Treatment of a Human Breast Cancer Xenograft with an Adenovirus Vector Containing an Interferon Gene Results in Rapid Regression due to Viral Oncolyis and Gene Therapy

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We propose that the complete regression of the breast cancer cell line (MDA-MB-435) in nude mice with a recombinant adenovirus containing the human interferon (IFN) consensus gene, IFN-conl (ad5/IFN), resulted in tumor regression in 100% of the animals. Tumor regression occurred when virus was injected either within 24 hr of tumor cell implantation or with established tumors. However, regression of the tumor was also observed in controls in which either the wild-type virus or a recombinant virus containing the luciferase gene was used, although tumor growth was not completely suppressed. Tumor regression was accompanied by a decrease in p53 expression. Two other tumors, the human myelogenous leukemia cell line K562 and the hamster melanoma tumor RPMI 1846, also responded to treatment but only with ad5/IFN. In the case of K562 tumors, there was complete regression of the tumor, and tumors derived from RPMI 1846 showed partial regression. We propose that the complete regression of the breast cancer cell line with the recombinant virus ad5/IFN was the result of two events: viral oncolysis in which tumor cells are being selectively lysed by the replication-competent virus and the enhanced effect of expression of the IFN-conl gene. K562 and RPMI 1846 tumors regressed only as a result of IFN gene therapy. This was confirmed by in vitro analysis. Our results indicate that a combination of viral oncolysis with a virus of low pathogenicity, itself resistant to the effects of IFN and IFN gene therapy, might be a fruitful approach to the treatment of a variety of different tumors, in particular breast cancers.

MATERIALS AND METHODS

Cell Lines and Virus. MD-MBA-435 cells, a human breast ductal carcinoma cell line, were kindly supplied by G. Weber (Indianapolis, Ind.). and were maintained in Eagle’s minimal essential medium (EMEM) supplemented with nonessential amino acids, EMEM vitamins, sodium pyruvate, and 10% fetal calf serum. RPMI 1846 cells, a Syrian hamster melanoma cell line, were grown in McCoy’s medium supplemented with 20% fetal calf serum. K562, a human myelogenous leukemia cell line, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. 293 cells, a human embryonic kidney cell line transformed with the left end of the adenoviral genome and containing the adenovirus E1a and E1b genes was maintained in EMEM supplemented with 10% fetal calf serum.

Adenovirus-5 wild-type ad5-Luc3 (ad5/lfu) were kindly provided by F. Graham (Hamilton, ON, Canada). ad5-Luc3 contains the luciferase gene flanked by the simian virus 40 regulatory sequences in the E3 region of the adenovirus. ad5/IFN contains the human consensus IFN gene inserted between 78.5 and 84.7 map units of the E3 region. All of these viruses are replication competent on human HeLa cells.

Construction of Plasmids and Recombinant Virus. Standard cloning techniques were used to construct the plasmids. The plasmid pIFNSS contains a 0.6-kb EcoRI/BamHI fragment coding for the signal peptide and coding sequence of IFN-conl (11). The plasmid pAd5/IFN was generated by inserting the 0.6-kb fragment from pIFNSS into the Xba I site of the pFG-dx1 by blunt end ligation. Restriction fragments used for ligation were purified from agarose gels using GeneClean (Bio 101). PFG-dx1 contains 40% of the right end of the adenovirus genome with a deletion from 78.5 to 84.7 map units. This 1.9-kb deletion removes most of the E3 region but leaves the E3 promoter and termination site. 293 cells were cotransfected with the plasmid pAd5/IFN and the EcoR1 digested adenovirus-5 DNA to rescue the recombinant virus (12), which was plaque purified twice and analyzed by restriction enzyme digestion.

ad5/wt, ad5/IFN, and ad5/IFN were added to monolayers of 293 cells at a multiplicity of infection (moi) of 50. The cells and

Abbreviations: IFN, interferon; pfu, plaque-forming unit(s); moi, multiplicity of infection; ISEL, in situ DNA end-labeling.

