

Brief Report

Development of Oncolytic Adenovirus Armed with a Fusion of Soluble Transforming Growth Factor- β Receptor II and Human Immunoglobulin Fc for Breast Cancer Therapy

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ABSTRACT

We have developed an approach to cancer gene therapy in which the oncolytic effects of an adenoviral vector have been combined with selective expression of a soluble form of transforming growth factor (TGF)- β receptor II fused with Fc (sTGF β RIIFc). We chose to use adenoviral *dl*01/07 mutant because it can replicate in all cancer cells regardless of their genetic defects. An oncolytic adenovirus expressing sTGF β RIIFc (Ad.sT β RFc) was constructed by homologous recombination. Infection of MDA-MB-231 and MCF-7 human breast cancer cells with Ad.sT β RFc produced sTGF β RIIFc, which was released into the media. The conditioned media containing sTGF β RIIFc could bind with TGF- β_1 and inhibited TGF- β -dependent transcription in target cells. Infection of MDA-MB-231, MCF-7, and 76NE human breast cancer cells with Ad.sT β RFc resulted in high levels of viral replication, comparable to that of a wild-type *dl*309 virus. Although some viral replication was observed in actively dividing normal human lung fibroblasts, there was no replication in nonproliferating normal cells. Direct injection of Ad.sT β RFc into MDA-MB-231 human breast xenograft tumors grown in nude mice resulted in a significant inhibition of tumor growth, causing tumor regression in more than 85% of the animals. These results indicate that it is possible to construct an oncolytic virus expressing sTGF β RIIFc in which both viral replication and transgene expression remain intact, and the recombinant adenovirus is oncolytic in a human tumor xenograft model. On the basis of these results we believe that it may be feasible to develop a cancer gene therapy approach using Ad.sT β RFc as an antitumor agent.

INTRODUCTION

NEARLY 200,000 WOMEN are diagnosed with breast cancer each year in the United States (American Cancer Society, 2006). Considering the high level of mortality associated with breast cancer, there is a need to develop novel approaches to treat this disease. In this study we have investigated the feasibility of developing a novel recombinant oncolytic adenovirus as an antitumor agent. Although several conditionally replicating adenoviral mutants have been developed, we have chosen to use adenoviral mutant *dl*01/07, because it can

replicate in all cancer cells regardless of their genetic background (Howe *et al.*, 1990, 2000). *dl*01/07 produces an adenoviral E1A protein that is defective in binding both p300 and retinoblastoma protein (Rb) and is therefore ineffective for S-phase progression in primary cells (Seth, 1999) (Fig. 1A). Because most human tumors are heterogeneous, with varied genetic background, and can be easily infected with human adenoviruses (Seth *et al.*, 1996; Rakkar *et al.*, 1998), we believe recombinant oncolytic adenoviruses based on the *dl*01/07 mutant can potentially be developed as gene therapy drugs.

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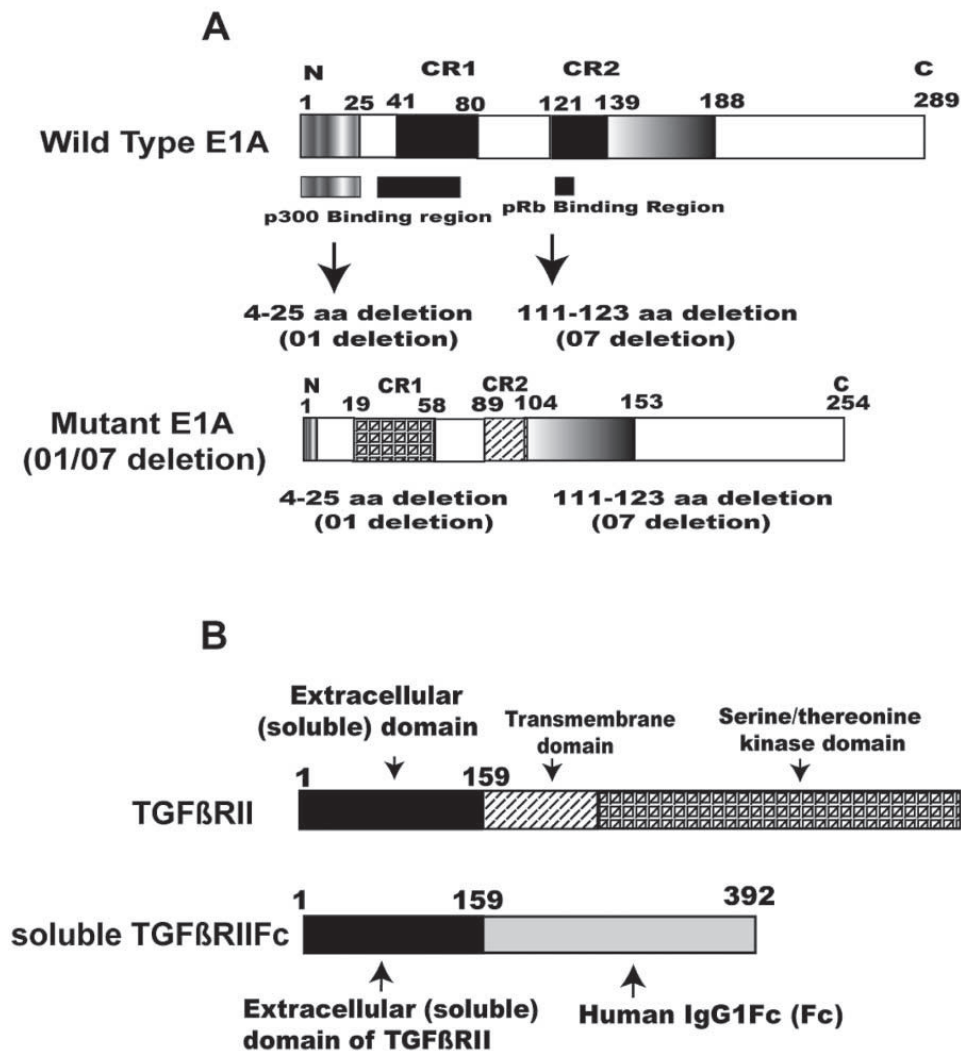


