Systemic Delivery of an Oncolytic Adenovirus Expressing Soluble Transforming Growth Factor- β Receptor II–Fc Fusion Protein Can Inhibit Breast Cancer Bone Metastasis in a Mouse Model

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Abstract

We have investigated whether systemic delivery of an oncolytic adenovirus, Ad.sT β RFc, expressing the soluble form of transforming growth factor- β receptor II fused with human immunoglobulin Fc fragment (sTGF β RIIFc), could inhibit breast cancer bone metastasis in a mouse model. MDA-MB-231 (human breast cancer) cells were inoculated into the left heart ventricles of nude mice. Once the skeletal tumors were visible by X-rays, mice were intravenously injected with either buffer, Ad.sT β RFc, Ad(E1⁻).sT β RFc (a replication-deficient adenovirus expressing sTGF β RIIFc), or Ad.luc2 (a replicating adenovirus expressing firefly luciferase gene). On days 2 and 7 after viral injections, viral replication and sTGF β RIIFc expression were detected in the skeletal tumors in Ad.sT β RFc-treated group; only viral replication in Ad.luc2 group, and sTGF β RIIFc expression in the Ad(E1⁻).sT β RFc group, were detected. To examine the therapeutic effects, buffer or various viral vectors were administered on days 4 and 7 after intracardiac injection of MDA-MB-231 cells. On day 28, X-ray radiography showed a highly significant reduction in lesion size by Ad.sT β RFc, a significant reduction by Ad.luc2, and some reduction by Ad(E1⁻).sT β RFc. Goldner's trichrome and hematoxylin–eosin staining of the bone sections revealed a significant reduction of tumor burden in the Ad.sT β RFc group, but not in the Ad(E1⁻).sT β RFc or Ad.luc2 group. There were significant reductions in free calcium levels by Ad.sT β RFc, Ad(E1⁻).sT β RFc, and Ad.luc2; however, only in the Ad.sT β RFc group were calcium levels reduced to the normal values. These results suggest that concomitant viral replication and sTGF β RIIFc production are important to inhibit bone metastasis and osteolysis, and that Ad.sT β RFc could be developed for targeting breast cancer bone metastases.

Introduction

NEARLY 200,000 WOMEN are diagnosed with breast cancer, resulting in approximately 40,000 deaths, each year in the United States (American Cancer Society, 2010). During the advanced stage of breast cancer, the majority of patients develop bone metastases, which is a major cause of morbidity and mortality in these patients. Thus, there is an urgent need to develop novel therapies for the treatment of bone metastases of breast cancer. Our laboratory has been interested in developing recombinant adenoviruses for the treatment of breast cancer (Katayose *et al.*, 1995; Seth, 1999; Seth *et al.*, 2006; Wang *et al.*, 2006; Hu *et al.*, 2010). Although oncolytic adenoviruses have been developed in many laboratories, their

application in targeting bone metastasis has not been described (McCormick, 2005; Crompton and Kirn, 2007). The literature suggests that aberrant activation of transforming growth factor (TGF)- β pathways are associated with breast cancer bone metastases (Kang *et al.*, 2005; Steeg, 2006; Shipitsin *et al.*, 2007; Akhtari *et al.*, 2008; Padua and Massague, 2009), and that downregulation of TGF- β signaling in the tumor cells is considered a viable approach to control bone metastasis (Yin *et al.*, 1999; Yang *et al.*, 2002; Iyer *et al.*, 2005; Korpai *et al.*, 2009; Serganova *et al.*, 2009). Human breast cancer cells expressing a dominant negative transforming growth factor- β receptor II (TGF- β RII; lacking the kinase domain) or transgenic mice expressing soluble TGF β RII–Fc fusion protein (sTGF β RIIFc) have also been shown to reduce

