Adenovirus-mediated Gene Transfer to Human Breast Tumor Cells: An Approach for Cancer Gene Therapy and Bone Marrow Purging

Prem Seth, Ulrich Brinkmann, Gretchen N. Schwartz, Dai Katayose, Ronald Gress, Ira Pastan, and Kenneth Cowan

ABSTRACT

To examine the potential use of adenovirus vectors in cancer gene therapy as a mechanism for purging bone marrow cells of possible breast cancer contaminants, we compared the infection efficiency of adenovirus and the transfection efficiency of plasmid DNA in the presence of adenovirus in human breast cancer and bone marrow cells. Following infection of breast cancer cells with an adenovirus expressing β-galactosidase gene, high levels of β-galactosidase activity were observed. No β-galactosidase activity was observed in low-density human bone marrow cells. A replication-deficient adenovirus mutant dl312 enhanced the transfection efficiency of a plasmid DNA-expressing β-galactosidase gene into breast cancer cells, and addition of a liposome, lipofectamine, further enhanced the transfection efficiency. In contrast, human bone marrow cells treated under the same conditions expressed very low levels of the transfected β-galactosidase DNA. Transfection of cells with plasmid DNA expressing a truncated but fully active Pseudomonas exotoxin gene in the presence of cation-deficient adenovirus mutant dl312 enhanced the transfection efficiency of cells. Ad-mediated enhancement of toxicity of plasmid DNA using methods described previously (15).

INTRODUCTION

In recent years various approaches for ex vivo tumor cell killing for the purposes of bone marrow purging have been devised (1). These include the use of monoclonal antibodies against cell surface molecules, with or without linking to toxins, cytotoxic drugs, lectin agglutination, phototherapy, and biological modifiers (2–12). Recently, recombinant human adenoviruses have been shown to be effective vectors for gene transfer to eukaryotic cells (13–23). This can be accomplished either by utilizing the replication-defective Ad vector cointernalized with the plasmid expression vector DNA (13–19) or by generating the recombinant Ad expressing the transgene followed by infecting the target cells with the Ad vector (20–23). Both of these mechanisms require receptor-mediated endocytosis of Ad and are observed in cells expressing relatively high number of Ad receptors (13, 16, 23). When the Ad receptor number was measured in different cells, breast cancer cells were found to have a high number of Ad receptors (6–9 × 10^6/cell), whereas low-density bone marrow cells expressed undetectable levels. Breast tumor cells should therefore be much better targets for Ad-mediated gene transfer compared to bone marrow cells. Furthermore, Ad in combination with appropriate toxic genes could kill breast tumor cells while sparing bone marrow cells. In this report, we have compared the infectability of human breast cancer cells and human bone marrow cells with a replication-deficient Ad-expressing β-gal gene. We also examined the ability of a replication-deficient Ad, dl312, to transfect plasmid DNAs and have used a model system of breast cancer cell lines and normal bone marrow and CD34+ mobilized peripheral blood cells for comparison. In addition, we also have examined the effect of dl312 on the toxicity of a cointernalized plasmid DNA-encoding catalytic domain of the Pseudomonas exotoxin gene on human breast cancer and bone marrow cells. Finally, we compared the cytotoxicity of an Ad vector-expressing human wild-type p53 on these cells. The work described herein suggests that breast cancer cells are excellent targets for Ad-mediated cytotoxic effects, while human bone marrow cells are relatively resistant to Ad-dependent effects. Therefore, Ad vectors alone or in combination with the toxin genes could be potentially useful for ex vivo breast tumor cell killing and can therefore be considered for human bone marrow purging.