FIG. 1. (A) Schematic diagram depicting key features of the E1A-encoding region of *dl01/07*. *Top*: Structure of E1A region of wild-type adenovirus 5. It contains 289 amino acids (aa) and encodes proteins that can bind with p300 and Rb proteins, the regulators of cell cycle progression. *Bottom*: E1A structure of 01/07 mutant virus. The *dl01/07* virus has two deletions. One deletion is aa 4–25 (*dl01*), and the second deletion is aa 111–123 (*dl07*). Because of these mutations, the E1A region of *dl01/07* is shorter (254 aa). The resultant *dl01/07* E1 proteins cannot bind with p300 or Rb protein. Thus, *dl01/07* is ineffective for S-phase progression in primary cells and cannot replicate. However, cancer cells are able to progress to S phase, permitting viral replication in these cells. (B) Schematic diagram of sTGFβRIIFc cDNA. *Top*: TGFβRII structure. It contains three domains: an extracellular domain (amino acids 1–159), a transmembrane domain, and a serine/threonine kinase domain. *Bottom*: Extracellular domain fused with human IgG1 Fc (Fc). The resulting sTGFβRIIFc cDNA (kindly provided by R&D Systems, Minneapolis, MN) was cloned into the oncolytic *dl01/07* adenovirus backbone.

To further augment the effectiveness of *dl01/07* adenovirus as an antitumor agent, we have armed it with a soluble form of transforming growth factor (TGF)-β receptor II fused with Fc (sTGFβRIIFc). sTGFβRIIFc protein is expected to bind with TGF-β₁ and inhibit TGF-β signaling in target cells (Yang *et al.*, 2002). We chose to target the TGF-β pathway because high levels of TGF-β₁ have been shown to have tumor growth-promoting activities by enhancing angiogenesis, invasion, and metastasis, and by inhibiting immune functions (Inge *et al.*, 1992; Yin *et al.*, 1999; Akhurst and Derynck, 2001; Derynck *et al.*, 2001; Hiraga *et al.*, 2001; Teicher, 2001; Iwasaki *et al.*, 2002; Wakefield and Roberts, 2002; Zhao *et al.*, 2002; Guise and

Chirgwin, 2003; Roberts and Wakefield, 2003; Tang *et al.*, 2003; Iyer *et al.*, 2005; Thomas and Massague, 2005).

We constructed a *dl01/07*-based oncolytic adenovirus expressing sTGFβRIIFc (Ad.sTβRFc), and examined it in various *in vitro* and *in vivo* assays. We report that infection of breast cancer cells with Ad.sTβRFc produced high levels of sTGFβRIIFc that was subsequently released into the extracellular space. sTGFβRIIFc produced in the medium was shown to bind with TGF-β₁, and to inhibit TGF-β signaling in target cells. Infection of breast cancer cells also produced high-level viral titers. Direct injection of Ad.sTβRFc into human xenografts established in nude mice caused significant inhibition of tumor

growth, resulting in tumor regression in more than 85% of the animals. These results suggest that it is feasible to develop Ad.sT β RFc as a potential gene therapy vector for breast cancer therapy.

MATERIALS AND METHODS

Cell culture

HEK-293, MDA-MB-231, WI-38, and IMR-90 cells (from the American Type Culture Collection [ATCC], Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS; Mediatech) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). MV1Lu and MCF-7 cells (ATCC) were grown in Eagle's minimum essential medium (EMEM; ATCC) containing 10% FBS and 1% penicillin-streptomycin (Invitrogen).

Construction of Ad.sT β RFc

A 1.2-kb *HindIII*-*ApaI* fragment from pcDNA3/SR2F (Yang *et al.*, 2002) containing cDNA of the soluble form of TGF- β receptor II fused to human IgG Fc γ was first cloned in *HindIII*- and *ApaI*-digested pBS-SK(+). The sTGF β RIIFc cDNA was then cloned in p309 plasmid to produce shuttle vector p309/sT β RFc. The 15.7-kb *PacI*-*NruI* fragment from p309/sT β RFc was then cotransformed in *Escherichia coli* BJ5183 with *BstBI*- and *SpeI*-cut adenoviral backbone plasmid pAd01/07. Homologous recombination of the shuttle vector and plasmid pAd01/07 produced adenoviral genome plasmid p01/07/sT β RFc. *PacI*-cut p01/07/sT β RFc was transfected into HEK-293 cells to produce the recombinant adenovirus Ad.sT β RFc. Adenovirus was collected after 10 days, amplified in HEK-293 cells, and purified by double cesium chloride gradient ultracentrifugation as described previously (Katayose *et al.*, 1995; Craig *et al.*, 1997).