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the incidence of metastases in mice (Yin *et al.*, 1999; Yang *et al.*, 2002), and life-long exposure to sTGF β RIIFc in transgenic mice did not cause any apparent harmful effect to the mice (Yang *et al.*, 2002). To combine the capacity of oncolytic viruses to kill cancer cells, and the function of sTGF β RIIFc to inhibit TGF- β signaling, we have developed Ad.sT β RFc, an oncolytic adenovirus expressing sTGF β RIIFc (Seth *et al.*, 2006). Ad.sT β RFc is derived from an adenoviral mutant, dl01/07, that has two deletions in the E1A region, amino acids 4–25 and amino acids 111–123. The resultant E1 proteins are not able to bind with p300 or Rb proteins and cannot induce S-phase progression in primary cells. Therefore, dl01/07 is attenuated for viral replication in normal cells, but can replicate in cancer cells regardless of their genetic background (Howe *et al.*, 2000). We have previously shown that human breast tumor cells (cell line MDA-MB-231) possesses nearly 10,000 adenoviral receptors per cell, and are easily infectable by human adenoviruses (Seth *et al.*, 1996). Ad.sT β RFc can replicate in the MDA-MB-231 cell line (Seth *et al.*, 2006), which harbors a missense mutation in the p53 gene, in codon 280, changing arginine to lysine (Lacroix *et al.*, 2006). Infection of MDA-MB-231 cells with Ad.sT β RFc produces sTGF β RIIFc and inhibits TGF- β signaling, and its direct administration into subcutaneous tumors in nude mice results in significant inhibition of tumor growth (Seth *et al.*, 2006).

In the present study, we have examined whether systemic administration of Ad.sT β RFc could target breast cancer bone metastases in a nude mouse model. Our results presented here indicate that intravenous delivery of Ad.sT β RFc produced viral replication and sTGF β RIIFc in skeletal tumors and resulted in significant inhibition of tumor growth and osteolytic bone destruction. Ad(E1⁻).sT β RFc, a replication-deficient adenovirus expressing sTGF β RIIFc; and Ad.luc2, a replicating virus expressing the firefly luciferase gene, had some effects on tumor growth and tumor-induced hypercalcemia; but the therapeutic effects were weaker than that of Ad.sT β RFc. These findings suggest that both viral replication and sTGF β RIIFc production are critical in mediating the *in vivo* effects of Ad.sT β RFc in inhibiting bone metastases, and that Ad.sT β RFc can be developed for the treatment of breast cancer bone metastases.

Materials and Methods

Cell lines and viruses

HEK293 (American Type Culture Collection [ATCC], Manassas, VA) and MDA-MB-231 cells (Yin *et al.*, 1999) were maintained as described earlier (Katayose *et al.*, 1995; Yin *et al.*, 1999). Ad.sT β RFc (Seth *et al.*, 2006) is a replicating adenovirus in which the cytomegalovirus (CMV) promoter drives the sTGF β RIIFc gene cloned in the E3 region, while retaining the adenovirus death protein sequence. Ad.luc2 is identical to Ad.sT β RFc except that the luc2 gene is cloned in the E3 region. Ad(E1⁻).sT β RFc (Hu *et al.*, 2010) is a nonreplicating virus expressing the CMV promoter driving sTGF β RIIFc in the E3 region. All adenoviral vectors were amplified in HEK293 cells and purified as described earlier (Katayose *et al.*, 1995).

Breast cancer bone metastasis model and radiography

Five-week-old female athymic *nu/nu* mice were purchased from Charles River Laboratories (Wilmington, MA).

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at North-Shore University HealthSystem (Evanston, IL). MDA-MB-231 cells were trypsinized, washed twice, and resuspended in phosphate-buffered saline (PBS) to a final concentration of 1.5×10^5 cells in 100 μ l of PBS. Animals were anesthetized with ketamine-xylazine and positioned ventral side up. MDA-MB-231 cells were inoculated into the left ventricle (day 0) by percutaneous injection, using a 27-gauge needle (100 μ l per mouse), as previously described (Yin *et al.*, 1999). To evaluate the effects of viral vectors, viruses were injected via the tail vein (2×10^8 plaque-forming units [PFU] in 0.1 ml of buffer) on day 4, followed by a second dose (1×10^8 PFU in 0.1 ml of buffer) on day 7. Osteolytic lesions were analyzed by radiography, using an LX-60 system with digital camera (Faxitron X-Ray, Lincolnshire, IL). Mice were imaged in a prone position on day 28. The osteolytic lesion area was quantified with ImageJ software (National Institutes of Health [NIH], Bethesda, MD). Mice were anesthetized and blood was collected by cardiac aspiration on day 28 before euthanasia.