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media were harvested 2 to 3 days after infection and virus was released by sonication. Cell debris was removed by centrifugation, and the clarified lysate carefully layered over CsCl (1.43 g/cm³) and centrifuged in a Beckman SW-28 rotor at 20,000 rpm for 1 hr at 4°C. The visible virus band was collected and adjusted to 1.34 g/cm³, mixed with three times volume CsCl (1.34 gm/cm³), and centrifuged at 30,000 rpm for 24 hr. The resulting band of virus was collected and dialyzed against two changes of 200 vol 10 m M Tris-HCl/1 mM EDTA at 4°C for 4 hr. The purified virus was diluted in tris-saline-glycerol and stored at −70°C. The titer of the virus stocks were determined by plaque assay on 293 cells.

**IFN Activity Assay.** IFN produced by recombinant adenovirus-5 infected cells was determined by the inhibition of cytopathic effect assay with HeLa cells and vesicular stomatitis virus as challenge virus. For detection of IFN-con1 in ad5/IFN infected cells, the medium was collected at intervals between 6 and 72 hr after infection and tested. Human IFN standard (Namalwa Sendai, Ga 23-901-532) was used as a control. Human recombinant IFN-con1 had a specific activity of 1 × 10⁶ units/mg protein and was supplied by Amgen Biologicals. A polyclonal antibody to this IFN was also supplied by Amgen Biologicals.

**Growth Rate of Cell Lines.** Cells were plated at a density of 1 × 10⁵ cells per well in 48-well plates and infected the following day with wild-type or recombinant adenovirus-5 at a moi of 100. Culture medium alone was used in mock controls. Cells were counted daily from 1 to 6 days after infection. The growth rate was compared to uninfected cells, and cell viability assessed by trypan blue exclusion.

**In Vivo Treatment with Recombinant Adenovirus.** Female athymic BALB/c mice (3 to 4-week old) were purchased from Harlan-Sprague-Dawley. The mice were injected with 1 × 10⁸ MDA-MB-435 cells into the breast area, or 5 × 10⁶ K562 cells into the thigh area in 100 μl PBS per mouse. In some cases, 1 × 10⁸ plaque-forming units (pfu) per recombinant virus, or 10,000 or 100,000 units IFN-con1, or 100 μl PBS was injected into the site where the MDA-MB-435 cells were injected. In cases where established tumors were treated, injections of recombinant virus or IFN was at the dosage described above (at different times) and administered every 3 days. Female Golden Syrian hamsters (65–75 g; 5 to 6 weeks old) were purchased from Harlan-Sprague-Dawley. The hamsters were anesthetized by injection of 80–100 μl ketamine HCl at a concentration of 100 mg/ml. RPMI 1846 melanoma cells (10³) in 100 μl of McCoy’s medium were injected s.c. into the thigh region of the hamster using a 1-ml syringe and a 22-gauge needle. Adenovirus-5 and recombinant virus were injected into the center of the tumor nodule or into the tumor area, depending on time of injection. Recombinant virus or IFN was injected into the tumor once every 2 or 3 days for the length of the experiment.

**Histological Techniques.** The tumors were dissected, fixed in formalin, and embedded in paraffin. Percentage of tumor necrosis was calculated on hematoxylin/eosin stained sections at ×400 magnification using a 10 mm × 10 mm ocular grid on several representative cross sections of the tumor. Immunohistochemistry for the proliferation associated markers Ki-67 (clone MIB 1, dilution 1:5; Oncogene Science) (13) and PCNA (clone PC10, dilution 1:1000; Dako) (14) and the tumor suppressor gene product p53 [Pab 1801, dilution 1:10 (15), PB53–12 dilution 1:40 (16), NovaCasta, Newcastle, U.K.] was performed on paraffin section using a microwave oven-based antigen retrieval before treatment as described (17). Other proteins investigated by immunostaining in this study included apoptosis inhibitor oncoprotein Bel-2 (clone 124, dilution 1:30; Dako) (18), c-erb-2 oncoprotein (clone CB11, dilution 40; NovaCasta) (19), and estrogen receptor (clone ERID5, dilution 1:50; AMAC, Westbrook, ME) (20, 21). Following overnight incubation with the primary antibodies, the slides were stained with a biotin-conjugated goat anti-mouse antibody (30 min; Kirkegaard & Perry Laboratories) followed by peroxidase-conjugated streptavidin (30 min; Kirkegaard & Perry Laboratories). The enzyme was developed with 3,3′-diaminobenzidine (Sigma). The results were expressed as a percentage of positively stained neoplastic cell nuclei.