MATERIALS AND METHODS

Cells and Cell Culture. Breast cancer cell lines MDA-MB-231, MDA-MB-157, MDA-MB-453, and MCF-7 cells (all obtained from American Type Culture Collection) were cultured in MEM (Zn^2+ option) containing 10% FBS (GIBCO-BRL, Gaithersburg, MD). 293 cells (obtained from American Type Culture Collection), an Ad-transformed human kidney cell line, was propagated in improved MEM (Biofluids, Rockville, MD) containing 10% FBS. Human bone marrow cells were obtained from normal donors after informed consent as a part of a National Cancer Institutional Review Board protocol. Approximately 10 ml bone marrow aspirates were collected in a 20-ml syringe containing preservative-free heparin (Lymphomed; Deerfield, IL) and then diluted in HBSS without CaCl_2 or MgCl_2 (GIBCO-BRL). Cells with a density of ≤ 1.077 g/cm^3 were separated on a Ficoll-sodium diatrizoate gradient (LSM: Organon Teknika Corp., Durham, NC), washed three times with HBSS, and suspended in an enriched Iscove’s modified Dulbecco’s medium (GIBCO-BRL; Ref. 24). CD34+ cells were enriched from apheresis samples of G-CSF-mobilized peripheral blood cells from normal donors using an approved NIH protocol. If required, low-density bone marrow cells were exposed to 15 Gy γ-radiation (1.14 Gy/min).

Ad Vectors. dl312, a replication-deficient mutant of Ad (25), Ad.RSVβ-gal vector (26), and Ad.WTP53 (23) were propagated in 293 cells and purified by two rounds of CsCl density centrifugation. Ad titers were determined, and viral stocks were stored in Tris-Cl (pH 7.5) buffer containing 20% glycerol using methods described previously (15).

Plasmid DNAs. A plasmid expressing the bacterial β-gal gene driven by the CMV early gene promoter (CMV-β-gal) was used to measure the transfection efficiency of cells. Ad-mediated enhancement of toxicity of plasmid DNA was determined using a plasmid pUL1I00, which is a derivative of CMV-β-gal in which the β-gal coding region was replaced by a gene encoding the active (cytotoxic) domain of Pseudomonas exotoxin A. This leads to CMV promoter-driven expression of the toxin in transfected cells. A recombinant adenovirus expressing human wild-type p53 protein (Ad.WTP53) was also highly cytotoxic to breast tumor cells. Infection of breast cancer cells with Ad.WTP53 (100 plaque-forming units/cell) resulted in 100% loss of the clonogenicity of breast tumor cells. However, colony formation from CFU-GM was relatively resistant to the cytotoxic effects of Ad.WTP53 alone or in the presence of pUL1I00 plasmid and lipofectamine. On the basis of these results, it is proposed that human adenoviruses are potentially useful for cancer gene therapy and bone marrow purging.

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1 To whom requests for reprints should be addressed, at Medicine Branch, Building 10, Room 12 N 226, National Cancer Institute, NIH, Bethesda, MD 20892. Phone: (301) 496-9517; Fax: (301) 402-0172; E-mail: preethi@box-p.nih.gov.

2 The abbreviations used are: Ad, adenovirus; β-gal, β-galactosidase; FBS, fetal bovine serum; G-CSF, granulocyte colony-stimulating factor; Ad.WTP53, adenovirus expressing human wild-type p53 protein; CFU-GM, colony-forming unit granulocyte-macrophage; moi, multiplicity of infection; pfu, plaque-forming units; Ad.RSVβ-gal, recombinant Ad vector expressing the β-gal gene; CMV-β-gal, plasmid DNA expressing β-gal.

3 U. Brinkmann and I. Pastan, unpublished data.
as described previously (15, 16). In brief, 0.2 × 10^6 cells were used to bind
35S-radiolabeled d312 Ad (10^6 cpm, 0.1 μg Ad protein) at 4°C for 1 h in the
presence of unlabeled d312 (0–100 μg). Binding assays were conducted in
triple. 35S-radiolabeled d312 was produced in 293 cells according to a
previously published protocol (16).

Ad Infections: General Description. The experimental details including
the number of cells, moi of Ad used, and the length of incubations are
described below for each experiment. In general, cells were plated at
the appropriate density. After 24 h, the medium was changed to OPTI-MEM
(GIBCO-BRL), and the cells were infected with various moi of Ad. After 2 h,
FBS was added to a final concentration of 10%, and the incubation was
continued at 37°C.