Western blot analysis

Cells were plated (2×10^5 cells per well in a 6-well plate) in medium containing 10% FBS and incubated at 37°C overnight. The next morning cells were infected with adenovirus at 100 plaque-forming units (PFU)/cell for 48 hr. Medium and cells were separately dissolved in sodium dodecyl sulfate (SDS) sample buffer, and subjected to Western blot analysis as previously described (Katayose *et al.*, 1995; Craig *et al.*, 1997). Blots were probed with antibody reactive against TGF β RII (Santa Cruz Biotechnology, Santa Cruz, CA) or actin (Santa Cruz Biotechnology).

TGF- β_1 binding with sTGF β RIIFc

MDA-MB-231 cells (1×10^6 per well of a 6-well plate) were uninfected or infected with various adenoviruses (100 PFU/cell) for 3 hr in growth medium. Cells were washed and incubated in 1.7 ml of serum-free DMEM for 20 hr. Culture media were collected and 200- μ l volumes of culture media were mixed with TGF- β_1 (40 ng; Sigma, St. Louis, MO) for 1 hr at 4°C. Wheat germ agglutinin (WGA)-Sepharose agarose beads (Vector Laboratories, Burlingame, CA) were added, followed by incubation

for 1 hr at 4°C. The beads were then washed six times with buffer (50 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100; pH 7.4) and subjected to sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) and probed with rabbit anti-TGF- β_1 polyclonal antibody (Promega, Madison, WI).

Viral production assay

To assay for viral replication, cells were plated in 6-well plates (2×10^5 cells per well) and infected with Ad(E1-).Null, Ad.sT β RFc, or dl309 (100 PFU/cell) for 3 hr. They were then washed once with DMEM and incubated in 1 ml of DMEM at 37°C. One hour later, cells were washed and incubated in growth medium for another 48 hr. Medium and cells were collected and frozen-thawed three times to release the viruses. The lysates were then serially diluted and added to monolayers of HEK-293 cells. After 3 hr of incubation at 37°C, the infected HEK-293 cells were overlaid with 3 ml of 1.25% SeaPlaque agarose (Cambrex, East Rutherford, NJ) in growth medium. Plaques were counted after 7 days of incubation.

Luciferase assay

MV1Lu cells (1×10^6 per well) were transfected with 2 μ g of firefly luciferase reporter plasmid p3TP-Lux and 0.2 μ g of *Renilla* luciferase plasmid pRL-TK, using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The next day, the cells were serum-starved for 24 hr and incubated with various conditioned media in the absence or presence of TGF- β_1 (2 ng/ml) for 24 hr. Proteins were extracted with 1 $\times$ Passive Lysis Buffer (Promega). Luciferase activity was then measured in cell lysates, using a Dual-Luciferase reporter assay kit (Promega). Bioluminescence was measured with a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA).

Analysis of tumor growth in vivo

MDA-MB-231 cells were injected subcutaneously (5×10^6 cells per mouse) as described earlier (Li *et al.*, 1997; Rakkar *et al.*, 1998). Once palpable tumors were established, oncolytic viruses (Ad.sT β RFc and Ad.TK) were administered intratumorally (2×10^8 PFU in 0.1 ml of buffer per injection, three injections total on days 0, 2, and 4). Another group of animals received sTGF β RIIFc (2.5 μ g per injection, three injections total on days 0, 2, and 4). Control animals received buffer alone. Tumor volumes were measured with a digital caliper on various days as indicated. Animals were monitored weekly for the presence or absence of tumors. The animal experiments were conducted according to protocols approved by the Evanston Northwestern Healthcare Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

Construction of Ad.sT β RFc

sTGF β RIIFc cDNA (Fig. 1B) driven by the cytomegalovirus (CMV) promoter was cloned into a shuttle vector permitting in-

section of the cDNA at the E3 site of *dl01/07*. Ad.sT β RFc was generated by homologous recombination of the shuttle vector with an adenoviral plasmid containing the *dl01/07* E1A gene (Fig. 2A). The schematic structure of Ad.sT β RFc is shown in Fig. 2B.

Infection of human breast tumor cells with Ad.sT β RFc produces sTGF β RIIFc protein

To investigate Ad.sT β RFc-mediated expression of sTGF β RIIFc protein, MDA-MB-231 and MCF-7 breast tumor cells were infected with Ad.sT β RFc (100 PFU/cell) for 48 hr. Both media and cells were subjected to Western blot analysis and probed with anti-TGF β RII. Cells infected with Ad.sT β RFc, as well as the extracellular medium, showed a diffuse protein band of 50–70 kDa (Fig. 3A). These results indicate that infection of breast cancer cells with Ad.sT β RFc results in expression of sTGF β RIIFc, which is subsequently secreted by the cells.

sTGF β RIIFc can bind with TGF- β_1 , resulting in inhibition of TGF- β signaling

The success of the proposed cancer gene therapy strategy depends on the ability of vector-mediated sTGF β RIIFc to bind with TGF- β protein, causing the inhibition of TGF- β signaling in target cells. To examine the binding of TGF- β with sTGF β RIIFc, pure recombinant TGF- β_1 was incubated with culture medium derived from Ad.sT β RFc-infected MDA-MB-231 cells. The mixture was combined with WGA–Sepharose beads, which can bind with glycosylated proteins including sTGF β RIIFc. The complexes were analyzed by Western blotting, using anti-TGF- β_1 . Figure 3B shows the presence of TGF- β_1 in the precipitate when the medium from Ad.sT β RFc-infected cells was used. However, TGF- β_1 was not present when the medium from Ad.TK-infected cells was used (Ad.TK is a *dl01/07*-based oncolytic virus expressing the herpes simplex virus thymidine kinase [HSV-TK] gene, a kind gift from M.