Apoptosis was measured by a nonisotopic, in situ DNA end-labeling (ISEL) technique (ApopTag: Oncor) applied to paraffin sections obtained from the same blocks used for immunostaining. With the ISEL technique, residues of dioxigenin-nucleotides (dioxigenin-dUTP) were catalytically added to the fragmented DNA (31OH ends) by adding terminal deoxynucleotidyl-transferase (Oncor) to the slides (22–24). The added residues were subsequently immunostained by anti-dioxigenin peroxidase-conjugated antibody (Oncor). The localized peroxidase enzyme was then revealed by routine diamobenzidine (Sigma) staining. The number of apoptotic nuclei and bodies were quantified as a percentage of the neoplastic cell nuclei.

**Statistical Analysis.** Statistical analysis of the in vivo animal tumor model data and in vitro cell growth analyses were produced by pairwise comparisons of the raw data and significance was evaluated by the standard Student’s t test. Unless otherwise stated in the text, sets of data were considered to be significantly different at a predetermined level of P ≤ 0.001. The error bars designating standard deviation were omitted from the graphs for the purpose of clarity. Error can be estimated from the scatter of data away from a smooth curve or the level of confidence (P value) indicated for comparisons between adjacent curves. Histochemical analysis was performed using Student’s t test for unpaired data to compare different results obtained within the various diagnostic groups.

**RESULTS**

**Treatment of MDA-MB-435 Breast Cancer Cells Shortly After Transplantation.** MDA-MB-435 cells were injected into the breast area of 3- to 4-week-old nude mice at a concentration of 1 × 10⁶ cells per mouse. On the following day either IFN-con1 (10,000 units or 100,000 units) or recombinant viruses at 1 × 10⁸ pfu or PBS were injected into the same area and tumor growth followed for 60 days. As can be seen from Fig. 1A there was no visible growth of tumors when mice were infected with ad5/IFN. Tumors directly treated with IFN grew significantly slower than control tumors, although by day 45 there was no significant difference between those treated with IFN and the controls. Mice that were infected with wild-type adenovirus, or a replication competent recombinant adenovirus containing the luciferase gene, produced smaller tumors that grew significantly slower than similar tumors in control or IFN-treated animals.

**Treatment of Established MDA-MB-435 Tumors with Recombinant Virus.** In a clinical situation one would want to treat an established tumor mass with the recombinant virus. We thus tested the effect of adding virus to established tumors at various times after transplantation. Virus was injected at 3-day intervals directly into established tumors beginning at days 15, 21, and 27 after the initial inoculum. Regression occurred even in large tumors (40 mm²). The rate of tumor regression is presented in Fig. 1B. In mice from group 1 (15 days) and group 2 (21 days) there was complete regression, whereas in the case of more advanced tumors, group 3 (27 days), there was complete regression in two of five animals and partial regression in the other 3. When wild-type adenovirus or ad5/ifu was injected 20 days after transplantation of the xenograft a significant decrease in tumor progression occurred (Fig. 1C).

This was significantly less effective than using ad5/IFN. Treatment with IFN-con1 itself at clinically relevant doses at 3-day intervals (100,000 units/ml) resulted in a significant decrease in growth, although less than with any of the other treatments.
FIG. 1. Effect of adenovirus-5 wild-type and recombinant viruses on growth of MDA-MB-435 breast carcinoma in athymic nude mice. Cells (10⁶) were injected into the breast area of 4-week-old female mice. Each experimental group contained five mice. (A) Twenty-four hours later ad/wt, ad/lfu, and ad/IFN were injected into the breast area at 10⁶ pfu per mouse, and 0.1 ml IFN-con1 was injected at 10⁵ and 10⁶ units/ml. PBS (0.1 ml) was injected into control tumors. Treatment was only done once. (B) Virus was injected at 2-day intervals starting at day 15 for group 1, day 19 for group 2, and day 27 for group 3. (C) Recombinant virus and IFN (0.1 ml of 10⁵ units/ml) were injected at day 70 at 2-day intervals. Virus was injected until tumors were no longer visible. (D) Growth curve of MDA-MB-435 cells infected with wild-type or recombinant adenovirus. The cells were plated at a density of 1 × 10⁵ per well in 48-well plates. Twenty-four hours after infection ad/wt or recombinant viruses were added at a moi of 100 pfu per cell. Four wells were counted daily from day 1 to day 6 after infection and the average number of cells was calculated.