Bone histology and histomorphometry

Hind limbs of mice were collected on euthanasia on day 28. The left or right hind limb of each mouse, picked randomly, was fixed in 10% neutral buffered formalin for 48 hr and decalcified in 10% EDTA for 2 weeks. After decalcification, tissues were processed and embedded in paraffin wax for sectioning. Longitudinal, midsagittal sections (thickness, 3.5 μ m) from the tibia and femur were obtained. Tissue sections were stained with hematoxylin and eosin (H&E) or Goldner's trichrome stain and prepared for histomorphometric analysis (Yin *et al.*, 2003). Images were captured with a DMLB microscope (Leica Microsystems, Wetzlar, Germany) equipped with a charge-coupled device (CCD) camera (Roper Scientific, Tucson, AZ) and analyzed with IPLab image acquisition software (BD Biosciences, San Jose, CA). Tumor burden per hind limb was calculated at the tibia and femur, using sections (magnification, $\times 25$) stained with H&E or Goldner's trichrome stain.

Viral replication and sTGF β RIIFc expression in vivo

Animals bearing skeletal tumors as revealed by radiography were injected with viral vectors (2×10^8 PFU in 0.1 ml of buffer, day 0). Hind limbs and blood were collected on days 2 and 7. Immunohistochemical analysis was performed on decalcified paraffin-embedded bone sections for adenoviral hexon protein and sTGF β RIIFc expression. Antigen retrieval was performed with proteinase K (Dako, Carpinteria, CA). Primary antibody against adenovirus 5 hexon (Chemicon, Temecula, CA) or IgG Fc γ fragment (Jackson ImmunoResearch, West Grove, PA) was used to detect hexon and sTGF β RIIFc respectively. Immunodetection was conducted with a 3,3'-diaminobenzidine (DAB) substrate kit (Invitrogen, Carlsbad, CA) and counterstained with hematoxylin.

Measurements of blood calcium and sTGF β RIIFc levels

Serum calcium levels were measured with a QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA). Blood sTGF β RIIFc levels were determined by enzyme-linked immunosorbent assay (ELISA), using antibodies against the

human IgG Fc γ fragment (Jackson ImmunoResearch) as described earlier (Hu *et al.*, 2010).

Statistical analysis

Results are expressed as means $\pm$ SE. The two-tailed *t* test was performed for group comparisons, using Prism software version 5 (GraphPad Software, San Diego, CA). *p* < 0.05 was considered significant.

Results and Discussion

Systemic administration of Ad.sT β RFc results in replication and simultaneous production of sTGF β RIIFc *in vivo*

To examine Ad.sT β RFc replication and Ad.sT β RFc-mediated sTGF β RIIFc production *in vivo*, nude mice bearing skeletal tumors were injected with buffer, Ad.sT β RFc, Ad(E1⁻).sT β RFc, or Ad.luc2. After 2, 5, and 7 days of various

treatments, hind limbs were harvested and bone sections were analyzed for hexon and sTGF β RIIFc expression by immunohistochemical staining. Figure 1A and B shows the results obtained from day 2 and day 7 samples, respectively. In animals that received buffer, no hexon- or sTGF β RIIFc-positive cells were detected (Fig. 1A and B). For animals that received Ad.sT β RFc, a number of hexon- and sTGF β RIIFc-positive brown tumor cells were present in the tumors. Administration of Ad(E1⁻).sT β RFc resulted in sTGF β RIIFc production, whereas no hexon-positive cells were detected in the tumors. Animals that received Ad.luc2 produced hexon-expressing cells, but did not produce sTGF β RIIFc (Fig. 1A and B). These results indicate that intravenous injection of Ad.sT β RFc and Ad.luc2 viruses results in virus uptake and replication in skeletal tumors; and intravenous injection of Ad.sT β RFc and Ad(E1⁻).sT β RFc produces sTGF β RIIFc for 7 days after vector administration.