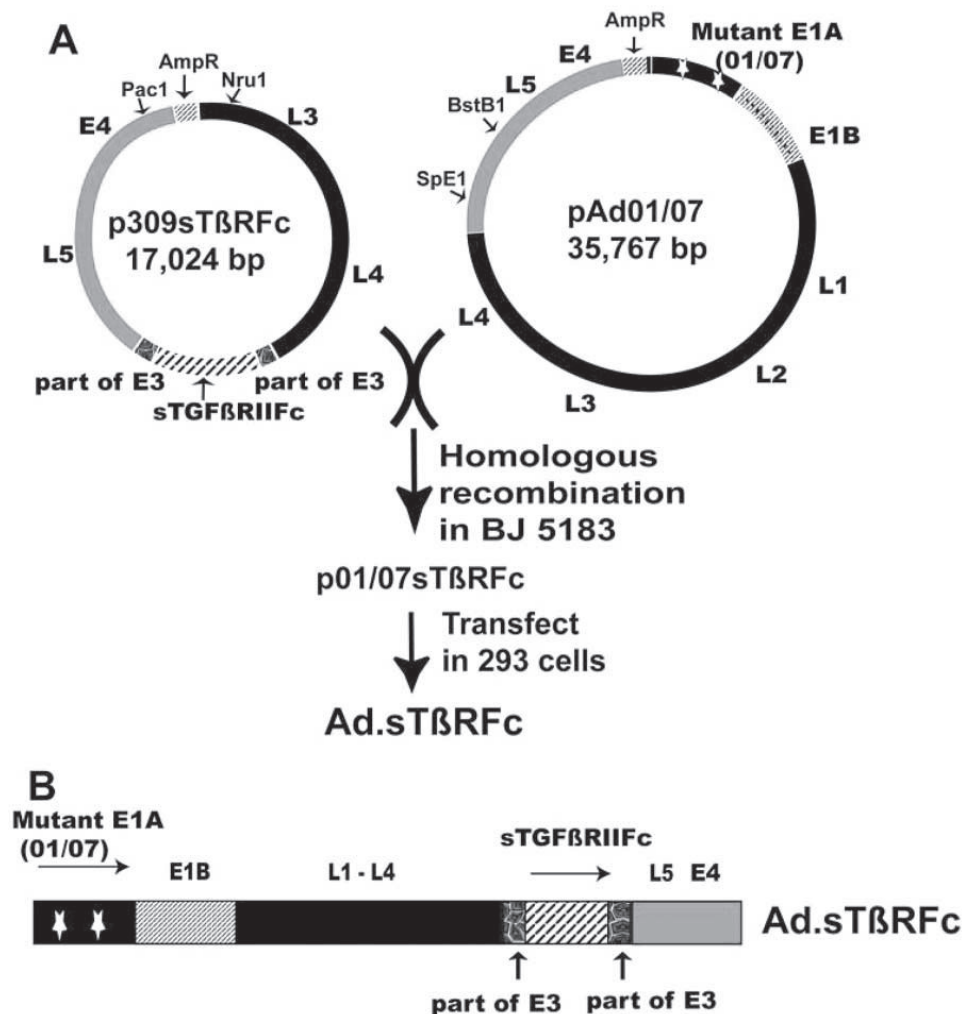
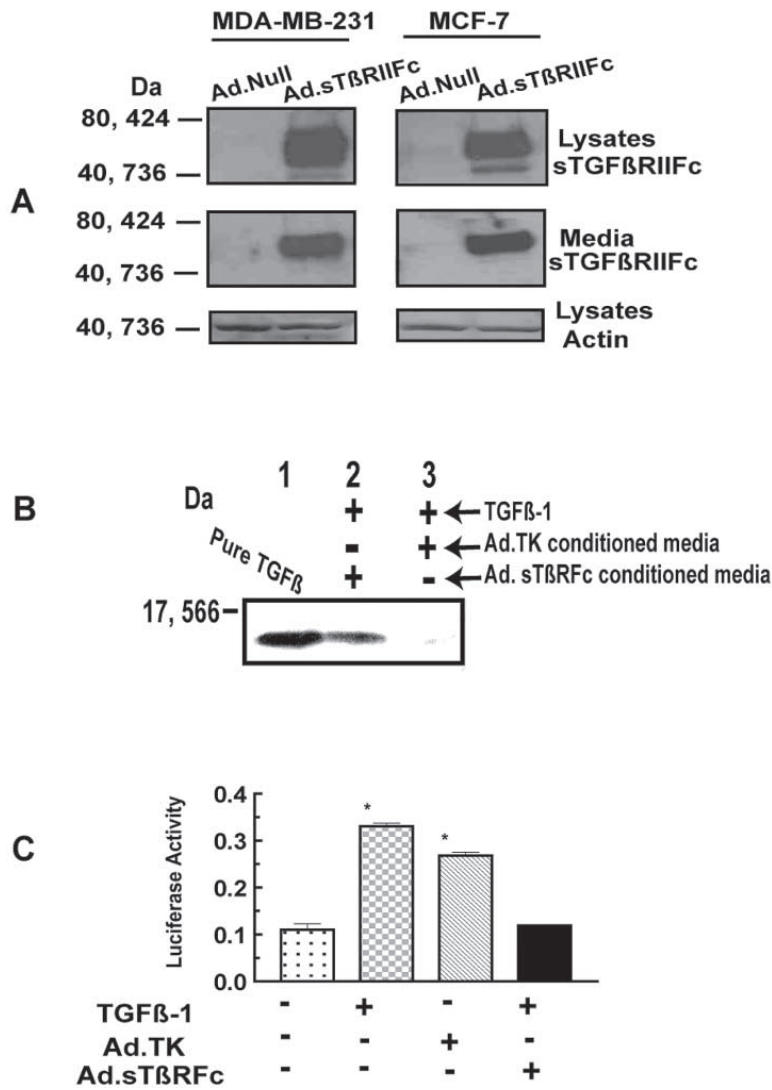