Treatment of K562 and RPMI 1846 Tumors with Recombinant Virus. To explore the generality of this phenomenon, athymic nude mice were injected with the myelogenous leukemic cell line K562. This cell line is quite resistant to IFN in vitro (see below) and forms large tumors in vivo within 1 week of transplantation into nude mice. There was complete lack of growth when ad5/IFN was injected into the area previously injected with tumor cells 24 hr after tumor cell injection (Fig. 2A). Although there was significant inhibition of growth rate with both ad5/lfu and wild-type adenovirus this was not as pronounced as in the case of MDA-MB-435 cells.

FIG. 2. Effect of adenovirus-5 wild-type and recombinant viruses on growth of K562 myelogenous leukemic cells in athymic nude mice. (A) K562 cells (5 × 10⁵) were injected into the right thigh of the mice. Twenty-four hours later, ad/wt, ad/lfu, and ad/IFN were injected into the same area; results are from a single injection of virus. Tumor growth is presented. (B) Growth curve of K562 cells infected with wild-type or recombinant adenovirus. The cells were plated at a density of 1 × 10⁵ per well in 48-well plates. Twenty-four hours after infection ad/wt or recombinant viruses were added at a moi or 100 pfu per cell. Four wells were counted daily from day 1 to day 6 after infection and the average number of cells was calculated.
RPMI 1846 is a fast growing hamster melanoma and develops into a tumor in Syrian hamsters. Hamster cells do not support a productive infection with adenovirus (12, 25). However when melanoma cells were transplanted into the thighs of hamsters and challenged 24 hr later with wild-type adenovirus and both recombinant viruses, only ad5/IFN inhibited tumor growth (Fig. 3A). There was no significant difference between controls and ad5/wt or ad5/ifu. Similar experiments to those described above were done with established tumors. ad5/IFN did inhibit the development of the established tumor and temporarily inhibited growth, whereas both wild-type virus and ad5/ifu had no effect on tumor growth. IFN-con1 at 100,000 units every 2 or 3 days had a slight effect on tumor development (data not shown).

In Vitro Effects of Recombinant Virus. To examine whether the differences seen in the response to wild-type and recombinant virus could be seen in culture, MDA-MB-435 cells were infected with adenovirus-5 wild-type and recombinant viruses at a moi of 100. Fig. 1D illustrates that all three viruses inhibited the growth of MDA-MB-435 cells and lysed the cells. The recombinant virus ad5/IFN was significantly (P < 0.001) more potent than the other viruses. K562 cells (Fig. 2B) did not show cell lysis. Although there appears to be a greater effect of ad5/IFN than the other viruses, this difference was not significant (P > 0.05); the ad5/IFN treatment was significantly different (P < 0.005) from the control. This cell line appeared to be semipermissive for the virus. That this is not resistance to uptake of virus was indicated by the IFN effect. If cells were resistant to the virus there would not be expression of the recombinant gene (see below). RPMI 1846 is of hamster origin and was expected to be nonpermissive or semipermissive for virus replication. As shown in Fig. 3B, neither wild-type virus nor ad5/ifu had any effect on cell viability. However, RPMI 1846 cells were very sensitive to ad5/IFN.

IFN-con1 Production. The amount of IFN-con1 being produced by infected cells was measured from 6 to 72 hr after infection in vitro every 6 hr. MDA-MB-435, RPMI 1846, and K562 all produced IFN-con1. The amount produced varied slightly at each time point. MDA-MB-435 cells and RPMI 1846 cells produced between 40,000 and 600,000 units of IFN-con1 per day for each 10^6 cells. On the other hand K562 cells produced between 5,000 and 20,000 units per day for each 10^6 cells. All three cell lines are resistant to low levels of IFN (100–1000 units/ml) under our growth conditions.

Histological Analysis of MDA-MB-435. Table 1 summarizes the results of histological analysis of the control and treated breast tumors. MDA-MB-435 tumor samples obtained from untreated control mice were characterized by less necrosis, stronger expression of p53 oncoprotein, and higher proliferative activity as measured by Ki-67 (P < 0.01) and PCNA (P < 0.01) immunostaining than the ad5/IFN and ad5/wt infected tumors. No significant differences were observed between the ad5/IFN and ad5/wt treated tumors, although the ad5/IFN infected group showed a slightly higher reduction in their tumor proliferative activity. No significant differences in the histological and immunohistological parameters were observed between the IFN-treated and control groups. ISEL for fragmented DNA in the different sample groups showed similar results. This suggests that the regressive effects that are observed in the virus-infected specimens are not apoptosis-mediated but may be related to down regulation of p53. MDA-MB-435 cells were also estrogen-receptor negative and Bcl-2 negative.