Ad.RSVβ-gal Expression. Cells (2 × 10^4) were plated in 96-well plates
and infected with increasing concentrations of Ad.RSVβ-gal (0.16–500 pfu/
cell) for 24 h. Cells were then lysed in 100 μl 20 mM Tris-Cl containing 0.1% 
Triton X-100, and 30-μl aliquots were used to determine β-gal activity by a
colorimetric assay (23). An 

Ad and Lipofectamine-mediated Enhancement of CMVβ-gal Expression.
Cells (2 × 10^4) were plated in 96-well plates and exposed to CMVβ-gal
plasmid (1 μg), in the absence and presence of d312 (0.01–100 pfu/cell) in
a final volume of 0.2 ml. To test the effects of lipofectamine, CMVβ-gal plasmid
DNA (1 μg) was preincubated with lipofectamine (1 μg) at room temperature
for 20 min and used for transfection assays. Following exposure of cells to
these reagents for 2 h, FBS was added to the final concentration of 10%, and
the cells were incubated at 37°C for 24 h. Breast cancer cells were then washed
twice with PBS and lysed in 20 mM Tris-Cl (pH 7.5) containing 0.1% Triton
X-100. For bone marrow cells, the same protocol was used except cells were
centrifuged at 1500 rpm in an Eppendorf centrifuge, washed twice with PBS,
and then lysed. Aliquots (50 μl) of cell lysates were used to determine β-gal
activity (23).

Ad and Lipofectamine-mediated Enhancement of pUL1100 Cytotoxicity.
Breast cancer cells were plated in 96-well plates (500 cells/well) and
exposed to pUL1100 plasmid (1 μg/well) in the absence or presence of d312
(10 pfu/cell) for 24 h. To examine lipofectamine effects, plasmid DNA was
preincubated with lipofectamine (1 μg lipofectamine/1 μg plasmid DNA).
Cells were then exposed to these reagents in OPTI-MEM for 2 h, after which
the serum concentration was raised to 10%. Cells were incubated for 7 days
at 37°C for proliferation. The cells were then fixed in 10% trichloroacetic acid,
stained with 0.4% sulforhodamine B (Sigma, St. Louis, MO). An A540 was taken
using a Bio-Kinetic Reader EL 340 (Bio-Tek Instruments; Ref. 23) as a
measure of cell number at the end of the 7-day period. Cytotoxicity to
CFU-GM following transfection in the presence of d312 and pUL1100 was
estimated using the clonogenic assays described below.

AdWtp53-mediated Cytotoxicity Assays. Freshly trypsinized breast can-
cer cells were mixed with enriched CD34+ cells, and the total cell mixture was
exposed to different moi (0.1–10,000 pfu/cell) of AdWtp53 for 2 h. Cell
cultures were then split; one third of the cells (500 cells) were plated in each
60-mm dish and grown in improved MEM containing 10% FBS to form
colonies of breast cancer cells. After 14 days, the colonies were stained with
5 mM methylene blue and counted. The remaining cells were cultured in 0.8%
methylcellulose medium containing 5% phytohemagglutinin-leukocyte-condi-
tioned medium (Stem Cell Technologies, Vancouver, British Columbia, Can-
da) to stimulate colony formation from CFU-GM. One ml methylcellulose
medium containing 1000 cells was placed in each 35-mm gridded tissue
culture dish (Nunc, Naperville, IL), incubated at 37°C in 5% CO2 and room air,
and after 14 days colonies of ≥50 granulocytes or macrophages or both were
counted as derived from CFU-GM.

Table 1  Ad receptor numbers on breast cancer cells and human bone marrow cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Low-affinity</th>
<th>High-affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>6.2 × 10^3</td>
<td>1.1 × 10^3</td>
</tr>
<tr>
<td>MCF-7</td>
<td>5.3 × 10^3</td>
<td>7.7 × 10^2</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>7.5 × 10^3</td>
<td>8.8 × 10^3</td>
</tr>
<tr>
<td>Low-density bone marrow cells</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

RESULTS

Breast Tumor Cells Have a Greater Number of Ad Receptors
Than Human Bone Marrow Cells. Ad receptor number was determined
in breast cancer cell lines and human bone marrow cells. As
shown in Table 1, all of the breast cancer cells tested (MDA-MB-231,
MDA-MB-453, and MCF-7) expressed Ad receptors. The receptor
number in each of these cell lines was in the range of 5–7 × 10^3
low-affinity and 10^3 high-affinity Ad receptors. In contrast, receptors
on low-density human bone marrow cells were undetectable using this
method.