FIG. 2. (A) Construction of Ad.sT β RFc. Ad.sT β RFc was constructed by homologous recombination as described in Materials and Methods, and purified by double cesium chloride gradient ultracentrifugation as described previously (Katayose *et al.*, 1995; Craig *et al.*, 1997). (B) Schematic structure of Ad.sT β RFc. The key regions of recombinant Ad.sT β RFc shown are as follows: a mutant E1A region (01/07), the expression cassette containing sTGF β RIIFc inserted at the E3 site of the adenoviral genome (the part of E3 encoding the adenoviral death protein is left intact), E1B, L1–L5, and E4. (Note: Adenoviral E2 sequences are not shown here.)



nescence was measured with a Turner Designs luminometer. Results shown represent luciferase activities when medium from control or Ad.TK- or Ad.sTβRIIFc-infected MDA-MB-231 cells was used. * $p < 0.05$ versus basal-level luciferase activity.

Ramachanda, Canji, San Diego, CA). These results indicate that secreted soluble TGFβRIIFc in conditioned medium can bind with TGFβ₁.

To test whether Ad.sTβRIIFc-mediated expression of sTGFβRIIFc was capable of blocking TGFβ functions, inhibition of the TGFβ-responsive luciferase reporter gene was examined. Plasminogen activator inhibitor-1 (PAI-1) is a known target gene of TGFβ signaling. TGFβ-mediated transcriptional activity was assessed in mink lung epithelial MV1Lu cells, which are known to be TGFβ sensitive (Kanamoto *et al.*, 2002). Plasmid p3TP-Lux (kindly provided by J. Massague, Memorial Sloan-Kettering Cancer Center, New York, NY) (Wrana *et al.*, 1992), containing multiple response elements from the promoter of the gene encoding plasminogen activator inhibitor, was used in a reporter assay. MV1Lu cells were transiently transfected with p3TP-Lux reporter plasmid (with and without TGFβ₁), and luciferase activity was measured. As shown in Fig. 3C, in the absence of TGFβ₁ basal-level luciferase was detected.

However, addition of TGFβ₁ resulted in a 3-fold increase in luciferase activity. Coincubation of medium from Ad.TK-infected cells with TGFβ₁ did not inhibit the TGFβ₁-dependent increase in luciferase activity. However, TGFβ-dependent luciferase activity was significantly decreased when culture medium from Ad.sTβRIIFc-infected cells was used, indicating that medium containing sTGFβRIIFc inhibited TGFβ₁ transcriptional activity (Fig. 3C).

Ad.sTβRIIFc is replication competent in human breast tumor cells, but exhibits low-level replication in normal human cells

Next, we examined the replication potential of the Ad.sTβRIIFc oncolytic virus by measuring viral titers in Ad.sTβRIIFc-infected tumor cells. Human breast tumor cells were exposed to either Ad.sTβRIIFc or control adenovirus—either a replication-deficient virus [Ad(E1-).Null] or a fully replication-competent wild-type adenovirus (d1309)—for 48 hr. Cell lysates