DISCUSSION

Adenovirus vectors containing the HSVtk gene have been used in the treatment of a variety of tumors in mouse model systems (26–28). In these cases there was partial or complete tumor regression following treatment of the animal by i.p. injection of gancyclovir. In this paper we present an alternative mode of treatment that we found to be very effective in the treatment of human breast cancer and a human myelogenous leukemia in athymic nude mice.

In particular, breast cancer of humans has been refractile to IFN and other cytokine treatments. Treatment of MDA-MB-435 cells with high doses of type I IFN had very little inhibitory effect on the final growth of the tumors, although there was a significant decrease in cell growth initially (Fig. 1A and Table 1). Direct in vivo application of the recombinant virus either early after transplantation of tumor cells or at later stages resulted in complete tumor regression in the case of MDA-MB-435. When MDA-MB-435 cells were infected in culture with wild-type ad5, ad5/ifu, or ad5/IFN there was complete lysis of the cells, significantly faster with ad5/IFN than in the other two. Thus, the in vitro results correlated with in vivo data and suggest that tumor regression was primarily due to an oncolytic effect of the virus that is enhanced by the presence of the IFN-con1 gene.

In vitro infection of K562 cells results in minimum cell lysis, so that these cells appeared to be semipermissive for adenovirus. However, there was some inhibition of cell growth in vitro when the cells were infected with ad5/IFN. This probably reflects the large amounts of IFN being produced because K562 cells are refractory to IFN-con1 levels of 100 to 1000 units/ml (Y.G., unpublished results). However, when K562

![Image](image_url)

**FIG. 3.** Effect of ad5/IFN and other viruses on the Syrian hamster melanoma RPMI 1846. (A) RPMI 1846 cells (10^6) were injected into the thighs of hamsters. Twenty-four hours later, wild-type and recombinant viruses were injected into the area. Tumor growth was followed. (B) Growth curve of RPMI 1846 cells infected with wild-type or recombinant adenovirus. The cells were plated at a density of 1 x 10^4 per well in 48-well plates. Twenty-four hours after infection ad/wt or recombinant viruses were added at a moi of 100 pfu per cell. Four wells were counted daily from day 1 to day 6 after infection and the average number of cells was calculated.
tumors were treated with ad5/IFN in vivo there was complete suppression of tumor growth and regression of the tumors (4, 11); there was little affect of wild-type virus or ad5/IFN on tumor growth.

There was partial regression of the hamster melanoma RPMI 1846 following treatment with ad5/IFN, but not with the wild-type ad5 or ad5/IFN. As shown in Fig. 3C, RPMI 1846 cells were sensitive to ad5/IFN in vitro. Hamsters have previously been shown to be responsive to IFN-con1, although in vivo data indicate that this response is not as efficient as in the case of human cells (29). The inhibition seen here may be explained by the very high levels of IFN produced by the recombinant virus.

The histological and immunohistochemical analysis was made with advanced tumors treated at day 20 after tumor transplantation and removed 1 month later. The increased percentage of necrosis and the reduced frequency of p53 and proliferation associated markers observed in the ad5/IFN- and ad5/wt 4 5.5 ± 1.1
ad/IFN 5 3.5 ± 0.4
4 5.5 ± 1.1
5 7.6 ± 2.3
3 7.6 ± 2.3
6 9.9 ± 2.3
ad/wt 4 5.5 ± 1.1
5 7.6 ± 2.3
3 7.6 ± 2.3
6 9.9 ± 2.3
IFN 3 7.6 ± 2.3
4.3 ± 1.2
82.7 ± 8.4
89.3 ± 3.5
Untreated 6 9.9 ± 2.3
19.8 ± 4.0
84.3 ± 4.0
84.4 ± 8.1

Histochemical analysis was done as described in Materials and Methods.
35. Lindenman, J. & Klein, P. A. (1967) Recent Results Cancer Res. 9 (monogr.).