Breast Cancer Cells Are Easily Infected by Recombinant Ad as
Compared to Low-Density Human Bone Marrow Cells. Ad-me-
diated transgene expression was examined in breast cancer cells and
bone marrow cells following infection with different moi of a recom-
binant Ad-expressing β-gal gene (Ad.RSVβ-gal). As shown in Fig. 1,
following 24 h infection of MDA-MB-231, MDA-MB-453, MDA-
MB-157, and MCF-7 cells with Ad.RSVβ-gal, β-gal activity was
observed which was dependent on the moi of Ad.RSVβ-gal used.
Maximum enzyme activity in the range of 5–7 units was observed in
cells infected with 100 pfu/cell, and saturation kinetics were observed
at higher moi. With the infection of cells with Ad.RSVβ-gal (100
pfu/cell) for 24 h, followed by staining of the cells using X-gal, nearly
100% of the cells were stained for blue color, indicating a high
expression of β-gal activity in every cell (data not shown). In contrast,
following infection of bone marrow cells with Ad.RSVβ-gal with up
to 500 pfu/cell, no detectable β-gal activity was observed (Fig. 1). By
infecting low-density bone marrow cells with up to 500 pfu/cell of
Ad.RSVβ-gal, more than 80% of the cells were viable, as judged by
the trypan blue exclusion test (data not shown). Since all of the breast
cancer cells had nearly the same number of Ad receptors and ex-
pressed a foreign transgene, all subsequent experiments were
conducted using a representative breast cancer cell line, MDA-MB-231.
Ad and Lipofectamine Significantly Increased the Expression of Plasmid DNA in Breast Cancer Cells but not in Human Bone Marrow Cells. Using CMVβ-gal, a plasmid expressing the β-gal gene, we next investigated the Ad-mediated enhancement of the plasmid DNA delivery to MDA-MB-231 and bone marrow cells. MDA-MB-231 cells transfected with plasmid DNA alone expressed low levels (<0.01 unit) of β-gal activity. However, in the presence of increasing concentrations of dl312, there was an increase in β-gal activity with maximum β-gal expression (0.65 unit) observed in the presence of 100 pfu/cell (Fig. 2). On the other hand, β-gal activity was undetectable in low-density bone marrow cells in the presence of CMVβ-gal plasmid alone or in the presence of CMVβ-gal plasmid and dl312 (Fig. 2).

Since previous studies have shown that an Ad-mediated increase in plasmid DNA uptake and expression are augmented by the addition of a monocationic liposome, lipofectin (17), we next examined the effect of a polycationic liposome, lipofectamine, on dl312-mediated CMVβ-gal plasmid expression in both human breast cancer cells and human bone marrow cells. As shown in Fig. 3, when plasmid DNA alone was used a low level (<0.01 unit) of β-gal activity was observed for both MDA-MB-231 and bone marrow cells. dl312 or lipofectamine when used individually increased the β-gal activity in MDA-MB-231 cells to 0.65 and 0.58 units, respectively, and the combination of both dl312 and lipofectamine increased the β-gal expression in MDA-MB-231 cells to 3.8 units (Fig. 3). On the other hand, low-density bone marrow cells showed only low levels of β-gal activity in the presence of either dl312 (100 pfu/cell) or lipofectamine alone. Even in the presence of both dl312 and lipofectamine, only 0.04 units of β-gal activity was obtained (Fig. 3), which is much lower than that obtained in MDA-MB-231 cells under the same conditions.