FIG. 3. (A) Ad.sTβRIIFc-mediated expression of sTGFβRIIFc protein in breast cancer cells. MDA-MB-231 cells (left) and MCF-7 cells (right) were plated in 6-well plates (2×10^5 cells per well). The next day, cells were infected with Ad.sTβRIIFc or Ad.Null at 100 PFU/cell. Both cells and media were collected and subjected to Western blot analysis. Blots were probed with anti-TGFβRII antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin antibody (Santa Cruz Biotechnology), and developed by enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ). Molecular weight is indicated to the left. (B) TGFβ binding with conditioned medium containing sTGFβRIIFc. Conditioned medium derived from Ad.sTβRIIFc-infected MDA-MB-231 cells was incubated with TGFβ₁. The TGFβ-TGFβRIIFc complexes were bound with WGA and subjected to Western blot analysis, probed with rabbit anti-TGFβ₁ polyclonal antibody (Promega). Lane 1 received pure TGFβ₁ as a positive control. Note that the TGFβ₁ band is present when the medium from Ad.sTβRIIFc-infected cells was used (lane 2), but not when medium from Ad.TK-infected cells was used (lane 3). (C) Inhibition of TGFβ transcriptional activity. MV1Lu cells (5×10^5 per well) were transfected with 2 μg of firefly luciferase reporter plasmid p3TP-Lux and 0.2 μg of Renilla luciferase plasmid pRL-TK, using Lipofectamine 2000 (Invitrogen). The next day, cells were serum-starved for 24 hr and incubated with conditioned medium derived from control Ad.TK- or Ad.sTβRIIFc-infected MDA-MB-231 cells (infection time, 24 hr) in the absence or presence of TGFβ₁ (2 ng/ml). Proteins were extracted and luciferase activity was measured in cell lysates, using a Dual-Luciferase reporter assay kit. Biolumi-

were prepared and aliquots were used to evaluate viral titers by plaque-forming assay in 293 cells. In MDA-MB-231 cells, the Ad(E1-).Null titer was 1.1×10^6 PFU (burst size, 1.0). The wild-type *dl309* viral titer reached 2.7×10^9 PFU, with a 2454-fold increase in burst size (Fig. 4A). The Ad.sT β RFc titer reached 1.12×10^9 PFU, which represents a 1018-fold increase in burst size. Thus, the Ad.sT β RFc viral titer was comparable to that of *dl309* (Fig. 4A and D). In MCF-7 breast tumor cells the Ad.sT β RFc viral titer was 9.7×10^8 PFU, 3.36-fold lower than that of *dl309* (Fig. 4D). Another human breast tumor cell line, 76NE, produced an Ad.sT β RFc titer of 3.98×10^8 PFU,

which is 2.5-fold higher than that of *dl309* (Fig. 4D). These results indicate that Ad.sT β RFc is replication competent in human breast tumor cells.

Viral replication was also investigated in normal human lung fibroblast WI-38 and IMR-90 cells. In WI-38 cells, Ad(E1-).Null produced a titer of 1.96×10^6 PFU (burst size, 1), Ad.sT β RFc produced a titer of 4.35×10^6 PFU (burst size, 2.2), whereas the *dl309* titer was 2.95×10^8 PFU (burst size, 151), indicating that the replication potential of Ad.sT β RFc is 69 times lower than that of *dl309* (Fig. 4B and D). In IMR-90 cells, Ad(E1-).Null produced a titer of 7.95×10^5 PFU (burst