Amounts of sTGF β RIIFc present in blood samples obtained from day 2 and 7 treatments were determined by ELISA. Sera derived from day 2 treatment showed sTGF β RIIFc at $17.46 \pm$

0.56 and 8.76 ± 1.07 mg/ml in Ad.sT β RFc- and Ad(E1⁻).sT β RFc-treated groups, respectively (Fig. 1C). sTGF β RIIFc levels on day 7 were 18.95 ± 0.40 and 96.78 ± 10.04 mg/ml in sera derived from the Ad.sT β RFc and Ad(E1⁻).sT β RFc groups, respectively. Mice from all other groups had less than 8 ng of sTGF β RIIFc per milliliter on day 2 or 7 (Fig. 1C). The large amounts of sTGF β RIIFc in the blood in Ad.sT β RFc- and Ad(E1⁻).sT β RFc-treated animals is probably due to protein production from tumors as well as other organs such as liver; systemic administration of adenoviruses is known to result in viral uptake by the liver (Waddington *et al.*, 2008).

Intravenous injection of Ad.sT β RFc into nude mice inhibits bone metastases

The effect of Ad.sT β RFc, Ad(E1⁻).sT β RFc, and Ad.luc2 in inhibiting bone metastases was examined by X-ray radiography on day 28 after cell inoculation. A normal hind leg bone, along with a typical bone bearing tumors (marked with arrows), from each of the groups is shown in Fig. 2A.

FIG. 2. Effect of systemic administration of Ad.sT β RFc on bone metastasis. MDA-MB-231 cells were inoculated into the left cardiac ventricle on day 0. Buffer, Ad.sT β RFc, Ad(E1⁻).sT β RFc, or Ad.luc2 was injected via the tail vein on days 4 and 7. On day 28, radiographs and blood were obtained from nine mice in each group. **(A)** Representative radiographs from a normal mouse and mice injected with tumor cells followed by buffer, Ad.sT β RFc, Ad(E1⁻).sT β RFc, or Ad.luc2. **(B)** Average lesion area per mouse on day 28 is plotted ($n=9$ mice in each group). p values for comparison with the buffer group are shown (** $p < 0.01$; * $p < 0.05$). **(C)** Blood levels of sTGF β RIIFc on day 28 for each group are shown ($n=9$ mice in each group).

Animals that received tumor cells followed by buffer showed bone with large radiolucent lytic tumor lesions. Bones from Ad.sTβRfC-, Ad(E1⁻).sTβRfC-, and Ad.luc2-treated groups had smaller lesions. Lesion areas in groups treated with buffer, Ad.sTβRfC, Ad(E1⁻).sTβRfC, and

Ad.luc2 were 9.55 ± 1.67 , 3.69 ± 0.88 , 5.68 ± 1.70 , and $3.91 \pm 1.00 \text{ mm}^2$, respectively (Fig. 2B). There was a highly significant difference in lesion size between the buffer and Ad.sTβRfC groups ($p = 0.0066$), and significant differences between the buffer and Ad.luc2 ($p = 0.0106$) groups.

FIG. 3. Effect of systemic administration of Ad.sTβRfC on tumor burden and tumor cell-induced bone destruction. **(A)** Normal bone of mice that received tumor cells (day 0) followed by buffer, Ad.sTβRfC, Ad(E1⁻).sTβRfC, or Ad.luc2 was collected on day 28. Longitudinal, midsagittal sections (thickness, $3.5 \mu\text{m}$) from the tibia and femur were obtained and processed for Goldner's trichrome staining or hematoxylin and eosin (H&E) staining. Representative photographs of femur (Goldner's trichrome staining) or tibia (H&E staining) from each group are shown (original magnification, $\times 25$). In the Goldner's trichrome sections, bone matrix (M) stains green, bone marrow (BM) stains dark purple, and tumor cells (T) stain purple-gray. Arrows are directed toward tumors present in the various bone samples shown. Scale bars: $500 \mu\text{m}$. **(B)** Tumor burden in hind limbs was measured by quantitative histomorphometry (original magnification, $\times 25$); data were obtained from longitudinal, midsagittal sections from nine bone samples of each experimental group. Three randomly selected longitudinal sections were examined for each of the bone samples. p values for comparison with the buffer treatment group are shown in **(B)** ($*p < 0.05$). **(C)** Serum calcium levels were measured ($n = 9$ mice in each group). p values for comparison with the normal mouse group are shown in **(C)** ($***p < 0.001$; $**p < 0.01$).

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Author Disclosure Statement

No competing financial interests exist for any of the authors.

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