression of adenovirally encoded protein *in vitro* and *in vivo* when evaluating IL12 (*in vivo* and *in vitro*) and B7.1 (*in vitro*). The second possibility is that various proteins that play a role in antigen presentation like MHCI or gp96 may be upregulated after radiotherapy (34, 35). However, neither for 4T1 (MHC I+, B7.1–) nor for B16.F10 (MHC I–, B7.1–) was the expression of MHC I or B7.1 changed for doses up to 18 Gy in our study. The third possibility is that radiation-induced apoptosis may make more tumor specific antigen available to the immune system. Recent data suggest that uptake of apoptotic bodies by dendritic cells might be an important pathway of presenting antigens on DC (36). Along this line of thought, we did observe an increase in FasL expression in the B16 melanoma cells after 18 Gy of irradiation. Therefore It is possible that radiation induced apoptosis might facilitate the local immune response. Still another possibility is the synergistic interaction of the antiangiogenic effects of IL12 with radiation (22, 23, 30). However, the beneficial effect of AdIL12/B7.1 appears to be mainly mediated through improving immune response to tumor cells in our models. This is postulated for the following reasons. First, the timing of the vector injection would not matter so much if antiangiogenesis is the main mechanism because IL12-induced IFN-γ production responsible for the antiangiogenic properties does not solely depend on immune cells (22). Second, the dramatically reduced effects in a T-cell depleted environment and after NK-depletion point to the importance of those cell types.

Our study also confirms the complicated nature of IL12 function. Many previous reports on the effects of IL12 described a different relative impact of T-cells, NK-cells, or macrophages depending on the individual tumor model, the mode of application of IL12, the vector system and the timing of IL12 application (12, 20, 37). Since, however, no CTL response [as reported previously by Fernandez *et al.* (38)] and no persistent cures were observed in our models (although mild generalized depigmentation in some animals several weeks after treatment indicated some late CTL effect), the presumed T-cell role in the mediation of IL12 effects may be mediated by IFN γ, as others have suggested (39), rather than cytotoxic activities, which we and others actually found to be suppressed immediately after IL12 application (40). Considering that IL12 expression can indeed elicit long-term protection [our own results in Fig. 4 and others (11, 12)], these results are puzzling. However, it is possible that long-term antitumor CTL activity can more easily be elicited prophylactically or in the very early stages of tumor growth and requires longer duration expression of IL12, which a single AdIL12/B7.1 injection can not achieve. Indeed, others have stressed the benefit of multiple injections to achieve a maximum therapeutic effect and protective antitumor immunity (12, 29, 37). In our case this possibility was not addressed because we chose a single injection for the purpose of studying the timing of the treatment modalities. However, multiple local injections of adenoviral vectors are feasible and might

help create cures and protective immunity even in our nonimmunogenic tumor models.

Finally, we observed that elevated serum levels of IL12 were detectable even though the virus was intratumorally injected, which is most likely due to adenovirus leaking into the systemic circulation and infecting parenchymatous organs (41). Further restriction of transgene expression, for example through the use of inducible promoters, might be desirable. Furthermore, this systemic leakage might actually be increased after radiation because of enhanced vessel permeability as suggested by our experience with acute toxicity after radiation in one mouse strain (Balb/c).

In summary, our data support the concept of adding immunotherapy after radiotherapy in a combined regimen.

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