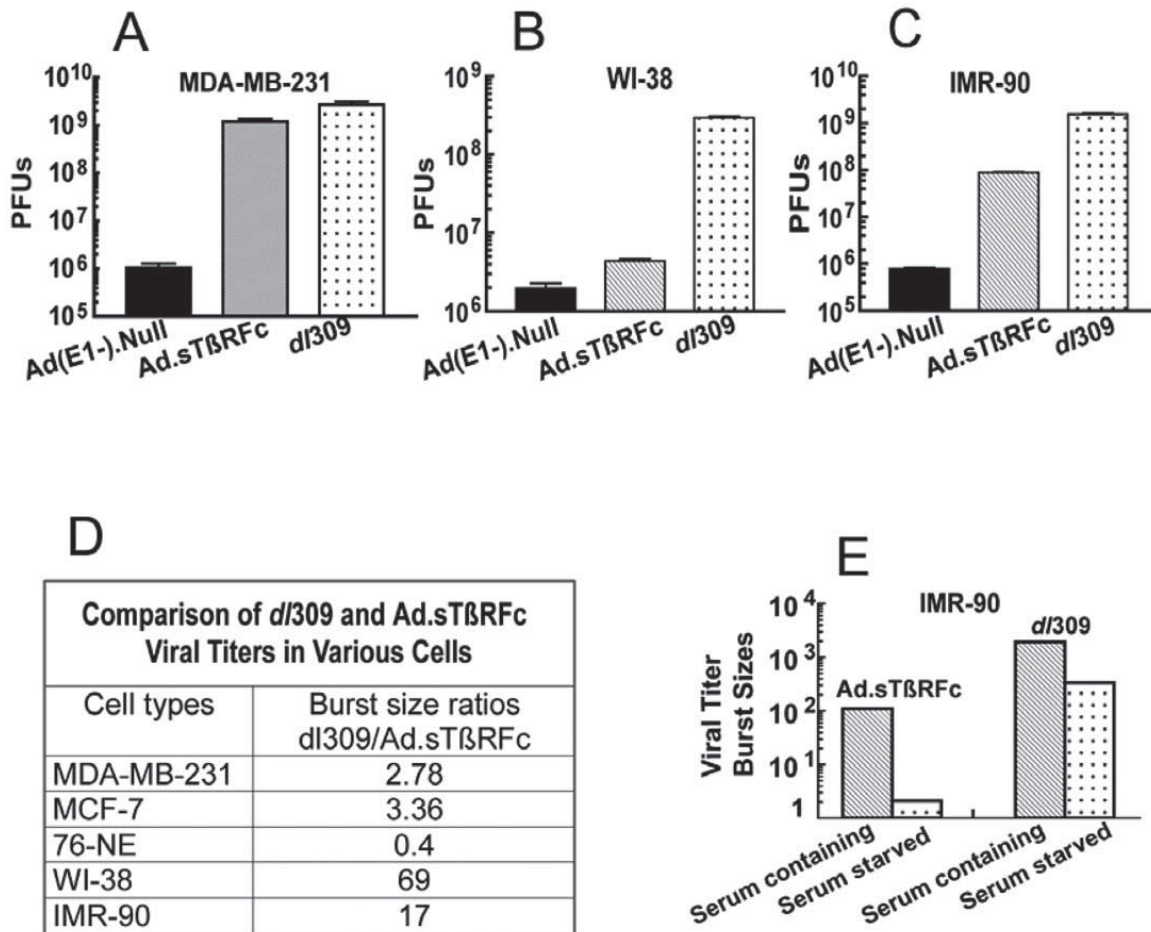


FIG. 4. (A) Ad.sT β RFc and *dl309* titers in MDA-MB-231 cells. MDA-MB-231 cells (10^5) were plated in 6-well plates and infected with Ad(E1-).Null, Ad.sT β RFc, or *dl309* (100 PFU/cell). After 3 hr, cells were washed to remove unbound virus and the incubations were continued for 48 hr. Media and cells were collected in 2-ml volumes. After three rounds of freeze-thawing, samples were serially diluted and incubated with 293 cells in 6-well plates. After 3 hr of incubation at 37°C, the infected cells were overlaid with 1.25% agarose in growth medium. Viral plaques were counted after an 8-day incubation, and are shown as plaque-forming units. Results shown represent the average (+SE) of three determinations. (B and C) Ad.sT β RFc and *dl309* titers in WI-38 and IMR-90 cells. WI-38 and IMR-90 cells were plated in 6-well dishes (4×10^4 and 1.5×10^4 cells per well, respectively). Cells were infected with Ad.sT β RFc or *dl309* (100 PFU/cell) for 48 hr. Cell lysates and supernatants were collected and viral titers were measured by plaque-forming assay in 293 cells. Results shown represent averages + SE. (D) Comparison of *dl309* and Ad.sT β RFc viral titers in various cells. The table shows the ratio of *dl309* and Ad.sT β RFc viral titers in three breast tumor cell lines (MDA-MB-231, MCF-7, and 76NE) and two normal cell lines (WI-38 and IMR-90). Results shown represent the average of three determinations. (E) Ad.sT β RFc and *dl309* viral burst sizes in IMR-90 cells grown under serum-containing and serum-starved conditions. Note that the Ad.sT β RFc burst size is reduced to 2.1 under low serum conditions, whereas *dl309* still produces a burst size of 334.

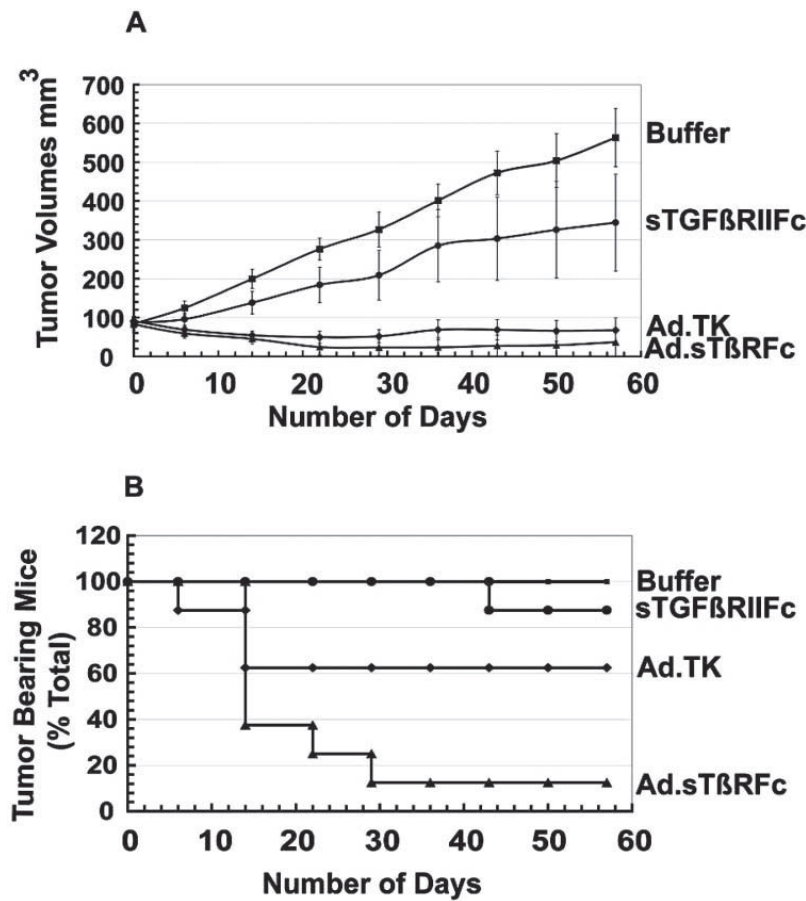


FIG. 5. (A) *In vivo* evaluation of effects of Ad.sTβRFc on MDA-MB-231 tumor xenografts in nude mice. MDA-MB-231 tumors were established subcutaneously in nude mice and treated with oncolytic virus (Ad.sTβRFc or Ad.TK), sTGFβRIIFc, or buffer as described in Materials and Methods. Tumor volumes were measured and calculated according to the formula $(a \times b^2) \times 0.52$. There were eight animals in each group. Compared with the buffer group, the *p* value for the Ad.sTβRFc group is <0.0001 ; for the Ad.TK group it is <0.0001 and for the sTGFβRIIFc group it is 0.16 (day 57). (B) Percentage of tumor-bearing mice in the various groups of animals (control and Ad.sTβRFc-, Ad.TK-, and sTGFβRIIFc-treated groups). Animals (from Fig. 4A) were monitored weekly for the presence or absence of tumors. The percentage of animals with tumors is shown here.