Ad-mediated Enhancement of the Cytotoxicity of a Plasmid DNA Expressing Pseudomonas Exotoxin Gene Is Greater in Breast Cancer Cells Than G-CSF-mobilized Cells. Next, we investigated whether low doses of dl312 could enhance the delivery of a plasmid DNA coding for a toxin gene as measured by cytotoxicity with the idea of developing a purging technique that will reduce the amounts of Ads to be used for purging. To test this, MDA-MB-231 breast tumor cells were transfected with pUL1100 DNA, a plasmid containing the cDNA encoding for the catalytic component of Pseudomonas exotoxin, which is cytotoxic to cells. Transfections were conducted in the absence and presence of dl312 and/or lipofectamine, and cell killing was examined by a colorimetric assay using sulforhodamine as described in “Materials and Methods.” As shown in Fig. 4, exposure of MDA-MB-231 cells to either dl312 (10 pfu/cell), lipofectamine (1 μg alone), or in combination had no significant effect on cell killing (p values > 0.05). Incubation of cells with pUL1100 (1 μg) alone also had no significant cytoxic effect (p > 0.05). However, pUL1100 (1 μg), in combination with either dl312 (10 pfu/cell) or lipofectamine (1 μg), resulted in approximately 70% killing of MDA-MB-231 breast cancer cells (p values < 0.05). Furthermore, when pUL1100 transfections were performed in the presence of both dl312 and lipofectamine, more than 95% cell killing was observed (p values < 0.05; Fig. 4). In contrast, exposure of CD34+ cells to similar concentrations of pUL1100 plasmid alone or in combination with dl312 or lipofectamine resulted in little toxicity to CFU-GM, as determined by colony formation (p values > 0.05; Fig. 4).

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*P. Seth, unpublished data.*
AdWTp53 Is More Cytotoxic to Breast Cancer Cells Than G-CSF-mobilized Cells. Next, we investigated the cytotoxic effects of an Ad vector expressing human wild-type p53 (AdWTp53). Overexpression of wild-type p53 has been previously shown to be toxic to different cancer cells (23). Following infection of a mixture of MDA-MB-231 breast cancer cells and CD34+ cells with increasing moi of AdWTp53, the cytotoxicity of AdWTp53 was measured using colony-forming assays. As shown in Fig. 5, following infection of MDA-MB-231 cells with AdWTp53, significant cytotoxicity (about 55% decline in colony numbers) was observed at a moi of 1 pfu/cell, and that at moi 10 pfu/cell or higher, no colony formation was observed. Bone marrow cells, on the other hand, were much more resistant to killing by AdWTp53. At a moi of up to 100 pfu/cell, there was essentially no decline in colony formation in CFU-GM. However, at a very high moi (1000 pfu/cell) of AdWTp53, there was about 50% reduction in colony numbers (Fig. 5). These results indicate that MDA-MB-231 breast cancer cells are at least three orders of magnitude more sensitive to the cytotoxic effects of AdWTp53 compared to human CD34+ human bone marrow cells.

We also tested the relative cytotoxic effects of AdWTp53 on breast tumor cells in the presence of bone marrow cells. Coincubation of 10^6 irradiated low-density bone marrow cells and MDA-MB-231 cells (in the range of 5 × 10^5-10^6 cancer cells); with 100 pfu/cell AdWTp53, 100% killing of the breast cancer cells was observed (Table 2). We also examined the cytotoxicity results using a combination of low moi of AdWTp53 (1 pfu/cell), pUL1100 DNA (5 μg/ml), and lipofectamine (1 μg/1 μg DNA). Although this treatment resulted in a 100% reduction in colony formation by MDA-MB-231 breast cancer cells, the same treatment (AdWTp53, pUL1100, and lipofectamine) resulted in only 12% reduction in CFU-GM colonies (data not shown). These results therefore indicate that while exposure of breast tumor...
cells to a low concentration of AdWTp53, lipofectamine, and a plasmid coding for a toxin gene is markedly cytotoxic to breast cancer cells, human bone marrow cells are relatively resistant to this treatment, and such treatment relatively purges a breast tumor cell population from normal marrow.