size, 1), Ad.sTβRFc produced a titer of 8.8×10^7 PFU (burst size, 111), and the dl309 titer was 1.53×10^9 PFU (burst size, 1924) (Fig. 4C and D). Thus, in IMR-90 cells, the replication potency of Ad.sTβRFc virus is 17 times lower than that of wild-type virus. These results indicate that although Ad.sTβRFc titer is lower than that of dl309 in both WI-38 and IMR-90 cells, Ad.sTβRFc clearly replicates in IMR-90 cells.

Because 01/07 E1A proteins are defective in binding with Rb and p300, the transcription factors responsible for cell cycle progression from G₀/G₁ to S phase, we hypothesized that Ad.sTβRFc would be further attenuated in nonproliferating normal cells. To investigate this, replication assays were performed in IMR-90 cells grown under either serum-containing or serum-starved conditions. Under serum-starved conditions, Ad.sTβRFc titers were reduced (burst size, 2.1), whereas dl309 produced a burst size of 334 (Fig. 4E). Thus low-serum conditions resulted in a dramatic increase in burst size ratio between dl309 and Ad.sTβRFc, to 159. These results indicate that Ad.sTβRFc replication is highly attenuated in nonproliferating

normal cells, whereas wild-type dl309 is still able to replicate under these conditions.

Thus, on the basis of the preceding observations, we conclude that Ad.sTβRFc replication in tumor cells is comparable to that of wild-type adenovirus, its capacity to replicate in normal cells is lower compared with wild-type virus, and Ad.sTβRFc replication is further reduced in nonproliferating normal cells. We are encouraged by these preliminary studies. However, these results also suggest the need to create oncolytic adenoviruses that would be further attenuated for viral replication in normal cells. Such investigations are currently underway in our laboratory.

Direct injection of Ad.sTβRFc into preestablished MDA-MB-231 breast tumors in nude mice causes significant inhibition of tumor growth and induces tumor regression

We have examined the effects of Ad.sTβRFc in the MDA-MB-231 xenograft model. MDA-MB-231 tumors were estab-

lished subcutaneously in nude mice. Once the tumors were about 80 mm³ in volume, Ad.sT β RFc, Ad.TK, purified sTGF- β RIIFc peptide, or buffer alone was injected directly into the tumors. Three injections of virus (2×10^8 PFU/injection), or 2.5 μ g of peptide per injection, were administered on alternate days (days 0, 2, and 4). Tumor sizes were monitored weekly, using a digital caliper. As shown in Fig. 5A, in animals that received buffer alone, tumors grew rapidly from an average tumor volume of 83 to 563 mm³ by day 57. In the Ad.sT β RFc group, after the vector treatment, there was a steady decline in tumor growth. On day 57, the average tumor size was 36 mm³, a 93% reduction in average tumor volume compared with the buffer control group ($p < 0.0001$). More importantly, seven of eight animals that received Ad.sT β RFc had complete tumor regression by day 28 (Fig. 5B). Ad.TK virus also showed significant inhibition of tumor growth ($p < 0.0001$); however, Ad.TK treatment caused tumor regression in only three of eight animals. sTGF β RIIFc peptide alone had a slight effect on tumor growth ($p = 0.16$ versus the buffer group), causing tumor regression in one of eight animals. These studies suggest that the antitumor effects of Ad.sT β RFc are perhaps largely due to viral replication, but vector-mediated protein production in combination with viral replication could potentially be involved in tumor regression. Clearly, a larger study is needed to examine Ad.sT β RFc-mediated antitumor effects *in vivo*, including the effects of systemic administration of the oncolytic viruses. Since the initial proposal of using conditionally replicating adenovirus dl1520 (Onyx 15), which has a mutation in E1B 55-kDa protein and replicates in cancer cells that lack wild-type p53 gene (Bischoff *et al.*, 1996), several adenoviruses that replicate in cancer cells harboring other genetic defects such as Rb mutations or deregulated E2F activity have also been used (Heise *et al.*, 2000; DeWeese *et al.*, 2001; Biederer *et al.*, 2002; Johnson *et al.*, 2002; Reid *et al.*, 2002a,b; Post *et al.*, 2003; Yan *et al.*, 2003; Wang *et al.*, 2006). Given that dl101/07-based oncolytic virus has the capacity to replicate in cancer cells regardless of their genetic background (Howe *et al.*, 1990, 2000), we believe that Ad.sT β RFc will be useful to treat breast cancers caused by various genetic alterations. Although a detailed *in vivo* investigation of Ad.sT β RFc is warranted, the results reported in this paper raise the possibility of considering a future human clinical trial in breast cancer patients.

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