**DISCUSSION**

The studies presented in this report indicate that recombinant Ads can be used for efficient gene transfer in human breast cancer cells and that human bone marrow cells are relatively poor targets for this viral vector. Therefore, the use of appropriate recombinant Ad vectors could be considered for purging for human bone marrow samples. The evidence for these conclusions is derived from several experiments. First, breast cancer cells were easily infected by a recombinant adenovirus expressing a transgene β-gal, whereas human bone marrow cells were resistant to infection by this vector. In these studies, high expression of β-gal following Ad.RSVβ-gal infection was observed in breast cancer cells relative to that in human bone marrow cells. Second, when Ad-mediated transfection of a plasmid DNA expressing the β-gal gene was examined, breast cancer cells expressed high levels of the β-gal gene. In contrast, bone marrow cells treated under the same conditions expressed low levels of β-gal activity. Even incubating in the presence of Ad and lipofectamine, bone marrow cells expressed much less β-gal activity as compared to breast tumor cells. Third, when the plasmid DNA containing a cytotoxic *Pseudomonas* exotoxin gene was used, Ad-mediated expression of this toxin gene resulted in much more cytotoxicity to breast cancer cells as compared to normal hematopoietic colony-forming cells. Fourth, a recombinant Ad vector expressing the human tumor suppressor gene p53 was approximately 10^3 times more cytotoxic to MDA-MB-231 breast cancer cells as compared to the bone marrow cells. This is likely to be associated with a high expression of wild-type p53 in breast tumor cells following infection with AdWTp53 compared to bone marrow cells. A high level of p53 production is known to be cytotoxic to breast cancer cells (23), which results in a marked difference in cytotoxicity in breast cancer cells relative to bone marrow cells.

Although there could be several reasons for the effectiveness of Ads to deliver genes to breast cancer cells as compared to bone marrow cells, the principal difference appears to be mediated at the level of Ad receptor expression. The human breast cancer cells examined contained a relatively high number of Ad receptors (in the range of 6–9 × 10^5) while bone marrow cells were relatively deficient in the cell surface Ad receptor. Because Ad-mediated gene transfer requires receptor-mediated uptake of adenovirus (13, 16) into the cells, breast cancer cells expressing Ad receptors would be better targets for Ads than the Ad receptor-deficient bone marrow cells. However, in a previous study, the adenosine deaminase gene could be transferred to about half of the human bone marrow cells using an adenoviral vector (27). Whether this is because of unique experimental conditions (such as the presence of certain growth factors in the incubating medium) which might favor the expression of Ad receptors on bone marrow cells needs to be investigated.

On the basis of the results presented here, it is proposed that Ads can be considered for purging breast cancer cells from bone marrow samples. One could either utilize a replication-deficient Ad in combination with plasmid DNAs coding for a toxin gene or an Ad vector expressing a cytotoxic gene. Ad vectors expressing wild-type p53 that have been shown to be cytotoxic to breast cancer cells can also be used in combination with a plasmid expressing a toxin gene. Each of these approaches resulted in at least a 1000-fold increase in cytotoxicity to breast cancer cells compared to bone marrow cells, suggesting the power of recombinant adenoviral vectors for bone marrow purging. Additional work is needed to determine whether this purging approach may be applied to the purging of fresh breast tumor cells as described here for cancer cell lines. Moreover, it will also be important to evaluate whether Ads can be used for purging other types of tumor cells such as lymphomas.

One recent study has shown a high incidence of breast cancer cell contamination in peripheral blood cell and bone marrow collections (about 60% bone marrow specimens positive for breast cancer cells) from patients undergoing chemotherapy and hematopoietic stem cell transplantation (4). Various approaches to purge tumor cells include negative selection by eliminating the contaminating cancer cells or positive selection in which the bone marrow cells are enriched from hematopoietic progenitor cells (1). To kill tumor cells, monoclonal antibodies against breast cancer cells’ receptors linked with toxin proteins have been used (5, 12). Others have utilized immunomagnetic bead separation (6) and photoirradiation for *ex vivo* purging of residual tumor cells from autologous bone marrow grafts (7). Hematopoietic progenitor and stem cells can also be enriched by the use of specific antibodies (e.g., anti-CD34 antibody) or lectins followed by concentrating the cells by biotin-avidin, immunomagnetic, or solid-phase binding (1, 9). Results presented here indicate that bone marrow purging using Ads should also be considered in the clinical setting. The potential purging protocols described in this article are simple and effective. The recombinant Ads are replication deficient (28), can be grown to high titer (20), and can be used in combination with plasmid vectors expressing toxic genes, which reduces the dose and increases the safety of Ad-mediated protocols.

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